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Applicability of short-lived radiometallic nuclide for high sensitivity two-site "sandwich" immunoradiometric assay: Human growth hormone assay

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The sensitivity of the IRMA method is limited by the specific activity (SA) of the conventionally employed radioisotopic label and high sensitivity radioimmunoassay should theoretically be attained by the use of short-lived radiometallic nuclides. Our group have achieved radiolabeling of high SA IgG by using the radiometal, gallium-67 (^{67}Ga) with a short half-life ($T^1/_2$ = 78 h) and deferoxamine (DF), a bifunctional chelating agent bound through a multispacer (dialdehyde starch, DAS) as the linker (*J Nucl Med* 32: 825, 1991). In the present work, the application of the approach is attempted by employing a two-site IRMA for human growth hormone (hGH); the monoclonal antibody to hGH (MAB2) is bound to DF via DAS and the coupled DF-DAS-MAB2 is radiolabeled with ^{67}Ga. The ^{67}Ga-DF-DAS-MAB2 of high SA (4,884 MBq/mg versus 370–518 MBq/mg calculated for radioiodinated MAB2) was thus used for the two site 'sandwich' ^{67}Ga-IRMA. Excellent correlation with the ^{125}I-IRMA was registered, and higher detection capability obtained by using ^{67}Ga over the ^{125}I in the hGH IRMA offered a good basis for the exploitation of short-lived radio-nuclides in the IRMA system.

Key words: high specific activity radiolabeled antibody, short half-life radiometal nuclide IRMA, ^{67}Ga-IRMA, high sensitivity hGH assay, radioactive waste disposal

INTRODUCTION

IT HAS OFTEN BEEN REPORTED that the high sensitivity of the immunoradiometric assay (IRMA) method is limited by the specific activity of the conventionally employed radioisotopic labels such as ^{125}I.[1] In our laboratory, the labeling of high specific activity IgG has been achieved,[2,3] by using deferoxamine (DF), a bifunctional chelating agent bound via a multi-site spacer (dialdehyde starch, DAS) to the protein as a means to increase the number of DF molecules per IgG molecule. The IgG-coupled DF was then labeled with the radiometal, gallium-67 (^{67}Ga) with a short half-life ($T^1/_2$ = 78 hr). In the present work, the enhancement of the assay sensitivity through the use of high specific activity ^{67}Ga labeled antibody is attempted with a two-site IRMA system for hGH and its performance compared with the radioiodinated IRMA. Attainment of a hGH assay with improved sensitivity may be of clinical interest, i.e. follow-up of growth hormone therapy in elderly people.[4] The data gathered by the short-lived (78 hrs) radiometallic nuclide of ^{67}Ga offered the basis for the high specific activity needed to increase the sensitivity of IRMA.

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MATERIALS AND METHODS

The following reagents were employed for this work: gallium-67-citrate (^{67}Ga) (187 MBq/5 m*l*) (Nihon Medi-Physics, Takarazuka, Japan), monoclonal antibody anti-hGH MAB652 (MAB2) (Chemicon International Inc., California, USA), deferoxamine mesylate (DF) (Ciba-Geigy, Basel, Switzerland and repurified by Ciba-Geigy, Japan), BSA RIA Grade (Sigma Chemical Co., Mo., USA), polymeric dialdehyde starch (DAS) (Sigma Chemical Co., Mo., USA and purified by Nihon Medi-Physics, Takarazuka, Japan, the final product containing 5.25 mmol of aldehyde group per mg of DAS). The following

high purity reagents were used: ascorbic acid (Fe less than 0.0005%), butyl acetate atomic absorption spectrometer grade, iron-free hydrochloride acid (Fe less than 0.000005%), (Nacalai Tesque Inc., Kyoto, Japan) and sodium citrate (Fe less than 0.0005%), (Wako Pure Chemicals Industries, Osaka, Japan). Other chemical reagents were of reagent grade: sodium dihydrophosphate, di-sodium monophosphate, sodium chloride, sodium borohydride, hydrobromic acid, barbital sodium, diethyl-barbituric acid (Nacalai Tesque Inc., Kyoto, Japan), triethylamine (TEA), (Wako Pure Chemicals Industries, Osaka, Japan), Sephadex G-50 medium (Pharmacia, Uppsala, Sweden). Also the following buffers were prepared:

