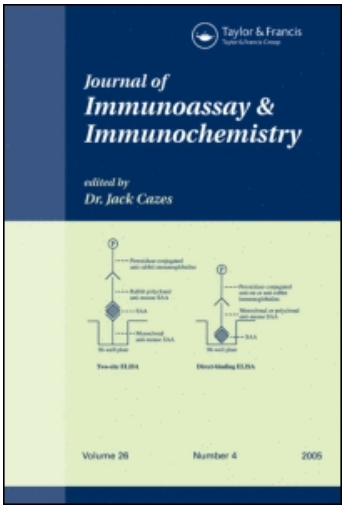

Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

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To cite this Article Matsuzawa, Shigetaka , Kimura, Hiroko and Suzuki, Hiroko(1988) 'Quantitation of Human Chorionic Gonadotrophin by the Planimetry of Latex Agglutination-Inhibition Results on Microtiter Plates', Journal of Immunoassay and Immunochemistry, 9: 1, 1 — 18
To link to this Article: DOI: 10.1080/01971528808053207
URL: http://dx.doi.org/10.1080/01971528808053207

JOURNAL OF IMMUNOASSAY, 9(1), 1-18 (1988)

QUANTITATION OF HUMAN CHORIONIC GONADOTROPHIN BY THE PLANIMETRY OF LATEX AGGLUTINATION-INHIBITION RESULTS ON MICROTITER PLATES

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ABSTRACT

A new type of heavy latex particle (LP) coated with the conjugates of human chorionic gonadotrophin (hCG) and bovine serum albumin (BSA) was used as indicator for the microtiter agglutination-inhibition assay of hCG using a monoclonal antibody (McAb) to hCG. The precipitation patterns of LP were measured by an automatic planimetry system. The detection limit of this method was 4 to 8 IU/l. Human luteinizing hormone (hLH) and follicle-stimulating hormone (FSH) caused hardly any cross-reaction in the presence of 3,350 IU/l and 1,500 IU/l, respectively. This planimetric immunoassay (PMIA) method provides a potentially useful method for hormone assay.
(**KEY WORDS**: Immunoassay, Latex particles, Microtiter, Planimetry, Human chorionic gonadotrophin)

INTRODUCTION

It has been established that the electronically measured agglutination of latex particles (LP) is a useful immunoassay method (1-8). The sensitivity of LP agglutination has been improved by the formation of precipitates on the bottom of the

reacting vessel and the application of antiglobulin processes (9,10). Electronic data processing analysis of the precipitated LP, produced by the latex antiglobulin method, was shown to have high sensitivity (9,11). The electronic data processing system has been further improved, and this assay method is now considered to be particularly suitable for research purposes (12–16). However, the method has not been broadly employed, probably because of the difficulty of reagent preparation and the high cost of the equipment.

This study concerns a simple and broadly applicable immunoassay method using LP reagents. Agglutination-inhibition results for urine human chorionic gonadotrophin (hCG) levels using a microtiter plate can be determined by an automated system. The system is generally applicable to the assay of substances such as hormones.

MATERIALS AND METHODS

Hormones

Crystallized hCG (8,700 IU/mg) was purchased from ZYMED Lab. (San Francisco). Alpha and beta subunits of hCG (α-hCG, β-hCG), human luteinizing hormone (hLH, 6,700 IU/mg) and follicle-stimulating hormone (FSH, 3,000 IU/mg) were obtained from UCB-Bioproducts (Brussels).

Urine

Specimens from pregnant and non-pregnant women, including those from patients with chorionic carcinoma, were kindly supplied by the Department of Gynecology and Obstetrics, Kanto Teishin Hospital, Tokyo. A pool of urine from a four-year-old boy was used as the diluent of test specimens, because urine from young boys contain no hormones cross-reacting to anti-hCG.

Anti-hCG antibodies

Mouse ascitic fluid containing IgG_1 monoclonal antibody (McAb) to β-hCG was obtained from Miles Lab. (Elkhert, Ind.). Polyclonal antibody (PcAb) to β-hCG was prepared in our laboratory by hyper-immunization of rabbits with β-hCG mixed with complete Freund's adjuvant.

