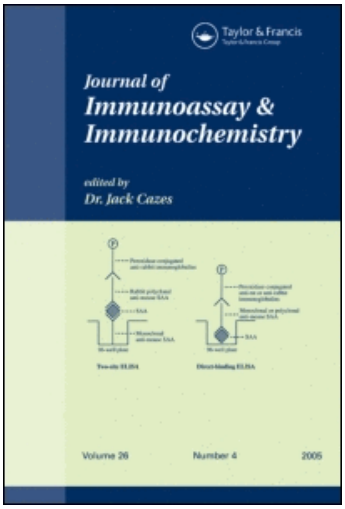

Journal of Immunoassay and Immunochemistry

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

Measurement of Free Insulin-Like Growth Factor-I Using Immunoradiometric Assay

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To cite this Article Takada, M. , Nakanome, H. , Kishida, M. , Hirose, S. , Hasegawa, T. and Hasegawa, Y.(1994) 'Measurement of Free Insulin-Like Growth Factor-I Using Immunoradiometric Assay', Journal of Immunoassay and Immunochemistry, 15: 3, 263 — 276
To link to this Article: DOI: 10.1080/15321819408009577
URL: http://dx.doi.org/10.1080/15321819408009577

JOURNAL OF IMMUNOASSAY, 15(3), 263-276 (1994)

MEASUREMENT OF FREE INSULIN-LIKE GROWTH FACTOR-I USING IMMUNORADIOMETRIC ASSAY

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ABSTRACT

The free Insulin-like growth factor-I (IGF-I) in plasma from normal adults was directly measured with a newly developed highly sensitive immunoradiometric assay (IRMA) for IGF-I. The capture antibody did not crossreact with IGF-I associated binding proteins which exist in plasma, and the assay was designed not to shift the equilibrium of the IGF-I and binding proteins.

Total IGF-I concentration was measured using this assay with preliminary acid-ethanol extraction. Approximately 1 percent of total IGF-I existed in the free form.

Gel filtration of plasma was also used to separate the free IGF-I from its bound form. The free/total ratio of IGF-I as determined by gel filtration was similar to that determined directly by IRMA with and without acid-ethanol extraction.

(KEY WORDS: insulin-like growth factor-I, free-form, binding protein, immunoradiometric assay, Western immunoblot)

INTRODUCTION

Insulin-like growth factor-I (IGF-I) is the main mediator of the action of growth hormone (GH) on somatic growth. The clinical significance of total serum IGF-I measurement has been demonstrated (1,2). IGF-I and insulin-like growth factor-II (IGF-II) are known to have at least six binding proteins (IGFBP-1~6) (3,4,5,6), with IGFBP-3 preponderating in plasma (7,8). It is thought that the free IGF-I reflects biological activity. There are few methods that provide information on the free IGF-I in plasma (9,10), and no analytical method has enough sensitivity to determine free IGF-I directly.

In this report, we describe a highly sensitive immunoradiometric assay (IRMA) for the measurement of free IGF-I that uses two monoclonal antibodies. We used an immobilized monoclonal antibody which did not crossreact with the IGF-I-IGFBPs complex as the capture antibody, with conditions designed not to disturb the equilibrium of the IGF-I and IGFBPs.

Free IGF-I in plasma from normal adults was compared with total IGF-I concentration as measured after acid-ethanol extraction. Gel filtration of plasma was also performed prior to assay (9).

MATERIALS AND METHODS

Materials

Recombinant IGF-I was purchased from Toyobo Co., Ltd. (Osaka, Japan) and

the concentration was calibrated by the international standard (WHO 87-518). Insulin-like growth factor-Ⅱ (IGF-Ⅱ) was obtained from Peptide Institute, Inc. (Osaka, Japan). IGF-I(24-41) was obtained from Peninsula Laboratories, Inc. (California). Proinsulin was obtained from Sigma Chemical Company (St.Louis). Superose 12 and Sephadex G-75 were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). The ECL Western blotting system was purchased from Amersham International (Buckinghamshire, England).