A) Phosphate buffer saline (PBS) for coupling (Na phosphate 0.01 M, pH 7.4 containing NaCl 0.15 M)
B) Phosphate buffer for purification of coupled protein (Na phosphate 0.01 M, pH 7.4 containing NaCl 1.0 M)
C) PBS for IRMA (sodium phosphate 0.1 M, pH 7.4 containing NaCl 0.15 M)
D) PBS-BSA for IRMA (buffer C containing 0.5% BSA) and
E) Veronal 0.05 M, pH 8.6 for electrophoresis.

The buffer used in the Sephadex G-50 column chromatography (15 × 250 mm, at 60 m*l*/h) is described, in each corresponding section.

I. Radioiodinated IRMA system (^{125}I-IRMA)

The radioiodinated IRMA system, named the Allégro hGH Kit was purchased from Nichols Institute Diagnostics, California, USA. According to the manufacture's report, two monoclonal antibodies obtained from mouse, the MAB1 and MAB2 (MAB652, Chemicon International Inc., California, USA), each specific for a different and distinct epitope on the hGH molecule, were employed. The MAB2, is radioiodinated (MAB2*) for detection, while the other MAB1 is coupled to biotin (Biotin-MAB1). Subsequent addition to the reaction mixture of an avidin coated plastic bead allows for a specific and efficient means of binding the sandwich complex to a solid phase via the high affinity interaction between biotin and avidin.

II. Radiogallium IRMA system (^{67}Ga-IRMA)

A. Preparation of DF-DAS-MAB2

The unlabeled anti-hGH MAB2 was identified by the kit manufacturer as the anti-hGH MAB652; it was purchased from Chemicon International Inc., California, USA (10 mg in 4 m*l* of buffer TRIS-acetate 0.05 M, pH 7.0 with 0.1% NaN_3) and it was concentrated to 1 m*l* at 4°C (MINICON system, Amicon Corporation, MA, USA) and had the buffer exchanged by Sephadex G-50 chromatography equilibrated with buffer A. 2 m*l* fractions were collected, the absorbance of protein measured at 280 nm (Spectrophotometer Hitachi 330-S, Tokyo, Japan) and those fractions containing the MAB2 were pooled and then concentrated to 1 m*l* for the coupling reaction (5–8 mg/m*l*).

The coupling of MAB2 with DF and DAS was performed as described by Furukawa.[3] Briefly, while the reaction was kept under continuous gentle stirring at 17.5°C, 0.4 μ*l* of DF-DAS solution (DF = 32 mg; DAS = 8 mg) and 14.4 μ*l* of TEA were slowly added to 1 m*l* of MAB2 (5.7 mg after buffer exchange) and the gentle stirring continued for 2 hours. Following this, 10 μ*l* of NaBH4 (0.1 g/m*l* in buffer A), freshly prepared was added to the mixture and stirred gently for another hour. The mixture was purified through a Sephadex G-50 column with Buffer A; the elution profile and the DF-DAS/MAB2 conjugation level were followed by the absorbance of DF-DAS at 311 nm. By using the same Sephadex G-50 column, the DF-DAS-MAB2 was repurified with buffer B and had the buffer changed to A for labeling. The DF concentration of the purified compound was determined by the addition of Fe^{3+}; then the absorbance of Fe^{3+}-DF at 430 nm was measured[5] and the number of DF molecules per DAS-MAB2 molecule was calculated by the Nitrilo Triacetic Acid (NTA) method, as described elsewhere.[6]

The purity and homogeneity of the coupled compound was analyzed by SDS-PAGE (Mini Protean II Kit, Bio-Rad, California, USA). Following the manufacturer's instructions, a 7.5% polyacrylamide gel slab was prepared and 10 μ*l* of the sample or of molecular markers were loaded. The electrophoresis was run at 150 V for 1 hour at room temperature and the gel was stained with Coomassie Blue solution.

