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Development of an Ultra-Sensitive Enzyme Immunoassay for Insulin and Its Application to the Evaluation of Diabetic Risk by Analysis of Morning Urine

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1. Introduction

1.1 Principle of immunoassay for peptides

Currently available enzyme immunoassay methods for peptides can be divided into two groups, homogeneous and heterogeneous methods. Homogeneous enzyme immunoassay methods, in which signals that are directly obtained from a mixture of test samples and reagents correlate with the amount of peptide in test samples, are simpler and quicker but less sensitive than heterogeneous enzyme immunoassay methods, in which free and bound forms of enzyme-labeled reactants are separated from each other.

Heterogeneous enzyme immunoassay methods can be divided into competitive and noncompetitive methods. In a typical competitive enzyme immunoassay method, a fixed amount of labeled peptide is reacted with the corresponding antibody in the absence and presence of the unlabeled peptide whose level is to be measured. The amount of the peptide to be measured correlates with the amount of labeled peptide that is bound to the antibody, which can only be measured within a certain range of error (approx. 5%). Assay sensitivity increases as the concentration of labeled peptide and antibody are decreased. However, the concentration of labeled peptide and antibody should be high enough so that more than 50% of the labeled peptides and antibody used are in bound form. In other words, the minimum concentration of labeled peptide that can be used is limited by the affinity of the antibody. The sensitivity of a competitive enzyme immunoassay that is modified using a labeled antibody is also limited in a similar manner. In this type of assay, it makes no difference whether radioisotopes or enzymes are used as labels. In most cases the detection limit for peptides using such a modified competitive enzyme immunoassay is at femtomole

($\times 10^{-15}$ moles) or higher levels. [Aikawa, 1979; Fyhrquist, 1976; Glänzer, 1984; Morton, 1975; Mukoyama, 1988; Scharpé, 1987; Tikkanen, 1985; Uno, 1985]

In contrast to competitive assay methods, in noncompetitive immunoassay methods, the excess of enzyme-labeled antibody is efficiently eliminated by simple washing, and the amount of enzyme-labeled antibody nonspecifically bound to an antibody-coated solid phase in the absence of the antigen to be measured (background noise) can be minimized. This reduction in background noise makes it possible to achieve attomole sensitivities, provided that antibodies with sufficiently high affinity are used. Indeed, the reported detection limit for peptides, using noncompetitive enzyme immunoassay methods with appropriate techniques, is at attomole ($\times 10^{-18}$ moles) levels, as described below [Hashida, 1991 & 1993; Ishikawa, 1983a & 1989].

Twenty years ago, a novel method (immune complex transfer method) was developed to lower the nonspecific binding of enzyme-labeled antibody without reducing the size of the solid phase surface or the reaction mixture volume used for immunoreactions [Hashida, 1988]. For this method, the antigen to be measured was reacted simultaneously with a 2,4-dinitrophenylated IgG antibody and a Fab' antibody that was labeled with β-D-galactosidase from Escherichia coli. The resulting immune complex, which was comprised of all three components, was trapped on polystyrene beads that were coated with affinity-purified anti-2,4-dinitrophenyl group IgG. These polystyrene beads were then washed to eliminate the excess Fab'-β-D-galactosidase conjugate. The immune complex was eluted from the polystyrene beads with an excess of *εN*-2,4-dinitrophenyl-L-lysine and was transferred to polystyrene beads coated with anti-IgG Fc portion IgG. This transfer resulted in more complete elimination of the Fab'-β-D-galactosidase conjugate that was nonspecifically bound to the polystyrene beads coated with the affinity-purified (anti-dinitrophenyl group) IgG, thereby markedly reducing the background noise of the two-site enzyme immunoassay. Since there was also a smaller decrease in specific binding than in previous types of assays, these two features of the assay significantly improved assay sensitivity. Using this two-site immune complex transfer enzyme immunoassay, the detection limit of human ferritin was 1 zmol (zeptomole; 1×10^{-21} moles) per assay [Hashida, 1990 & 1995].

1.2 Ultra-sensitive assay for insulin

Insulin levels have typically been measured in serum using ELISA, which is a noncompetitive assay method [Lindström, 2002]. In order to determine insulin secretion ability, an oral glucose tolerance test (OGTT) is performed for which blood samples have to be collected every 30 min for 2 h. However, blood sampling involves risks of pain and infection. In contrast, urine samples can be collected more easily than serum samples. The insulin level in urine, collected at a specific time after serum insulin changes, may reflect these serum insulin levels. Thus urinary insulin levels may provide useful information regarding insulin secretion.

In order to overcome the problem that urinary insulin levels are too low to be measured by conventional ELISA, we developed an ultra-sensitive immune complex enzyme immunoassay (ICT-EIA) to measure urinary insulin.

2. Materials and methods

2.1 Buffers

The following buffers were used. Buffer A: 10 mM sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl, 1.0 g/l bovine serum albumin (BSA), 1.0 mM MgCl2 and 1.0 g/l NaN3; buffer B: 10 mM sodium phosphate buffer (pH 7.0) containing 0.4 M NaCl, 0.1 g/l BSA, 1 mM MgCl2 and 1.0 g/l NaN3. Bovine serum albumin (BSA; fraction V) was obtained from Intergen Co. (Purchase, NY).

2.2 Human insulin, antibodies and ELISA

Recombinant human insulin was purchased from Millipore (St. Charles, MO). Human resistin and human Insulin-like growth factors-I (IGF-I) were purchased from R&D Systems, Inc. (Minneapolis, MN). Human chorionic gonadotropin (hCG) was purchased from Roche Diagnostics K.K. (Tokyo, Japan). Human growth hormone (hGH) was purchased from JCR Pharmaceuticals Co., Ltd (Hyogo, Japan)

Mouse anti-human insulin monoclonal antibodies (Ab-1; 16E9 and Ab-2; 6F7) were purchased from Japan Clinical Laboratories, Inc. (Nagoya, Japan). Guinea Pig anti-human insulin (Ab-3; N2PP05) antibody was purchased from Meridian Life Sci., Inc. (Saco, ME). Mouse anti-human resistin monoclonal antibodies (184305 and 184320) were purchased from R&D Systems. Rabbit (anti-2,4-dinitrophenyl (DNP)-BSA) serum was purchased from Shibayagi Co., Ltd. (Gunma, Japan). Rabbit anti-mouse Fc IgG was purchased from Thermo Sci. (Rockford, IL).

The ELISA for insulin was purchased from Mercodia (Uppsala, Sweden).

2.3 Subjects

A total of 141 Japanese non-diabetic, non-obese (NDNO) and non-diabetic obese (NDO) volunteers and 42 Japanese diabetic patients (DM) from our clinic participated in the study (Table 1). Criteria for obese subjects in this study included: body mass index (BMI) of >25 kg/m^2 or percentage body fat ratio of >25% for men and >30% for women. The study protocol was approved by the ethics committees of Tokushima Bunri University and Tokushima University and all participants gave written informed consent.