Collection of Plasma

Blood samples were obtained from 9 adult volunteers with no previous history of diseases. Samples were collected into ice-chilled tubes containing 1.0 mg EDTA per 1 ml of blood. Samples were centrifuged immediately and the plasma was stored at -40℃.

Characterization of Antibodies

In this study, an IRMA was designed to produce an immune complex of capture antibody, IGF-I and indicator antibody. Two monoclonal antibodies were characterized by RIA using IGF-I, IGF-Ⅱ, proinsulin and IGF-I(24-41). The RIA procedure used was as follows : to each incubation tube, monoclonal antibody solution (0.1 ml), peptide (0.1 ml) and ^{125}I-IGF-I (approximately 20,000 cpm; 0.1 ml) were mixed, the mixture was incubated for 20 hours at 4℃. Diluted

goat anti-mouse γ-globulin serum (0.1 ml), diluted normal mouse serum (0.1 ml) and 12.5% polyethylene glycol (PEG6000) (0.5 ml) were added. After incubation for 30 min at 4℃, the mixture was centrifuged at 2,000 rpm for 20 min at 4℃. The supernatant was aspirated, and the radioactivity of the precipitate was counted with a γ-counter. All samples were analyzed in duplicate.

Western Immunoblotting of Plasma

The crossreactivity of anti-IGF-I antibodies (39-11 and 39-12) to the IGF-I-IGFBPs complex was elucidated by Western immunoblotting method using commercially available reagents (Amersham). Plasma was electrophoresed and electroblotted onto a nitrocellulose membrane. Then the membrane was treated with unlabelled IGF-I. After washing, antibody (39-11) or antibody (39-12) was added as primary antibody. HRP labelled anti-mouse immunoglobulin and detection reagents were added according to the standard procedure.

Iodination of Monoclonal Antibody

Antibody (39-12) was dialyzed against phosphate buffer, pH 7.5, 500 mmol/L. Then the antibody was iodinated by the chloramine-T method (11) and the resulting labelled antibody was applied to a Superose 12 column (10x300 mm), and eluted with Tris-HCl buffer, pH 8.5, 100mmol/L. Specific activity of the iodinated antibody was 200 kBq/μg.

Preparation of Monoclonal Antibody (clone number 39–11) Immobilized on Polystyrene Beads

Antibody (39–11) was immobilized on polystyrene beads by a modification of the method of Belanger and coworkers (12). The monoclonal antibody (5 mg) dissolved in 300 ml carbonate buffer, pH 9.5, 100mmol/L was mixed with 1,000 polystyrene beads. After 20 hours at 20°C, the buffer was removed and the beads washed with phosphate buffer, pH 7.2, 100mmol/L, and then soaked in phosphate buffer, pH 7.2, 100mmol/L, containing 0.1% (w/v) bovine serum albumin (BSA) and 0.02% (w/v) sodium azide.

Direct assay for free IGF–I

IGF–I standard sample or plasma (0.2 ml) was added to each tube, and incubation for 30 min at 37°C. A capture antibody (39–11) coated bead was added, and incubated for 5 min at 37°C. The time–course of this reaction was followed previously (Figure 1). The bead was washed 2 times with distilled water. The indicator antibody (39–12), (approximately 200,000 cpm; 0.2 ml) was added, and the mixture incubated at room temperature for 3 hours with gentle shaking. The bead was washed 2 times with saline containing 0.1% (v/v) Triton X–100 and 0.02% sodium azide. The radioactivity of the bead was counted with a γ –counter. All standards and samples were analyzed in duplicate.