B. Purification of ^{67}Ga

The solution of ^{67}Ga was purified as reported by Furukawa[2] with some modifications regarding the final extracted product, the ^{67}Ga-citrate instead of the ^{67}Ga-chloride in the presence of ascorbic acid, as follows: the commercially available ^{67}Ga-citrate (185 MBq, 37 MBq/m*l*), 0.1 M ascorbic acid and the concentrated HBr were mixed at a volume ratio of 4 : 1 : 7 and then extracted with butyl acetate by vigorous mixing for 30 seconds. Then, the butyl acetate layer was carefully separated and the organic solvent was evaporated under a strong nitrogen stream while heating to more than 90°C; the clear residue was dissolved in 10 μ*l* of sodium citrate solution (2 mg/m*l*).

C. ^{67}Ga-Labeling of DF-DAS-MAB2

The radio-labeling of DF-DAS-MAB2 was carried out by adding 10 μ*l* of the above highly concentrated and purified ^{67}Ga solution (329.30–21,645.00 MBq/m*l*) to 100 μ*l* of the DF-DAS-MAB2 solution (100 mg/m*l*) prepared as described in A), and after mixing, the reaction was allowed to proceed for 1 hour at room temperature. The radiolabeled ^{67}Ga-DF-DAS-MAB2 was analyzed by elec-

trophoresis and diluted 10 times with buffer D until use (one to two ^{67}Ga half-life).

The electrophoretic analysis was carried out with acetate cellulose strips (SE-2, Toyo Co.; Separax, Jookoo Co., Ltd., Tokyo, Japan), in buffer E and run for 30 min at 0.8 mA/cm. At the end of the electrophoresis, the strip was dried, cut into 0.5 cm segments and the radioactivity of each one determined in a gamma counter (Cobra-TM 5003, Packard Instrument Co., CT, USA). The labeling efficiency (%) was estimated as the ratio of the radioactivity associated with DF-DAS-MAB2 to the total radioactivity of the strip.

D. Estimation of ^{67}Ga-DF-DAS-MAB2 specific activity

The specific activity (SA) of the radiolabeled conjugate was calculated by the equation below:

$$SA\ (MBq/mg) = \frac{*Radioactivity}{**[MAB2]}$$

*Radioactivity (MBq/m*l*) measured in a Curiemeter (IGC-3, Aloka, Japan).

**[MAB2] concentration (mg/m*l*) contained in DF-DAS-MAB2, estimated by the protein absorbance at 280 nm.

III. Two-site 'Sandwich' IRMA procedure

The standard pituitary hGH (0.50–50.00 ng/m*l*) was that provided with the commercial Allégro IRMA kit. The lower hGH concentration range of 0.03, 0.06, 0.12, 0.25, 0.50 ng/m*l* was prepared by diluting the 50 ng/m*l* standard of the kit with buffer D.

1) ^{125}I-IRMA: This is the commercially available two-site 'sandwich' IRMA, provided with: a) Reagent A: the avidin coated beads (Bead-Av) and b) Reagent B: the ^{125}I-antibody solution; that is, the radioiodinated MAB2, plus the biotin coupled MAB1 (Bio-MAB1). The assay is performed according to the manufacturer's instructions, without modification.