HCG-coated latex particles

Conjugates of hCG (1.7 mg or 15,000 IU) and bovine serum albumin (BSA, 60 mg) were prepared by the carbodiimide method of Likhite and Sehon (17). After the removal of free carbodiimide by passage through a Sephadex G-25 column, the conjugates were dissolved in 30 ml of pH 8.2 glycine-buffered saline (GBS). A

halogenated polystyrene LP used in the study (Type HD-08, size 0.9 μm, specific gravity 1.49) was developed in cooperation with the manufacturer, Takeda Chemical Ind. (Osaka). The LP of this type required only 3 hours to establish a firm precipitation pattern when tested by microtitration methods, whereas our availed conventional heavy LP (9-16) (type 59, size 0.9 μm, specific gravity 1.14) needed 6 hours.

The new type of LP physically adsorbed several times the quantities of hCG-BSA conjugates adsorbed by the conventional heavy LP. The detection limit of the new type of LP, coated with hCG, was 50 to 100 times lower than that of a reagent containing the conventional type of heavy LP coated with the same antigen. One volume of the 1% LP suspension in GBS was coated with an equal volume of hCG-BSA conjugate solution as described previously (10).

Planimetric immunoassay (PMIA)

Step one: Latex agglutination-inhibition on a microtiter plate

Two variations of the agglutination-inhibition reaction, the standard and delayed-addition techniques, were employed. Hormones (hCG, hLH and FSH) and urine specimens were serially diluted with the urine from a four-year-old boy. Into each well of a microtiter plate with U-shaped bottoms (Nunc, Denmark), 25 μl of the diluted hormones was dropped. The wells of the top and

bottom rows and at the right and left ends were not used, because of the occasional production of teardrop-shaped precipitates probably due to electro-static effects on the fringe.

For the standard competitive technique, a 0.08% suspension of the hCG-coated LP in GBS (25 μl) was added immediately after the addition of 25 μl of anti-hCG McAb (1:2,000) or PcAb (1:20,000) in GBS containing 0.1% BSA, and the plate was vibrated for 1 minute using a microtiter mixer (Fuji-Zoki, Tokyo). For the delayed-addition technique, the McAb was added and the plate was vibrated for 1 minute and allowed to stand for 1 hour in a 37°C incubator before the addition of the hCG-coated LP.

After standing for 3 hours at room temperature to settle the LP, the plate was placed on a viewing box (Jookoo, Tokyo) to read the degrees of agglutination-inhibition by comparison of the sizes of precipitated patterns with the unaided eye or with a magnifying glass.

Step two: Planimetry of the LP precipitation area

The agglutination pattern on the bottom of each well was observed under transmitted light at magnification (16x) with a stereoscopic microscope (Type SMZ 10, Nikon, Tokyo) equipped with a monocolor microscope television unit (Type WV-1410, Matsushita, Osaka) connected to a graphic computer (Type FS 5500, Matsushita) (Fig. 1). The computer program was written mainly in BASIC, and, for the calculation in machine language. The area of precipitated patterns could be measured either by a manual or by an automated procedure, but the automated measurement was utilized for this