Parameters measured	NDNO	NDO	DM
(mean ± SD)			
No. of subjects (male/female)	106 (10/96)	35 (12/23)	42 (27/15)
Age (years)	30.3 ± 18.1	48.4 ± 7.0a	55.2 ± 27.7a,b
BMI (kg/m2)	21.9 ± 2.9	27.0 ± 4.1a	25.1 ± 3.3a
Fasting plasma glucose (mg/dl)	80.0 ± 12.7	84.5 ± 9.4a	119.6 ± 25.5a,b
HbA1c (%)	4.8 ± 0.4	5.1 ± 0.3a	7.2 ± 1.5a,b

aP < 0.01, compared with NDNO; bP < 0.01, compared with NDO.
NDNO, non-diabetic, non-obese volunteers; NDO, non-diabetic obese volunteers; DM, diabetic patients

Table 1. Clinical characteristics of the study groups

2.4 Blood and urine sampling

Blood samples were drawn early in the morning from an antecubital vein of subjects who had fasted overnight. Samples were then transferred into chilled glass tubes and kept on ice for <30 min. Serum was separated from the samples by centrifugation at 1,500 x g for 15 min at 4 °C and kept frozen at -30 °C until analysis.

Urine samples were collected early in the morning from non-diabetic and diabetic subjects who had fasted for 16 h. The urine samples (10 ml) were mixed with 0.1 ml of both BSA (100 g/l) and NaN3 (100 g/l) and were kept frozen at -30 °C until analysis. Urinary albumin and creatinine were also measured using standard methods. Urine samples with micro- and macro-albuminuria were excluded. Urinary insulin levels were expressed as a ratio to the concentration (mg) of urinary creatinine (Cre).

2.5 Antibody preparation

IgG was prepared from serum by fractionation with Na_2SO_4 followed by passage through a column of diethylaminoethyl cellulose. Both polyclonal rabbit and monoclonal mouse IgGs were digested with pepsin to $F(ab')_2$, which was further reduced to obtain Fab' [Hashida, 1995].

2.6 Preparation of capture antibody and enzyme-labeled antibody

Mouse anti-insulin Fab' monoclonal antibody (Ab-1), guinea pig anti-insulin Fab' polyclonal antibody (Ab-3) and mouse anti-human resistin monoclonal antibodies (184305) were each conjugated with 6-maleimidohexanoyl-DNP-biotinyl-BSA and used as a capture antibody [Hashida, 1995; Ishikawa, 1983b]. Mouse anti-insulin Fab' monoclonal antibody (Ab-2) and mouse anti-human resistin monoclonal antibodies (184320) were conjugated with β-D-galactosidase from Escherichia coli using o-phenylenedimaleimide, or with horseradish peroxidase or alkaline phosphatase from calf intestine, using *N*-succinimidyl-6-maleimidehexanoate and was used as an enzyme-labeled antibody [Hashida, 1995; Ishikawa, 1983b].

Thiol groups were introduced into mouse anti-insulin IgG monoclonal antibody (Ab-4) using S-acetylmercaptosuccinic anhydride (Nacalai Tesque, Inc., Kyoto, Japan) and the antibody was then conjugated with 6-maleimidohexanoyl-DNP and 6-maleimidohexanoyl-biocytin and used as a capture antibody [Hashida, 1995; Ishikawa, 1983b].

2.7 Preparation of protein-coated polystyrene beads

Polystyrene beads (3.2-mm diameter; Immuno Chemical, Inc., Okayama, Japan) were coated with affinity-purified anti-DNP-BSA IgG (0.01 g/l), anti-mouse Fc IgG or biotinyl-BSA (0.01 g/l) by physical adsorption [Hashida, 1995; Ishikawa, 1983b]. Biotinyl-BSA-coated polystyrene beads were then reacted with streptavidin (0.01 g/l) [Hashida, 1995; Ishikawa, 1983b].

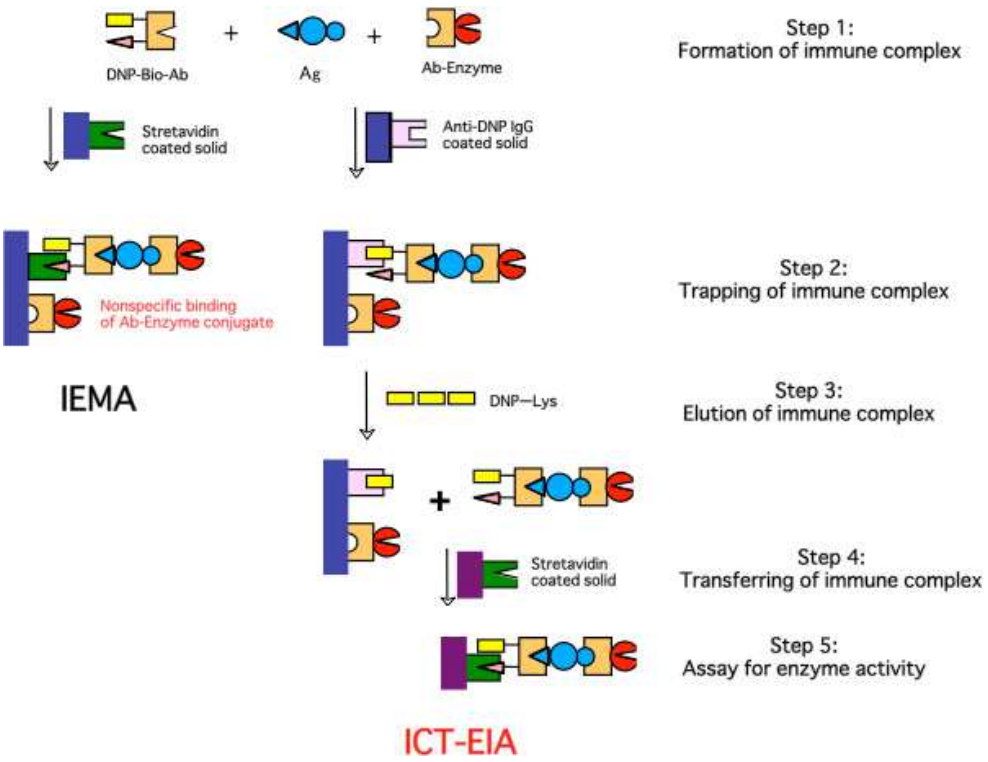
2.8 Immunoenzymometric assay (IEMA) for insulin

The protocol of the IEMA for insulin was as follows (Fig. 1): An aliquot (100 μl) of standard human insulin, or urine sample, was incubated overnight at 4 °C with 100 μl buffer B containing 100 fmol Ab-1 Fab' conjugated with DNP-biotinyl-BSA and 30 fmol Ab-2 Fab'-β-

D-galactosidase conjugate (Complex formation). Thereafter, one streptavidin-coated polystyrene bead was added and incubated for 30 min. The bead was then washed, and the bound β-D-galactosidase activity was assayed fluorometrically with 4-methylumbelliferyl-β-D-galactoside (0.2 mM) as the substrate for 1 h at 30 °C [Ishikawa, 1983b].