2) ^{67}Ga-IRMA: corresponds to the two-site 'sandwich' IRMA with the ^{67}Ga-DF-DAS-MAB2 prepared as described in the previous section (II) and modified reagents of the ^{125}I-IRMA system as follows: a) ^{125}I-MAB2 removal: 100 μl of the solution provided with the kit containing the biotin-MAB1 and the ^{125}I-MAB2 (reagent B) was mixed with 100 μl of buffer C, and then one avidin coated bead (reagent A) was added, followed by stationary incubation for 4 hours at room temperature. The supernatant was then aspirated and the bead was washed twice with 2 m*l* of buffer D each time. The MAB1 coated beads linked through the Avidin-Biotin system (Bead Avidin-Biotin-MAB1) were kept in buffer D at 4°C until their use (No more than 1–2 ^{67}Ga half-life). b)Two-site 'sandwich' ^{67}Ga-IRMA procedure: 100 μl of ^{67}Ga-DF-DAS-MAB2 (approximately 400,000 cpm diluted in buffer D) was mixed with 100 μl of the sample or standard sample followed by the addition of one previously prepared MAB1 coated bead as described in (a). After 4 hours of stationary incubation at room temperature, the supernatant was aspirated and the bead was washed twice with 2 m*l* of washing solution provided with the kit, and the ^{67}Ga radioactivity bound to the bead was measured. The standard curve, the assay data and the hGH sample concentration were compared with those obtained by the ^{125}I-IRMA, simultaneously performed.

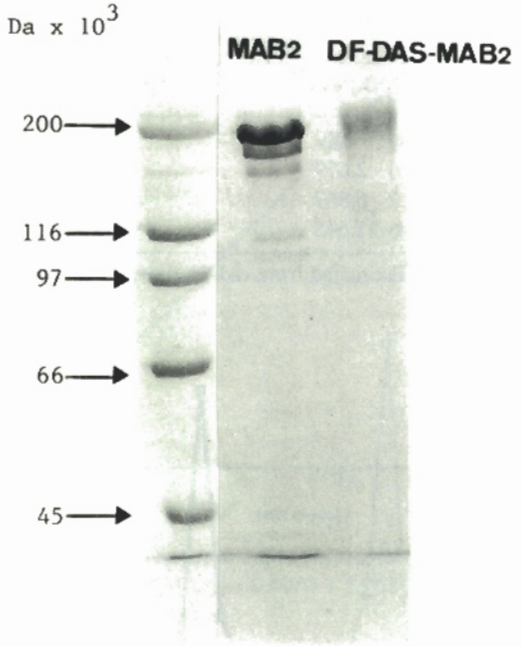

Fig. 1 Non-reduced SDS-PAGE analysis of monoclonal antibody anti-hGH (MAB2) before and after coupling with bifunctional chelating agent (DF) via dialdehyde starch (DAS), (DF-DAS-MAB2).

RESULTS

A. Coupling of DF-DAS-MAB2

The commercially supplied MAB anti-hGH (MAB652, named MAB2) was purified and after its coupling with the bifunctional chelating agent DF, via the water soluble spacer DAS, the non reduced SDS-PAGE analysis of DF-DAS-MAB2 indicated the presence of a single fraction at the position of the major fraction of uncoupled MAB2, closed to the molecular weight standard of 200,000 (Fig. 1).

The analysis of DF molecules bound per MAB2 calculated by the NTA method (6) indicated the DF-DAS-MAB2 conjugate as holding a conjugation level of DF/DAS-MAB2 of 11.81 to 13.45 and that of DF-DAS/MAB2 of 1.19 to 1.37. Approximately one molecule of DAS is therefore holding 9.30 to 11.00 DF molecules per MAB2. The conjugate obtained was then diluted in order to have 100 μg/m*l* of MAB2 before the radiolabeling. No change in stability was detected over a period of three

Table 1 Effect of purified ^{67}Ga concentration on DF-DAS-MAB2 labeling efficiency

Purified ^{67}Ga		Labeling efficiency (%)	^{67}Ga-DF-DAS-MAB2 (specific activity) (MBq/mg)
(MBq/m*l*)	(pmol/10 μ*l*)*		
a) 329	2.22	95.18	313
b) 2,679	18.90	91.80	2,459
c) 6,867	46.30	53.80	3,659
d) 21,645	146.00	22.71	4,884

*Estimated from radioactivity measurement.