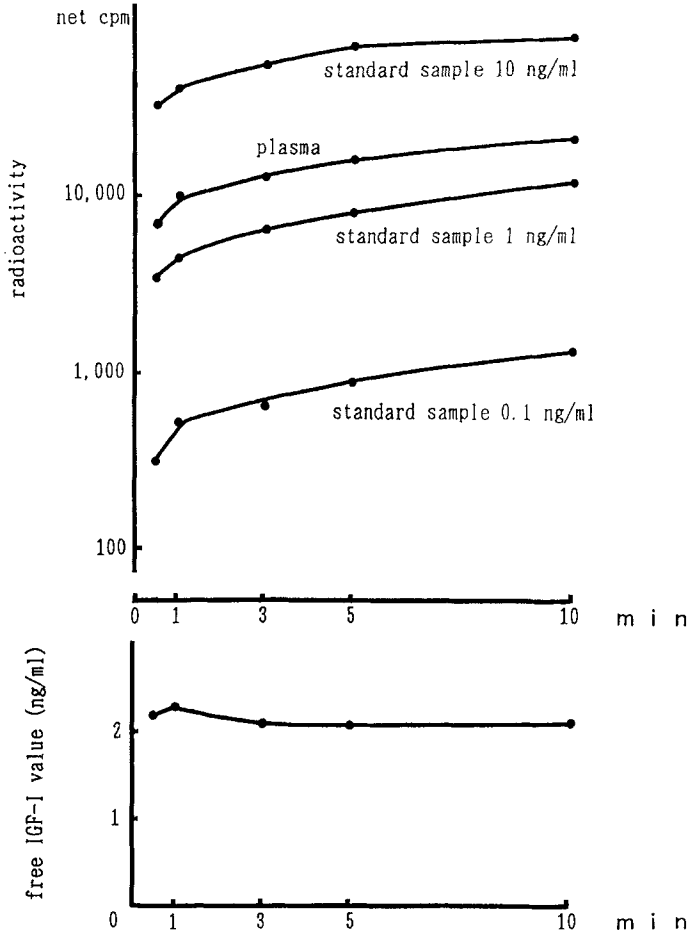

FIGURE 1. The time course of the free IGF-I value. Within 10 min of the first incubation, the radioactivity of the beads increase with time not only standard sample but also plasma. Free IGF-I value of plasma is stable.

Two-site IRMA for total IGF-I

To measure total IGF-I concentration in plasma, IGF-I was extracted with acid-ethanol by a modification of the method of Daughaday (13). In a 12x75 mm polypropylene tube, IGF-I standard or acid-ethanol extract (25 μl) was mixed with 0.3 ml labelled indicator antibody. Then the antibody coated bead was added to the mixture and incubated at room temperature for 2 hours with gentle shaking. The bead was washed 2 times with distilled water. The radioactivity of the bead was determined with a γ-counter. All standards and unknown samples were analyzed in duplicate. This assay could detect 300 pg/ml to 100,000 pg/ml.

Gel Filtration of Plasma

A 15x930 mm Sephadex G-75 column was equilibrated with sodium phosphate buffer, pH 7.5, 10 mmol/L, containing sodium chloride, 10 mmol/L, BSA, 0.025% (w/v), Tween 20, 0.025% (v/v), and sodium azide, 0.02% (w/v). The column was calibrated using ^{125}I-IGF-I and plasma. Plasma was applied to the column and eluted with phosphate buffer at a rate of 0.4 ml/min. Fractions of IGF-I-IGFBPs complex (P1 and P2) and the free IGF-I (P3) were collected. Each fraction was lyophilized.

The IGF-I concentrations in P1 and P2 were measured after acid-ethanol extraction by using the IRMA for total IGF-I. P3 was dissolved in distilled water and assayed for IGF-I immunoreactivity without an extraction process.