2.9 Two-site immune complex transfer enzyme immunoassay (ICT-EIA) for insulin

The protocol of the ICT-EIA for insulin was as follows (Fig. 1): An aliquot (100 µl) of standard human insulin diluted in buffer B, or urine sample, was incubated overnight at 4 °C with 100 µl buffer B containing 100 fmol of capture antibody conjugate; Ab-1 or Ab-3 Fab′ conjugated



The ICT-EIA assay entails five steps: 1. Complex formation, during which the three assay components of capture antibody, antigen, and enzyme-conjugated antibody form a complex; 2. Entrapment, during which the formed complex is trapped on an anti-DNP solid phase; 3. Elution, during which the bound immune complex is eluted with DNP-Lysine solution; 4. Transfer, during which the eluted immune complex is transferred onto a streptavidin solid phase; 5. Enzyme assay, during which the activity of the enzyme bound to the immune complex is assayed fluorescently. The IEMA assay is outlined at the left for comparison.
Ab, antibody; Ag, antigen; Lys, lysine.
Color key: Blue

Fig. 1. Schematic outline of the IEMA and ICT-EIA for insulin

with DNP-biotinyl-BSA or a DNP-biotinyl-Ab-1 IgG conjugate, and 30 fmol Ab-2 Fab'-β-D-galactosidase conjugate (Complex formation). Thereafter, one polystyrene bead coated with affinity-purified IgG (anti-DNP-BSA) was added to the mixture and incubated for 30 min (Entrapment). After removal of the incubation mixture, the polystyrene bead was washed twice with buffer A and then incubated in 150 μl buffer A containing 2 mM *eN*-2,4-DNP-L-lysine for 30 min (Elution). After removal of the polystyrene bead, one streptavidin-coated polystyrene bead was added to the eluate and incubated for 30 min (Transfer). The bead was then washed, and the bound β-D-galactosidase activity was assayed fluorometrically with 4-methylumbelliferyl-β-D-galactoside (0.2 mM) as a substrate for 20 h at 30 °C [Hashida, 1995; Ishikawa, 1983b]. All incubations with polystyrene beads were performed with shaking at 210 strokes/min at room temperature [Hashida, 1995; Ishikawa, 1983b].

To compare the assay of urinary insulin with assay of other urinary hormones derived from serum, ICT-EIAs for resistin were performed using a similar method as that described above for the ICT-EIA for insulin, including similar volumes of standard and samples, concentration of antibody conjugates, buffer, temperature and incubation times, except that, in the ICT-EIA for resistin, both DNP-biotinyl-BSA anti-resistin Fab' (184305) and anti-resistin Fab'(184320)-β-D-galactosidase antibody conjugates were used.

2.10 Measurement of urinary albumin and creatinine levels

Albumin and creatinine levels were measured in first morning urine samples. Albumin levels were assessed with an ICT-EIA as described above but using a specific anti-albumin monoclonal antibody as described in previous report [Umehara, 2009] and creatinine levels were assessed by reaction with picric acid using a commercial kit (Creatinine-test Wako, Wako Pure Chemical Industries, Ltd., Osaka, Japan).

2.11 Laboratory tests

Plasma lipoproteins, total cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), triglycerides (TG) and HbA1c levels were analyzed consecutively using standard laboratory techniques by Bio Medical Laboratories, Inc. (Tokyo, Japan).

2.12 OGTT test

Eleven healthy young subjects (3 males and 8 females) ingested 75 g glucose. Blood samples were then obtained at 0, 60 and 120 min and urine samples at 0 and 120 min following ingestion. The levels of blood glucose and insulin, as well as levels of urinary insulin, were measured.

2.13 Statistical analysis

All data are presented as means ± SD. The detection limit of ICT-EIA or ELISA for insulin was expressed as the minimal amount of insulin which gives a significant bound enzyme activity (fluorescence or absorbance signal) after subtraction of the background signal (no insulin). A significant difference from the background was confirmed using Student's t-test ($P < 0.001$, $n = 5$).

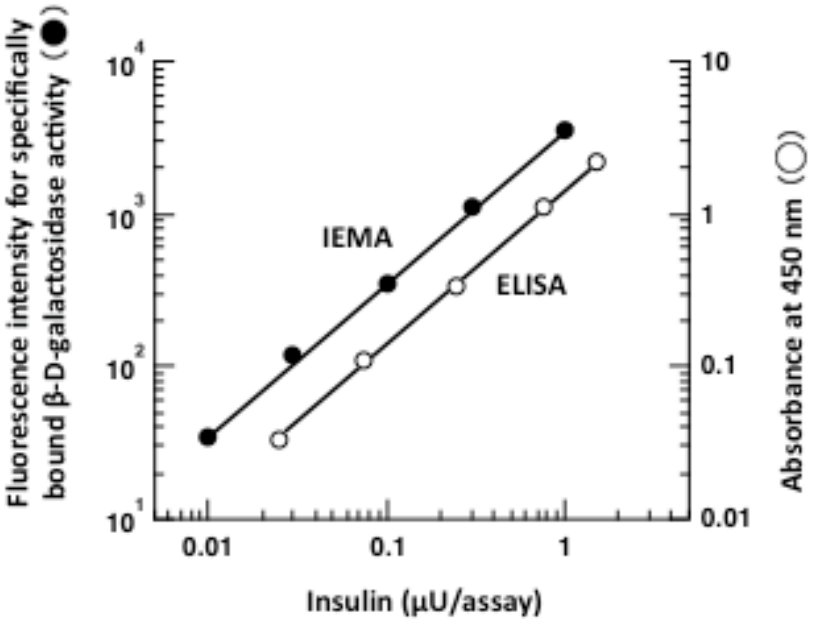
Statistical analysis was performed using SPSS version 20.0.0. Correlations were performed using Spearman's correlation coefficient. Differences between two and three groups were compared using the Mann-Whitney U test and the Kruskal-Wallis test, respectively.

3. Results

3.1 IEMA for insulin

To compare the newly developed ICT-EIA with other methods of insulin assay we performed an IEMA for insulin as outlined in Fig. 1. Insulin was simultaneously incubated with a mixture of a monoclonal (Mc) Ab-1 Fab' conjugated with DNP-biotinyl-BSA (Mc-DNP) and a monoclonal (Mc) Ab-2 Fab'-β-D-galactosidase conjugate (Mc-Gal), resulting in the formation of an immune complex consisting of all three components. This immune complex was then trapped onto a streptavidin-coated solid phase. The solid phase was washed to eliminate unbound conjugates and the bound β-D-galactosidase activity was measured fluorometrically.

Using this assay the detection limit for insulin was 0.01 μU, which was 3-fold lower than that of a conventional ELISA (Fig. 2).