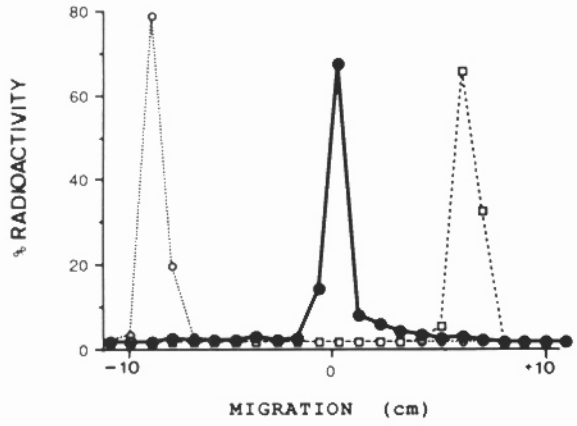

Fig. 2 Acetate cellulose electrophoresis of: ---○---, ^{67}Ga-DF; —●—, ^{67}Ga-DF-DAS-MAB2; ---□---, ^{67}Ga-citrate.

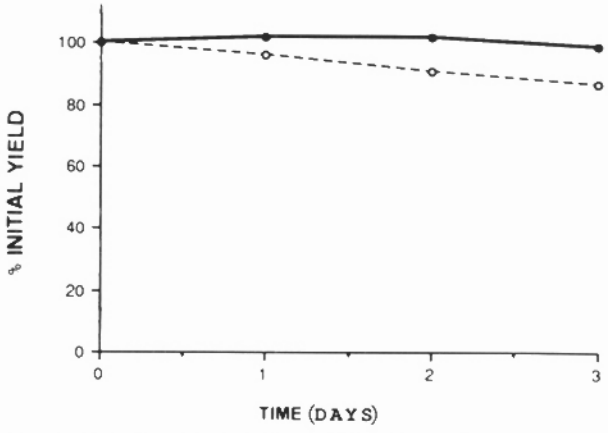

Fig. 3 Stability of ^{67}Ga-DF-DAS-MAB2 conjugate. ---○---, Control (undiluted); —●—, diluted (10 times, buffer D).

months.

B. Radiolabeling of DF-DAS-MAB2

The DF-DAS-MAB2 conjugate was easily radiolabeled with the purified iron-free ^{67}Ga-citrate by a simple mixing reaction. The purified ^{67}Ga solution (range 329.30 to 21,645.00 MBq/m*l*) was added to the DF-DAS-MAB2 conjugate preparation (100 mg/m*l*) reaching a labeling efficiency between 90 and 95% whenever the radioactivity of the purified gallium solution was lower than 3,700 MBq/m*l* (Table 1). In this range the electrophoretic analysis detected a single peak near the site of sampling at the center of the strip (Fig. 2), and neither a ^{67}Ga-DF peak nor a ^{67}Ga free peak was observed. The colorimetric Ponceau analysis demonstrated the peak as being that of protein. As the radioactivity of the ^{67}Ga solution increased, the electrophoretic analysis detected another peak in the anodic site, corresponding to unbound free ^{67}Ga; in fact, at the ^{67}Ga concentration of 21,645 MBq/m*l* the labeling yield decreased to 23% (with 72% of free ^{67}Ga fraction), requiring the purification of the radiolabeled conjugate.

C. Stability of ^{67}Ga-DF-DAS-MAB2

The stability of ^{67}Ga-DF-DAS-MAB2, followed through one half-life of the radionuclide (78 h), showed some sign of change if kept at a very high specific activity level (range 3,700–4,810 MBq/mg), but the simple mediation of a 10 fold dilution kept the radiolabeled conjugate stable, as seen in Figure 3, so that any following assay requiring a ^{67}Ga-DF-DAS-MAB2 conjugate with high specific activity was carried out immediately after its radiolabeling or stored in the diluted condition at 4°C for two half-lives at the most.