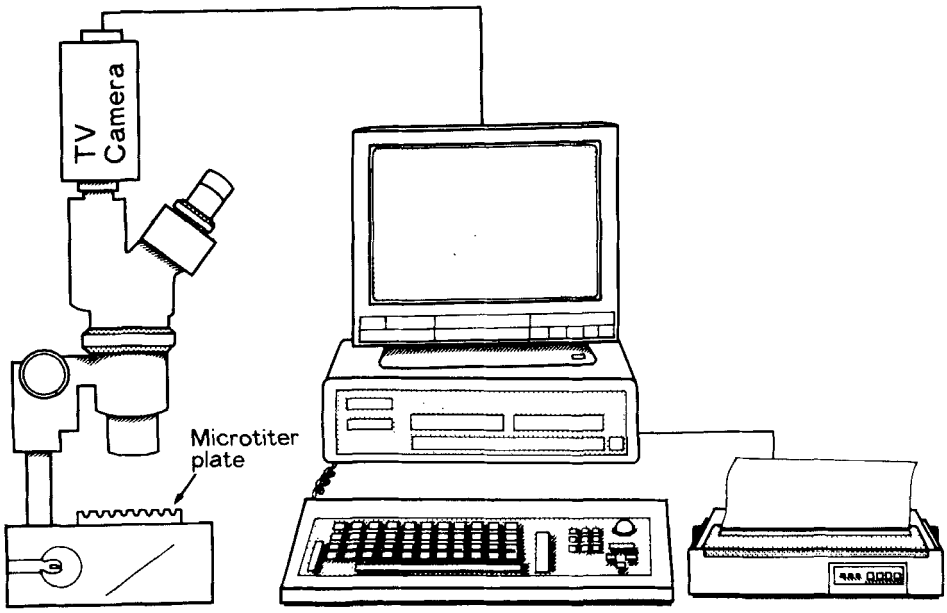

FIGURE 1. Planimetric system for microtiter latex agglutination.

study. The precipitated pattern to be measured, projected on the display unit, was converted into a digitized image with 8 levels of density gradation. The sensitivity was adjusted by selecting a countable density range from the 8 grades, and the area to be measured was limited so as to avoid the image of the well wall (Fig. 2). These conditions were established within about 30 seconds by the operation of the keyboard and were maintained for measurements on at least one microtiter plate. The automated sequential measuring process required about 20 seconds per well. Data was stored by the computer and printed out after the completion of measurement. As is described below, a decrease of

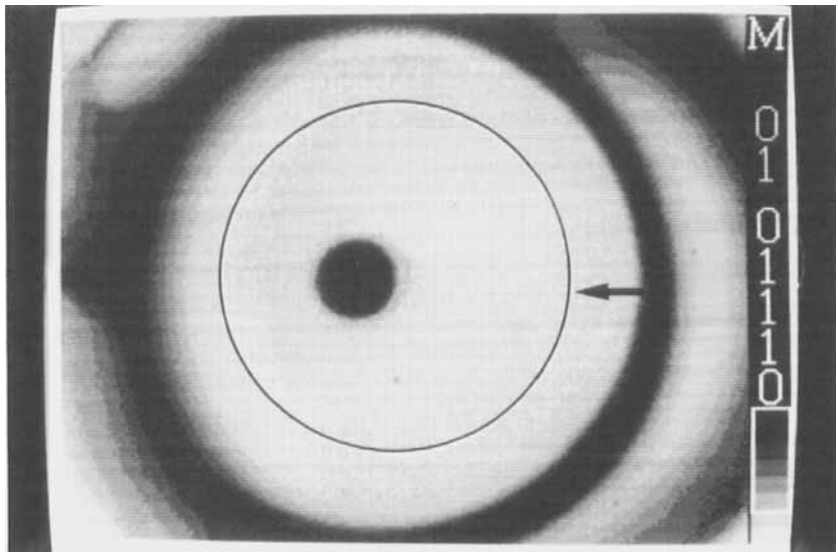

FIGURE 2. Photograph of a digitized pattern of a precipitated LP image on a display unit, with an added circle (marked by an arrow) within which all measurements were made in order to avoid the image of the well wall. At the right margin is shown the number of the measuring frequency (01), and the number of dots corresponding to the area of the precipitated LP (dark portion at the center of the well) (01110), and the level selected for sensitivity adjustment in this measuring system (6th degree in this Figure). Under the experimental conditions used, about 750 dots equal a 1 mm^2 LP precipitation area. Because the negative patterns in this series of experiments were 950 to 970 dots in size, the pattern in this Figure was considered to be very a weak positive.

10% or more in the precipitating area was considered to be significant, because S.D. values among different assays with the same hCG specimen were less than 5%.

Enzyme-linked immunosorbent assay (ELISA) by the avidin-biotin technique

A competitive double-antibody ELISA using microtiter plates was carried out according to the method of Voller et al. (18) with

minor modifications. The avidin-biotin technique of Guesdon et al. (19) was applied to increase sensitivity. An affinity chromatography-purified biotinylated horse anti-mouse IgG, second antibody, and avidin-conjugated horseradish peroxidase was obtained from Vector Lab. (Burlingame, CA). The absorbancy at 410 nm was measured using 2,2'-azinobis(3-ethylbenzthiazoline sulfonic acid) diammonium salt (Sigma) as a substrate.