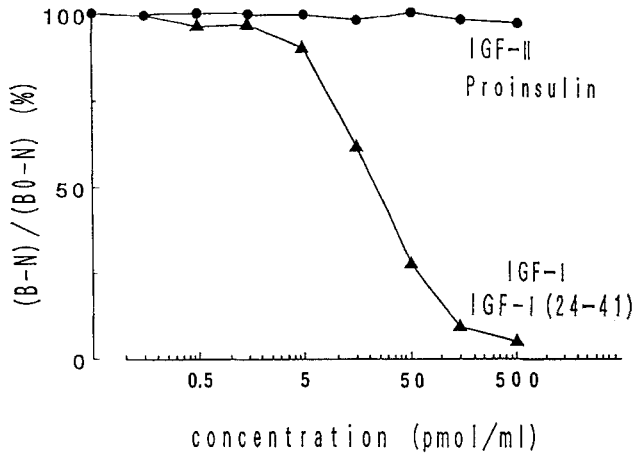

FIGURE 2. IGF-I RIA. Displacement curves of ^{125}I-IGF-I by IGF-I related peptides are shown. The capture antibody (39-11) recognizes IGF-I(24-41), which is the specific region of IGF-I. Intact IGF-I is also recognized. IGF-II and proinsulin do not crossreact with the antibody (39-11)

RESULT AND DISCUSSION

We set the IRMA for free IGF-I, not to disturb the equilibrium of IGF-I and IGFBPs. Samples were not diluted and temperature was maintained at 37℃. Furthermore, the incubation time of the first step was short. Within 10 min of the first incubation, the free IGF-I values were stable (Figure 1), suggesting the binding of the capture antibody to free IGF-I did not disturb the equilibrium of IGF-I and IGFBPs within 10 min. More than 1 hour of the first incubation resulted in an increment of free IGF-I levels (data not shown).

The capture antibody has high specificity for IGF-I and does not crossreact with IGF-I associated IGFBPs. Figure 2 shows the specificity of the capture

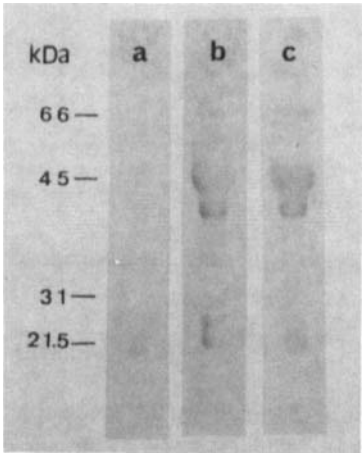

FIGURE 3. Western immunoblot of diluted normal adult plasma.
Plasma was electrophoresed and electroblotted onto nitrocellulose membrane. Lane **a** and **b** are treated with IGF-I. Then Lane **a** is treated with anti-IGF-I antibody (39-11). Lane **b** is treated with anti-IGF-I antibody (39-12). Lane **c** is treated with anti-IGFBP-3 antiserum. The capture antibody (39-11) does not recognize IGFBPs associated IGF-I.

antibody (39-11), which recognizes IGF-I(24-41), the specific region of IGF-I. The total crossreactivity of this IRMA to IGF-II is less than 0.03% (data not shown). Since Western ligand blot can detect IGFBP-1,2,3 and probably IGFBP-4 (7,14,15), the capture antibody (39-11) did not crossreact with IGF-I associated IGFBP-1,2,3 and 4 (Figure 3). We also proved the capture antibody did not crossreact with DSS-linked IGF-I-recombinant IGFBP-3 complex (data not shown).

Figure 4 shows the standard curve of the IRMA for free IGF-I. The procedure for free IGF-I can detect 30 pg/ml to 10,000 pg/ml. The intra- and interassay CV

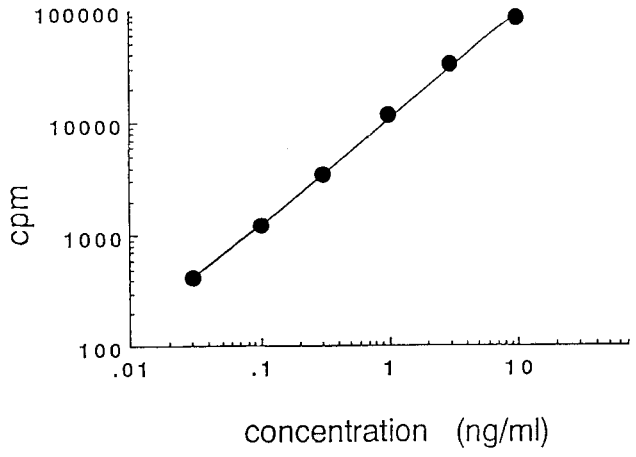

FIGURE 4. Typical standard curve of IRMA for free IGF-I.
Direct assay for free IGF-I exhibits linearity from approximately 0.03–10 ng/ml of IGF-I. Assay for total IGF-I follows 0.3 to 100 ng/ml (data not shown).