D. Two-site 'Sandwich' IRMA

The hGH two-site 'sandwich' IRMA was performed with 100 μ*l* of the 67Ga-DF-DAS-MAB2 at the highest specific activity obtained (4,884 MBq/mg) providing approximately 400,000 cpm per tube, as described in Material and Methods. In the ^{67}Ga-IRMA procedure, the percent bound/total counts (% B/T) versus the hGH concentration provided good linearity in both ranges, high (0.50–50.00 ng/m*l*) (Fig. 4A) and low (0.03–0.50 ng/m*l*) (Fig. 4B); the linearity range of the ^{125}I-IRMA kit (Fig. 4A) was that described in the Allégro kit brochure, up to 0.50 ng hGH/m*l*. The linearity of the ^{67}Ga-IRMA could reach 0.03 ng/m*l* (Fig. 4B). The highly significant correlation ($r = 0.9977$; $p < 0.001$) found between the hGH concentration of the sample measured by the ^{125}I-IRMA and the newly formulated ^{67}Ga-IRMA procedure is shown in Figure 5.

DISCUSSION

In recent years, the trend toward increasing the sensitivity of the isotopically based immunoassay method has led to

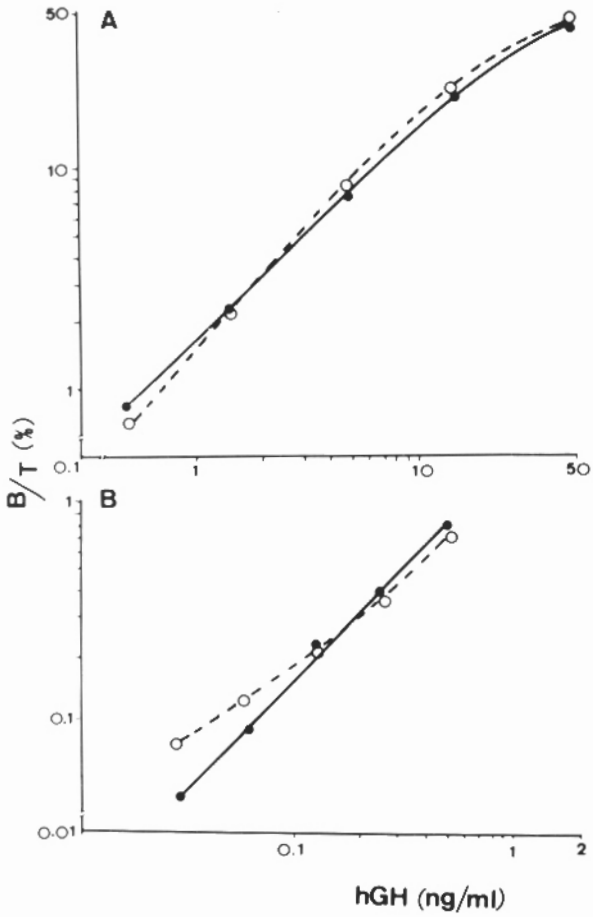

Fig. 4 Standard curves for hGH two-site 'sandwich' IRMA using: —●—, ^{67}Ga-DF-DAS-MAB2; ---○---, ^{125}I-MAB2. Assays plotted at two different hGH ranges: A) 0.50 to 50.00 ng/ml, B) 0.03 to 0.50 ng/ml.

the adoption of the noncompetitive approach. Among the methods, the two-site immunoradiometric assay (IRMA) has offered several advantages in comparison with the traditional IRMA or the competitive radioimmunoassay. On the other hand, the conventionally employed radioisotopic label, such as ^{125}I ($T^1/_2$ = 60.2 days), involves a limitation due to its slow desintegration rate.[1] The use of radionuclides, with faster desintegration rate, such as ^{67}Ga ($T^1/_2$ = 3.24 days) allows the labeling of IgG with higher specific activity (SA), particularly if a multi-site spacer like the DAS is inserted as the linker between the MAB and the metal chelating (DF) molecule[3] as a means to increase the number of DF per MAB molecule.