The open symbols indicate the specifically bound peroxidase activity in the conventional ELISA. The closed symbols indicate the specifically bound β-D-galactosidase activity in the IEMA performed using monoclonal capture and enzyme-conjugated antibodies

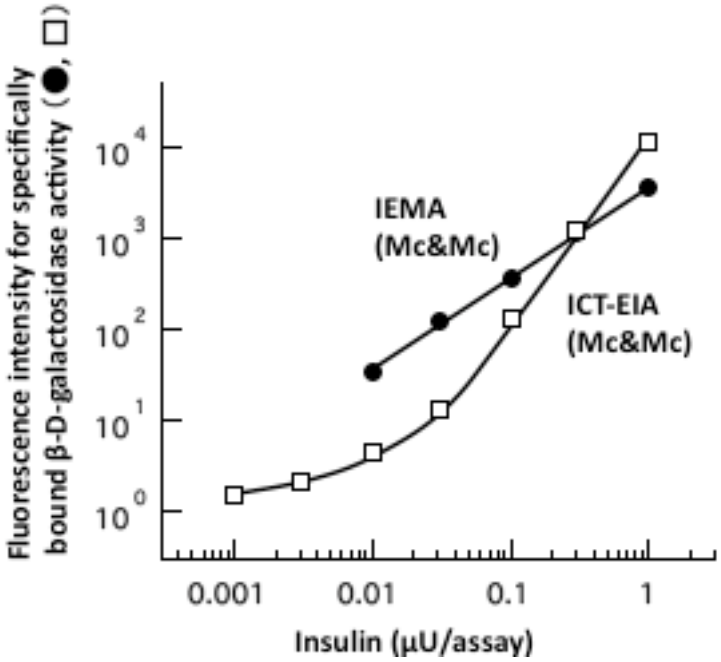
Fig. 2. Calibration curves for insulin assay using IEMA and ELISA.

3.2 ICT-EIA for insulin

The ICT-EIA for insulin was performed as outlined in Figure 1. Insulin was simultaneously incubated with a mixture of Mc-DNP and Mc-Gal, to form an immune complex as described

for the IEMA method. This immune complex was first trapped onto a solid phase coated with anti-DNP IgG. Subsequently, the solid phase was washed to eliminate unbound conjugates and the immune complex was then specifically eluted from this solid phase using DNP-Lys and transferred onto a second solid phase coated with streptavidin. Finally, the bound β-D-galactosidase activity was measured fluorometrically.

When the same two mouse monoclonal antibodies that were used for IEMA were used for ICT-EIA, the fluorescence signal from the bound β-D-galactosidase activity in the ICT-EIA was strongly decreased compared to that obtained in the IEMA at low insulin concentrations and was not linear with the lowest insulin concentrations. This effect resulted in a detection limit for insulin of 0.03 μU, which was 3-fold higher than that of the IEMA (Fig. 3). Similar results were obtained even if the high molecular weight β-D-galactosidase (540 kDa) enzyme with which the antibody was conjugated was exchanged for a lower molecular weight enzyme such as peroxidase (40 kDa) or alkaline phosphatase (120 kDa) (data not shown).

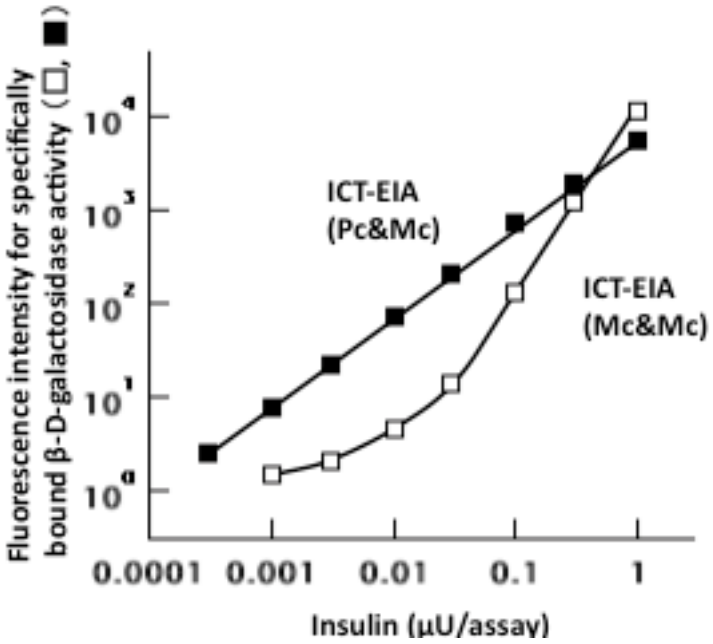


The closed symbols indicate the specifically bound β-D-galactosidase activity in the IEMA as described in Fig. 1 using monoclonal capture (Mc-DNP) and enzyme-conjugated (Mc-Gal) antibodies respectively. The open symbols indicate the specifically bound β-D-galactosidase activity in the ICT-EIA using the same two monoclonal antibodies that were used for IEMA.

Fig. 3. Calibration curves for insulin assay using IEMA and ICT-EIA.

We next tested the effect of using a combination of an enzyme labeled monoclonal mouse antibody (Mc-Gal) and a non-affinity purified polyclonal capture antibody (Pc-DNP) for

formation of the immune complex in ICT-EIA (Mc-Gal & Pc-DNP). The resulting fluorescence signal decreased linearly in accordance with insulin dilution. The detection limit for insulin was 0.0003 μU (12 fg; 1.8 amol) (Fig. 4), which was 30-fold lower than that of IEMA. When a polyclonal antibody was used for enzyme labeling and a monoclonal antibody for capture (Pc-Gal & Pc-DNP), or when both antibodies were the same polyclonal antibody, the insulin detection limit increased, but the linearity with insulin dilution was preserved (Fig. 5).

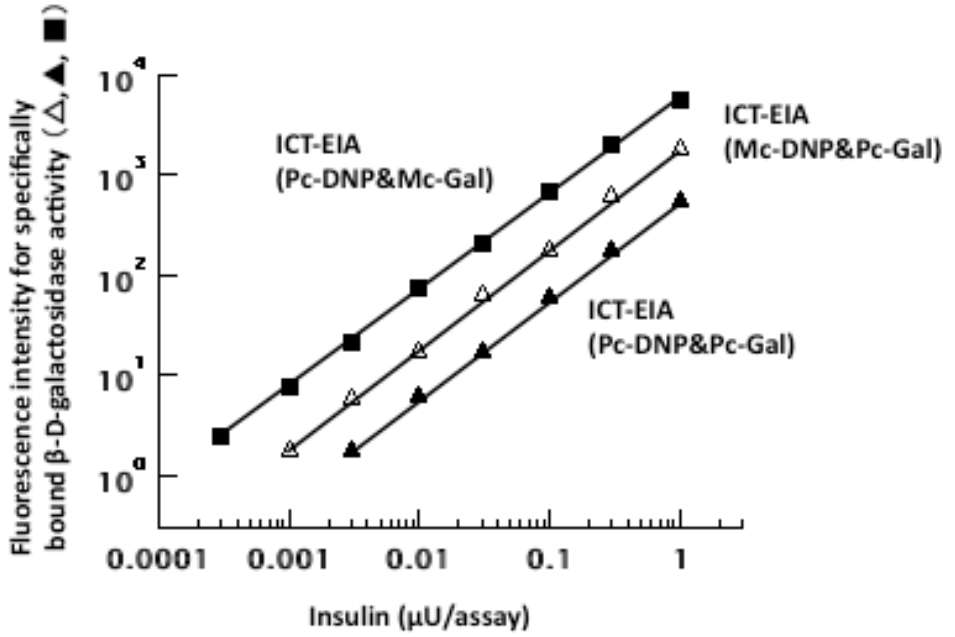


The open symbols indicate the specifically bound β-D-galactosidase activity in the ICT-EIA performed as described in Fig. 3. The closed symbols indicate the specifically bound β-D-galactosidase activity in the ICT-EIA in which a combination of a polyclonal capture antibody (Pc-DNP) and a monoclonal enzyme-labeled antibody (Mc-Gal) was used.