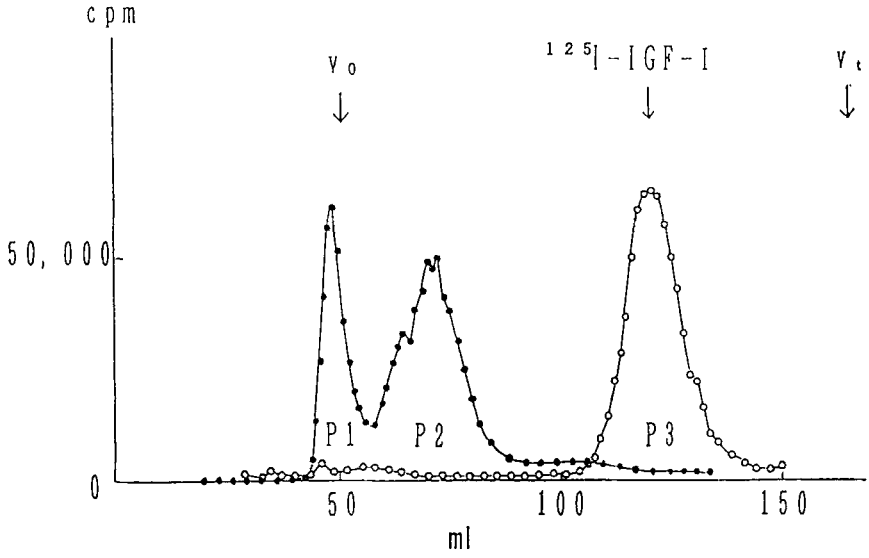

FIGURE 5. The elution profiles of ^{125}I-IGF-I (○) and the complex of ^{125}I-IGF-I and binding proteins (●). A Sephadex G-75 column was equilibrated with neutral buffer. Most ^{125}I-IGF-I bound to plasma binding proteins after coincubation with plasma. After calibration, plasma was applied to the column and eluted. Fractions of the IGF-I-IGFBPs complex (P1 and P2) and the free-form IGF-I (P3) were collected and measured.

TABLE 1

TOTAL AND FREE-FORM IGF-I IN NORMAL PLASMA FROM 9 ADULT VOLUNTEERS

	Total IGF-I (ng/ml)	Free form (ng/ml)	Free/Total (%)
A	146	0.8	0.55
B	183	2.5	1.37
C	172	1.1	0.61
D	115	1.4	1.25
E	201	1.1	0.54
F	374	1.4	0.39
G	197	0.7	0.35
H	116	0.9	0.81
I	227	1.3	0.56
mean	192	1.3	0.71
S.D.	78.1	0.54	0.36

of the assay for free IGF-I were less than 15%. These data suggest the IRMA for free IGF-I is accurate and reliable in a wide range of concentrations.

To validate the direct IRMA for free IGF-I, plasma sample was gel-filtered and IGF-I levels were measured. Each fraction (P1,2,3 in Figure 5) was pooled and lyophilized. P1 and P2 fractions were reconstituted and the IGF-I levels were measured after acid-ethanol extraction. P3 fraction was reconstituted and free IGF-I levels were measured without acid-ethanol extraction. These levels of the

free IGF-I and total IGF-I were compared with those measured without gel-filtration. The free and total IGF-I concentration obtained with gel-filtration were 2.0 ng/ml and 285 ng/ml, respectively. Those levels measured without gel-filtration were 2.1 ng/ml and 315 ng/ml. These data suggest that this IRMA is reliable for the evaluation of free IGF-I.

Finally, we measured free IGF-I and total IGF-I levels of 9 normal adult volunteers (Table 1). approximately 1 percent of total IGF-I was detected as the free form.

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