The antibody labeled with radiometallic nuclides at high specific activity appeared to be of interest in the IRMA procedure. Thus, to validate the presented conceptual approach, a commercial assay system containing MABs of facile acquisition was judged as primordial. In fact, in the selected two-site IRMA kit (Allégro), the anti-hGH mouse monoclonal antibody (MAB2) was commercially available. And, the MAB2 could be easily coupled with DF; the SDS-PAGE (Fig. 1) demonstrated the presence of a DF-DAS-MAB2 molecule with slightly slower migration rate than the free MAB2, probably due to the presence of approximately one DAS molecule (MW = 7,000) plus 11–13 DF molecules (MW = 656 × 11 or × 13 = 7,216 or 8,528) contributing to the change in the migration. Since as high a DF/MAB2 ratio as 11.80 was estimated in 100 μL of DF-DAS-MAB2 containing 10 mg MAB2 (MAB2 = 100 μg/ml), about 780 pmol of DF may be present, so it offered a MAB2 conjugate with capacity to coordinate a great amount of the radiometal per MAB2 molecule. As the coordinating metal, gallium-67 in a concentration range from 329 MBq/ml to 21,645 MBq/ml was used (theoretical calculation: 222 to 14,600 pmol/ml of ^{67}Ga) (1 MBq = 4.5 × 10^{-7} g; 1 mCi = 1.67 × 10^{-9} g).[7] Then, at a constant concentration of DF-DAS-MAB2 (100 μg/ml), the calculation indicated that in spite of sufficient DF coordination sites for the radiometal (DF approx 780 pmol/100 μL), the addition of a solution containing 46.30 pmol of ^{67}Ga (10 μL of 6,867 MBq/ml of ^{67}Ga) to the DF-DAS-MAB2 conjugate, induced a saturation (Table 1). A calculation can estimate the coordination of about 25 pmol of ^{67}Ga to the DF-DAS-MAB2; an amount 30 times lower than the calculated moles of DF. This achieved DF/^{67}Ga coordination ratio represented, nevertheless, an improvement of various order of magnitude over the ^{67}Ga-radiolabeling of fibrinogen previously reported by Takahashi,[6] which requires a 3,000 times excess of DF over ^{67}Ga.

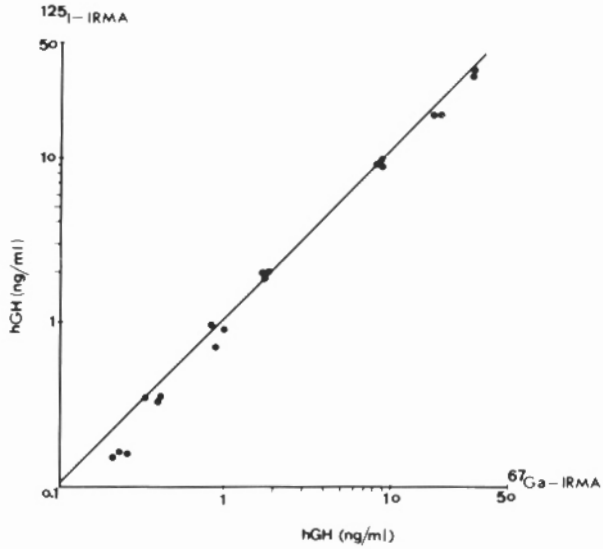

Fig. 5 Correlation between hGH values measured by ^{125}I-IRMA and by ^{67}Ga-IRMA ($y = 0.0084 + 1.0670x$).