Fig. 4. Calibration curves for insulin assay using two different ICT-EIAs.

3.3 Improved ICT-EIA for insulin and limit of urinary insulin detection

Figure 5 shows typical calibration curves of the ICT-EIAs for insulin using various combinations of polyclonal/monoclonal capture and enzyme-conjugated antibodies. The detection limit and sensitivity for insulin using a polyclonal capture antibody and a monoclonal enzyme labeled antibody (Pc-DNP & Mc-Gal) was 0.0003 μU (0.3 nU) and 0.15 μU/ml of urine, respectively, using 2-μl samples for complete recovery of the added insulin. This detection limit was 100-fold lower than that of a sensitive ELISA and of IEMA using Mc-DNP & Mc-Gal (Fig.2 and 3). The assay range (CV < 10%) for insulin in urine was 0.5-150 μU/ml, using urine samples with a volume of 2 μl. Based on these data the ICT-EIA using a polyclonal capture antibody and a monoclonal enzyme labeled antibody was used for the remainder of the study.



The closed squares indicate the specifically bound β-D-galactosidase activity in the ICT-EIA in which a polyclonal capture antibody (Pc-DNP) and a monoclonal enzyme-labeled antibody (Mc-Gal) were used as described in Fig. 4. The open triangles indicate the specifically bound β-D-galactosidase activity in an ICT-EIA in which the polyclonal (Pc-Gal)/monoclonal antibody (Mc-DNP) combination was reversed. The closed triangles indicate the specifically bound β-D-galactosidase activity in an ICT-EIA in which the same polyclonal antibody was used for the capture and enzyme-labeled antibody (Pc-DNP & Pc-Gal).

Fig. 5. Calibration curves for insulin assay using three different ICT-EIAs.

3.4 Assay precision

The reproducibility of ICT-EIA for analysis of insulin in urine was estimated using two or three samples containing different concentrations of insulin (range, 0.5-22.5 μU/ml). The within-assay and between-assay CVs for insulin were 2.5-6.2% (n = 10) and 3.5-7.5% (n = 10), respectively.

3.5 Recovery and dilution tests

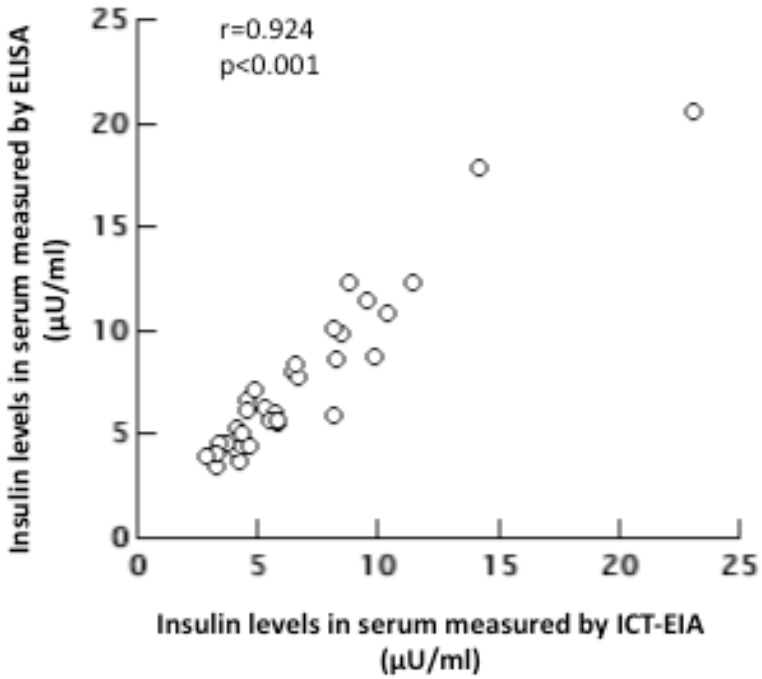
Analytical recovery of exogenously added insulin to 2-μl and 5-μl urine samples (range, 1.2-25.3 μU/ml) was 92.8-106% and 72.5-103%, respectively. Dilution curves for the urine samples paralleled those for standard insulin. Based on these data, 2 μl of urine was used for all experiments described below.

3.6 Specificity of ICT-EIA

The cross-reactivity of ICT-EIA for insulin and other hormones was evaluated. In the ICT-EIA for insulin, cross-reactions on a molar basis with hIGF-I, hCG and hGH were all <0.1%, indicating that ICT-EIA can specifically measure insulin concentrations.

3.7 Comparison with ELISA

Insulin levels measured in 35 serum samples (3.3-23.1 μU/ml) using ICT-EIA correlated well with those determined by ELISA ($r = 0.924$, $P < 0.001$) (Fig. 6).

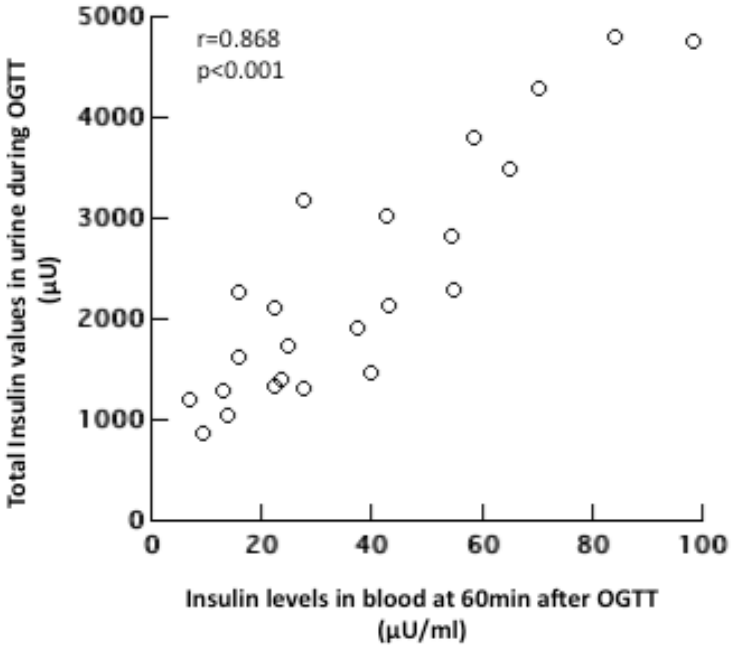


The regression equation and the calculated correlation coefficient ($n = 35$, $r = 0.924$, $P < 0.001$) are shown.

Fig. 6. Correlation between serum insulin levels measured by ELISA and by ICT-EIA.

3.8 Effects of increased blood glucose levels after OGTT on the insulin concentration in serum and urine

In order to determine whether the urinary insulin level reflects the blood insulin level, insulin levels in both serum and urine from non-diabetic healthy subjects were measured after OGTT using the ICT-EIA. Blood was collected at 0, 0.5, 1.0, 1.5 and 2.0 h, and urine was collected at 0 and 2 h after glucose ingestion. Blood glucose, serum insulin and insulin in the urine were measured (Table 2). Increases in the blood glucose level corresponded to significant increases in the blood insulin level, which were followed by increases in the urinary insulin level two hours later. The total quantity of insulin in urine was calculated based on the urine volume. A strong correlation was found between serum insulin levels at 60 min after glucose ingestion and total insulin values in urine during 2 h after ingestion ($r = 0.87$, $P < 0.001$), suggesting that measurement of insulin in urine may be useful for assessing the relative insulin levels in serum (Fig. 7).



Blood was collected at 1 h, and urine was collected at 0 and 2 h, after glucose ingestion. The regression equation and the calculated correlation coefficient (n = 23, r = 0.868, P < 0.001) are shown.

Fig. 7. Correlation between serum and urinary insulin levels after OGTT.

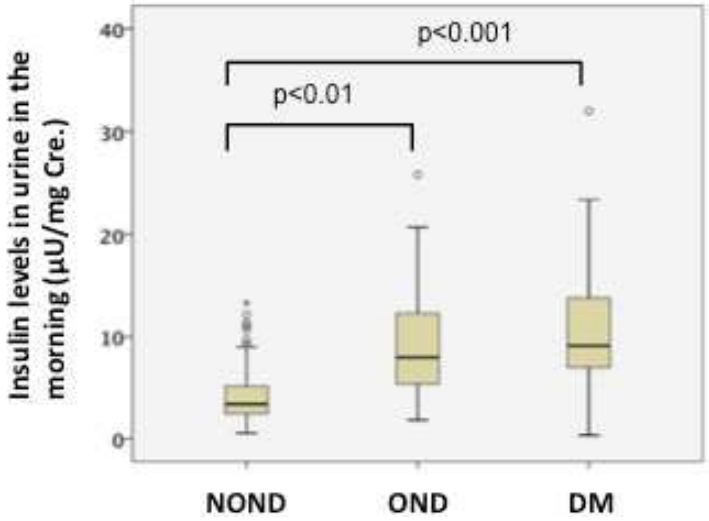
Serum and urine levels	Time after OGTT (h)		
	0	1	2
Blood glucose (mg/dl)	82 ± 11	135 ± 29*	118 ± 18*
Serum insulin (μU/ml)	3.1 ± 1.7	33.3 ± 25.9*	39.1 ± 45.0*
Urine insulin (μU/mg creatinine)	3.0 ± 1.9	-	15.2 ± 8.1*

*P < 0.01, compared with that at 0 h.
Urine: 0 h, before OGTT; 2 h, during 2 h after the OGTT.

Table 2. Concentration of glucose and insulin in serum, and of insulin in urine, in the oral glucose tolerance test (OGTT).

3.9 Measurement of insulin and resistin concentrations in urine

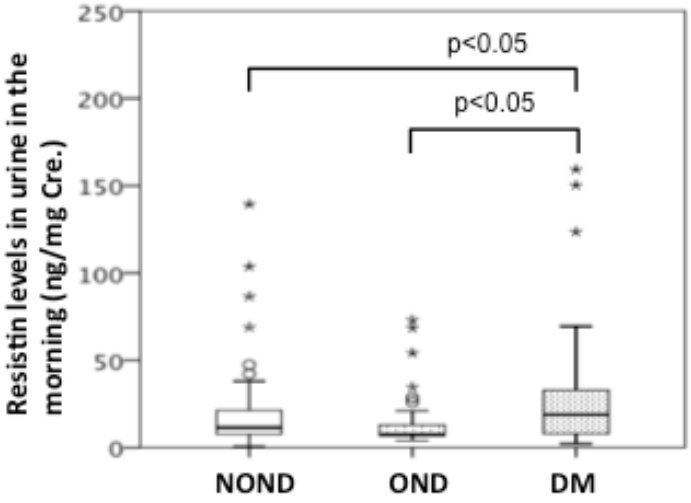
Urine samples were collected in the early morning from fasting NDNO and NDO subjects, and from fasting DM patients. Urinary insulin was measured by ICT-EIA and expressed in terms of μU/mg creatinine. The insulin concentrations determined in urine from NDNO and NDO subjects and from DM patients were 4.3 ± 2.8, 9.0± 5.5 and 13.9 ± 17.6 μU/mg creatinine, respectively (Fig. 8); the insulin concentration was significantly higher in DM patients than in NDNO subjects (P < 0.001).



Urinary insulin levels are expressed as a boxplot (box-and-whisker diagram); scale bars indicate sample minimum and maximum, boxes indicate lower quartile, median and upper quartile. DNO (n = 106), NDO (n = 35) and DM (n = 42). Insulin levels are expressed relative to urinary creatinine (Cre).

Fig. 8. Insulin levels in the urine of NDNO subjects, NDO subjects and DM patients.

Resistin concentrations were also measured in urine from NDNO and NDO subjects and from DM patients and were determined to be 18.6 ± 20.9, 15.4 ± 17.2 and 30.0 ± 36.2 ng/mg creatinine, respectively (Fig. 9); the resistin concentration in DM subjects was significantly higher than in NDO and NDNO subjects ($P < 0.05$) (Fig. 9).



Urinary resistin levels are expressed as a boxplot similar to that described in Fig. 8.

Fig. 9. Resistin levels in the urine of NDNO and NDO subjects and of DM patients.

3.10 Correlation of insulin level to resistin levels in urine

We analyzed the correlation of urinary insulin level to urinary resistin level in 42 DM patients. The urinary insulin level was significantly and positively correlated to urinary levels of resistin ($r = 0.426$, $P < 0.001$) (Fig. 10).

4. Discussion

We developed a new ultra-sensitive technique for the measurement of insulin by employing a non-competitive two-site binding method that utilizes two different types of anti-insulin antibodies and an immune-complex transfer method (ICT-EIA). This new method can measure insulin levels as low as 0.3 nU (12 fg; 1.8 amol), with a sensitivity of 0.15 μU/ml, using 2-μl urine samples. This assay is 100-fold more sensitive than a sensitive ELISA.

Various kinds of peptides can be measured at attomole levels by a non-competitive two-site enzyme immunoassay (ICT-EIA). For this purpose, the peptide molecules to be measured have to have two or more epitopes that are sufficiently separated from each other to allow simultaneous binding of two antibody molecules. A previous study using a single chain peptide provided a hint as to the distance required between epitopes for simultaneous binding of two antibodies. Human α-atrial natriuretic peptide (α-hANP) is a single chain polypeptide consisting of 28 amino acids with a ring structure that is formed by an intramolecular disulfide bound [Hashida, 1988b]. Ten amol of α-hANP were measured by a two-site enzyme immunoassay using a peroxidase-labeled antibody Fab' against the C-terminus of the peptide and a solid phase coated with IgG against the N-terminal half of the ring structure. The distance between the two epitopes recognized by the two antibody molecules in this single chain peptide appeared to correspond to 12-15 amino acids.