Labeling reactions dealing with radionuclides at high specific activity in the micro or nano concentration order is calling the attention of several disciplines.[8] The SA achieved for the anti-hGH antibody, although lower than that reached for the mouse CA-130-22,[3] was as high as 4,884 MBq/mg, almost 10 times higher than the theoretical one calculated from the radioiodinated antibody

Table 2 Comparative quality control parameters of IRMAs (range: 0.50 to 50.00 ng/m*l*)

	^{125}I-IRMA	^{67}Ga-IRMA
Total cpm (n = 3)	263,647 ± 694*	378,329 ± 10,506*
	(0.26%)**	(2.77%)**
Zero standard (n = 2)	208 ± 51	365 ± 58
	(24.50%)	(15.88%)
MDD*** (ng/m*l*)	0.06	0.05
Linear regression	$y = 0.1927 + 0.9198x$	$y = 0.2266 + 0.8712x$
	r = 0.9934	r = 0.9953
	p < 0.001	p < 0.001

*Mean ± SD; **(CV values); ***MDD = minimum detectable dose calculated by 2SD methodology (12).

(370–518 MBq/mg).[9] And when compared with other ^{67}Ga labeled preparations, the SA of the ^{67}Ga-DF-DAS-MAB2 was much higher than the values reported by Wu[10] of 37–111 MBq/mg at 1.8 mole DF/mole of antibody and by Motta-Hennessy[11] of 1.85–7.40 MBq/mg at 0.9 mole DF/mole antibody.

On the other hand, in spite of the various modifications introduced in the MAB2, the preservation of DF-DAS-MAB2 immunoreactivity could be assessed from the paralleled standard curves of the ^{67}Ga-IRMA to the ^{125}I-IRMA, in the higher range (Fig. 4A); this was strongly supported by the good correlation between the concentration of hGH measured by both methods, as shown in Figure 5. According to the theory of the IRMA kinetic, the limiting factor of the two-site 'sandwich' IRMA sensitivity is the SA of the labeled MAB rather than its affinity constant.[1] The theoretical calculation, based on ^{125}I and ^{67}Ga half-life alone, indicates a 20 times higher SA for ^{67}Ga (81.4 TBq/mmol vs. 1,480 TBq/mmol respectively). In fact, in the low range, as indicated in Figure 4B, the ^{67}Ga-IRMA with the synthesized ^{67}Ga-DF-DAS-MAB2 at high SA (> 3,700 MBq), enabled the measurement of a lower hGH concentration than that of ^{125}I-IRMA. Nevertheless, the higher CV values for the ^{67}Ga-IRMA induced a similar MDD range for both methods (Table 2), an indication of further experimental intervention, required in the separation procedure.

In conclusion, the coupling and the radiolabeling of monoclonal antibody for hGH assay with the radiometallic nuclide ^{67}Ga could be readily accomplished and, as predicted the radiolabeling of ^{67}Ga-DF-DAS-MAB2 at a very high SA was achieved, enabling the measurement of a smaller amount of hGH than the ^{125}I-IRMA counterpart. Above all, the data gathered indicated the feasibility of the theoretical approach to the increase in radiolabeled biomolecule specific activity, by the use of a short half-life radio-metallic nuclide and its consequent effect on the assay sensitivity. Another challenge involved in the use of short-lived radionuclides is the radioactivity waste disposal problem faced in today's clinical assay practice. The prompt availability of a generator produced ^{68}Ga is most desirable; due to its even shorter half-life of 68.3 min, approximately only 5,890 molecules of ^{68}Ga against the 4.02×10^5 molecules of ^{67}Ga is theoretically required to detect 1 dps (i.e. 7.5×10^6 labeled molecules for ^{125}I, $T^1/_2$ 60.2 d). Reactions with radiometals at microconcentrations are a challenging step toward the predicted goals and there are still many unknown physical and chemical factors to be determined, but the present results will serve as a basis on which to build a future understanding to facilitate better exploitation of short-half life radiometallic nuclides.

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