Since most polyclonal antibodies used in assays are now being replaced with monoclonal antibodies, a non-competitive two-site binding assay using two kinds of monoclonal antibody has recently been developed. When two kinds of monoclonal antibody were used in the ICT-EIA method, it was possible to measure most proteins at concentrations of less than attomole levels [Hashida, 1988b, 1990 & 1995].

However, it has not been possible to measure peptides, insulin, CRH, α-ANP, adrenomedullin or PAMP at less than attomole levels with an ultra-sensitive assay (ICT-EIA) using two monoclonal antibodies [Hashida, 2004; Katakami, 2002; Yamaga, 2003]. The major reason for this failure appeared to be due more to a decreased signal rather than because of dilution degree. Two factors have been considered that might contribute to this phenomenon. One factor is insufficient antibody affinity and the other is steric hindrance within an immune complex consisting of two antibodies (each of 50 kDa) and a peptide (6 kDa). Both of these factors would contribute to weak binding of the antibodies to the peptide in the immune complex resulting in dissociation of the peptide from antibody during the transfer from the first to the second solid phase.

We tested a number of combinations of ten different monoclonal anti-insulin antibodies, which varied in affinity and epitope, in the development of the ICT-EIA for insulin, but the results of all of the assays were the same (data not shown). We further examined the study due to the multi-antibody which several kinds of different monoclonal antibodies to insulin

were caused by, but the results of these assays were again the same (data not shown). When the same polyclonal antibody, which was not insulin affinity-purified, was used as both the capture and the enzyme labeled antibody for ICT-EIA, a linear standard curve in accordance with insulin dilution was obtained, but the assay was of lower sensitivity than the other ICT-EIA assays (Fig. 5). Subsequently, a combination of one monoclonal and one non-affinity purified polyclonal antibody to insulin was used and, in this assay, the detection limit of insulin was 1.8 amol and a linear standard curve in accordance with insulin dilution was obtained.

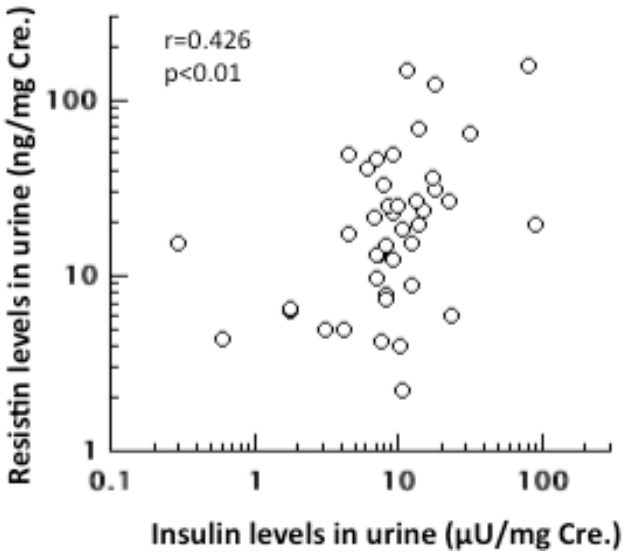
When an affinity-purified polyclonal rabbit antibody was used in an ICT-EIA for assay of ferritin (450 kDa), 1 zmol (0.001 amol; 1×10^{-21} moles) of ferritin was detected [Hashida, 1990]. The average number of β-D-galactosidase labeled antibody Fab' molecules bound per ferritin molecule was calculated to be 1.0-1.4. In addition, the detection limit of the β-D-galactosidase activity was 1 zmol. On the other hand, the average number of bound enzyme-labeled antibody molecules per peptide molecule was calculated to be 0.001-0.01. Therefore, the peptide detection limit of this immunoassay was estimated to be between 0.1-1 attomoles. Thus, it is likely that detection of 0.1 amol (0.02 μU) of a insulin will be possible if an affinity-purified antibody is used.

As described in the " Introduction ", blood samples have to be collected every 30 min for 2 h to assess the secretion of insulin in the OGTT. If the urinary insulin level reflects the serum insulin level, then the secretion of insulin after glucose ingestion may be easily assessed by measurement of urinary insulin levels. In the ICT-EIA of this study, the urinary insulin levels correlated well with the serum insulin levels at 1 h after glucose ingestion (Fig. 7). Thus, the level of insulin in urine appears to reflect the level in serum suggesting that measurement of insulin in urine may be useful for assessing insulin secretion.

A comparison of the insulin levels in urine collected early in the morning from NDNO and NDO subjects and from DM patients showed that the levels were significantly higher in NDO subjects and in DM patients than in NDNO subjects ($P < 0.01$ and $P < 0.001$ respectively) (Fig. 8). These results indicate that an increase in insulin levels may be a clinically important biomarker of obesity and diabetes risk.

We previously developed a highly sensitive ELISA for urinary growth hormone (GH) [Hashida, 1987, Sukegawa, 1988], which showed that assay of GH during nocturia was useful as a supporting diagnostic index of the pituitary dwarf. We have also recently developed an ultra-sensitive ICT-EIA for urinary soluble human insulin receptor ectodomain (sIR) and found that urinary sIR levels correlated well with sIR blood levels [Umehara, 2009]. Furthermore, the levels of urinary sIR in DM patients were significantly higher than those in NDO subjects and DM patients. Therefore, urinary biomarkers can be useful for both physiological and clinical studies.

In this study, urine samples with micro- and macro-albuminuria were excluded. Urinary insulin and resistin levels were expressed as ratios of urinary concentration to milligrams of urinary creatinine. Urinary insulin levels correlated well with urinary resistin levels ($r = 0.426$, $P < 0.01$) (Fig. 10). Further studies with larger numbers of patients will be needed to determine the cut-off value and criteria for insulin as a diabetic risk biomarker.



Insulin and resistin levels in the urine of 42 DM patients were measured and are expressed relative to urinary creatinine (Cre). The regression equation and the calculated correlation coefficient ($r = 0.426$, $P < 0.01$) are shown.

Fig. 10. Correlation between urinary insulin and resistin levels.

In summary, we have presented a new ultra-sensitive enzyme immunoassay for insulin, the ICT-EIA. This ICT-EIA can specifically measure insulin in urine, without any requirement for sample extraction or concentration. Urinary insulin and resistin levels were increased in patients with diabetes. ICT-EIA may be useful for both physiological and clinical studies of diabetic risk and urinary insulin may be used as a non-invasive maker that is relative to serum insulin. Therefore, measurement of urinary insulin levels will be useful in studies on dietary counseling and exercise guidance for patients with diabetes.

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6. References

Aikawa T, Suzuki S, Murayama M, Hashiba K, Kitagawa T, Ishikawa E. (1979). Enzyme immunoassay of angiotensin I. *Endocrinology*, 105, pp1-6

Fyhrquist F, Soveri P, Puutula L, Stenman U-H. (1976). Radioimmunoassay of plasma renin activity. *Clinical Chemistry*, 22, pp250-256

Glänzer K, Appenheimer M, Krück F, Vetter W, Vetter H. (1984). Measurement of 8-arginine-vasopressin by radioimmunoassay: development and application to urine

and plasma samples using one extraction method. *Acta endocrinologica*, 106, pp317-329

Hashida S, Ishikawa E, Kato Y, Imura H, Mohri Z, Murakami Y. (1987). Human growth hormone (hGH) in urine and its correlation to serum hGH examined by a highly sensitive sandwich enzyme immunoassay. *Clinica chimica acta*, 162, pp229-235

Hashida S, Ishikawa E, Mukoyama M, Nakao K, Imura H. (1988a). Direct measurement of a-human atrial natriuretic polypeptide in plasma by sensitive enzyme immunoassay. *Journal of Clinical Laboratory Analysis*, 2, pp161-167

Hashida S, Tanaka K, Kohno T, Ishikawa E. (1988b). Novel and ultrasensitive sandwich enzyme immunoassay (sandwich transfer enzyme immunoassay) for antigens. *Analytical Letters*, 21, pp1141-1154

Hashida S, Ishikawa E. (1990). Detection of one milliattomole of ferritin by novel and ultrasensitive enzyme immunoassay. *Journal of Biochemistry*, 108, pp960-964

Hashida S, Ishikawa E, Mukoyama M, Nakao K, Imura H. (1991). Highly sensitive two-site enzyme immunoassays for human atrial natriuretic polypeptides. in Atrial and Brain Natriuretic Peptides , *Proceedings of the Kyoto Symposium on ANP (1988),* pp. 97-119, Kodansha Scientific Ltd., Tokyo, Japan

Hashida S, Tanaka K, Yamamoto N, Ishikawa E. (1993). Ultrasensitive enzyme immunoassay for peptide. *Methods in neurotransmitter and neuropeptide research*, pp. 279-304, Elsevier Science Publishers B.V., Amsterdam, Netherlands.

Hashida S, Hashinaka K, Ishikawa E. (1995). Ultrasensitive enzyme immunoassay. *Biotechnology Annual Review Vol.1*, pp. 403-451, Elsevier Science Publishers B.V., Amsterdam, Netherlands.

Hashida S, Kitamura K, Nagatomo Y, Shibata Y, Imamura T, Yamada K, Fujimoto S, Kato J, Morishita K, Eto T. (2004). Development of an ultra-sensitive enzyme immunoassay for human proadrenomedullin N-terminal 20 peptide (PAMP) and direct measurement of two molecular forms of PAMP in plasma from healthy subjects and patients with cardiovascular disease. *Clinical Biochemistry*, 37, pp14-21

Ishikawa E, Imagawa M, Hashida S. (1983a). Ultrasensitive enzyme immunoassay using fluorogenic, luminogenic, radioactive and related substrates and factors to limit the sensitivity. *Developments in Immunology*, 18, pp219-232

Ishikawa E, Imagawa M, Hashida S, Yoshitake S, Hamaguchi Y, Ueno T. (1983b). Enzyme-labeling of antibodies and their fragments for enzyme immunoassay and immunohistochemical staining. *Journal of Immunoassay*, 4, pp209-227

Ishikawa E, Hashida S, Tanaka K, Kohno T. (1989). Ultrasensitive enzyme immunoassay for antigens: technology and applications — a review. *Clinical Chemistry and Enzymology Communications*, 1, pp199-215

Katakami H, Hashida S, Oki Y, Murakami O, Ikenoue T, Ono H, Matsukura S. (2002). Immunoreactive corticotropin-releasing hormone (CRH) in plasma and hypothalamic incubation media as assessed by a novel and highly sensitive immune complex transfer EIA. *Clincal Pediatrics Endocrinology*, 11(Suppl 17), pp93-97

Lindström T, Hedman CA, Arnqvist HJ. (2002). Use of a novel double-antibody technique to describe the pharmacokinetics of rapid-acting insulin analogs. *Diabetes Care*, 25, pp1049-1054

Morton JJ, Padfield PL, Forsling ML. (1975). A radioimmunoassay for plasma arginine-vasopressin in man and dog: application to physiological and pathological states. *Journal Endocrinology*, 65, pp411-424

Mukoyama M, Nakao K, Yamada T, Itoh H, Sugawara A, Saito Y, Arai H, Hosoda K, Shirakami G, Morii N, Shiono S, Imura H. (1988). A monoclonal antibody against N-terminus of a-atrial natriuretic polypeptide (a-ANP): a useful tool for preferential detection of naturally circulating ANP. *Biochemical and Biophysical Research Communications*, 151, pp1277-1284

Scharpé S, Verkerk R, Sasmito E, Theeuws M. (1987). Enzyme immunoassay of angiotensin I and renin. *Clinical Chemistry*, 33, pp1774-1777

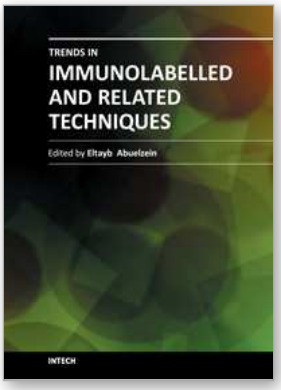
Sukegawa I, Hizuka N, Takano K, Asakawa K, Horikawa R, Hashida S, Ishikawa E, Mohri Z, Murakami Y, Shimizu K. (1988). Urinary growth hormone (GH) measurements are useful for evaluating endogenous GH secretion. *The Journal of clinical endocrinology and metabolism*, 66, pp1119-1123

Tikkanen I, Fyhrquist F, Metsärinne K, Leidenius R. (1985). Plasma atrial natriuretic peptide in cardiac disease and during infusion in healthy volunteers. *Lancet*, 2, 66-69

Umehara A, Nishioka M, Obata T, Ebina Y, Shiota H, Hashida S. (2009). A novel ultra-sensitive enzyme immunoassay for soluble human insulin receptor ectodomain and its measurement in urine from healthy subjects and patients with diabetes mellitus. *Clinical Biochemistry*, 42, pp1468-1475

Uno T, Uehara K, Motomatsu K, Ishikawa E, Kato K. (1982). Enzyme immunoassay for arginine vasopressin. *Experientia*, 38, pp786-787

Yamaga J, Hashida S, Kitamura K, Tokashiki M, Aoki T, Inatsu H, Ishikawa N, Kangawa K, Morishita K, Eto T. (2003). Direct measurement of glycine-extended adrenomedullin in plasma and tissue using an ultrasensitive immune complex transfer enzyme immunoassay in rat. *Hypertension research*, 26, pp45-53.



Trends in Immunolabelled and Related Techniques

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The book is coined to provide a professional insight into the different trends of immunoassay and related techniques. It encompasses 22 chapters which are grouped into two sections. The first section consists of articles dealing with emerging uni-and-multiplex immunolabelled methods employed in the various areas of research. The second section includes review articles which introduce the researchers to some immunolabelled techniques which are of vital significance such as the use of the conjugates of the Staphylococcus aureus protein "A" and the Streptococcus Spps. protein "G" in immunolabelled assay systems, the use of bead-based assays and an overview on the laboratory assay systems. The book provides technological innovations that are expected to provide an efficient channel for developments in immunolabelled and related techniques. It is also most useful for researchers and post-graduate students, in all fields, where immunolabelled techniques are applicable.

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