Radioimmunoassay (RIA)

The hCG test specimens were submitted to double-antibody RIA by hCG-radioimmunoassay kit (Commissariat à l'Energie Atomique, France) using ^{125}I-hCG and anti-β-hCG PcAb.

RESULTS

Table 1 presents the results of the PMIA by the delayed-addition technique. An increase or decrease of about 10% in the size of precipitated areas was considered to be significant because the S.D. values obtained in 4 parallel assays were about 5% or less, whereas differences of less than about 50% in size (30% in diameter) were not discernible with the unaided eye. Thus, the detection limits of hCG by the PMIA delayed-addition technique were 4 to 8 IU/l (0.46 to 0.92 ng/ml), and for the

TABLE 1

Size (Dot Numbers) of Precipitate Areas Measured for 4 Samples by PMIA by Delayed-Addition Technique

Conc. of hCG (IU/l)	Sample No. 1	2	3	4	Means ± S.D.
Control	4319	4314	4501	4437	4392.8 ± 79.6
1	4274	4144	4346	4218	4245.5 ± 74.1
2	3747	4010	3988	3942	3921.8 ±103.8
4	3675	3725	3876	3971	3811.8 ±118.0
8	3560	3622	3723	3608	3628.3 ± 59.3
16	3403	3704	3586	3267	3490.0 ±167.6
31	2185	2179	2171	2191	2181.5 ± 7.4
62	1140	1216	1211	1223	1197.5 ± 33.5
125	1139	1204	1090	1250	1170.8 ± 61.1
250	1170	1176	1289	1258	1223.3 ± 51.5
500	1107	1108	1135	1096	1111.5 ± 14.4

conventional method of determination were 20 to 40 IU/l (2.3 to 4.6 ng/ml).

Fig. 3 and Fig. 4 show the curves obtained by the planimetry of precipitated LP patterns. The curves with β-hCG showed more gentle slopes than those with hCG. No inhibition occurred in the presence of 3,350 IU/l (500 ng/ml) of hLH and 1,500 IU/l (500 ng/ml) of FSH.

As shown in Fig. 5, the detection limit for hCG by the competitive technique with the anti-β-hCG PcAb was 3 IU/l (0.34 ng/ml), a similar value to that obtained with the anti-β-hCG McAb. Weak cross-reactions occurred, with detection limits of 41.9 IU/l (6.25 ng/ml) for hLH and of 600 IU/l (200 ng/ml) or above for FSH.

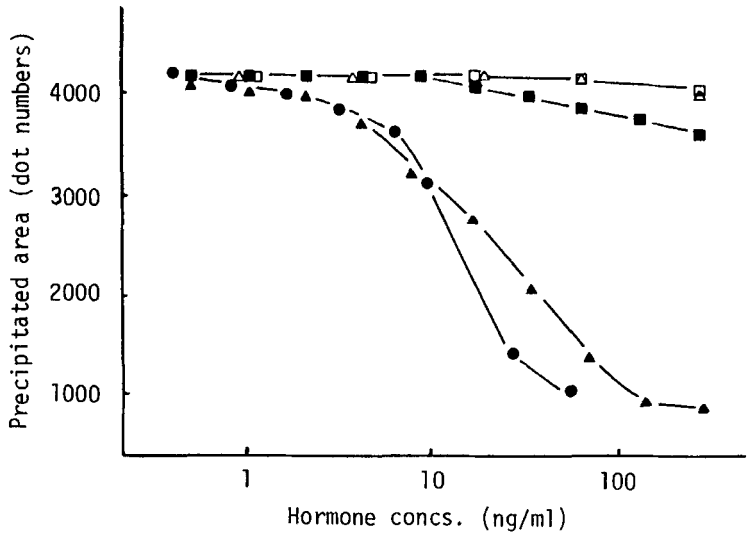

FIGURE 3. Decreasing PMIA curves obtained by competitive technique using an anti-β-hCG McAb. A mean of two measurements is plotted for each point for each hormone, as follows: hCG,● ; α-hCG,■ ; β-hCG,▲; hLH,△ ; and FSH,□ .

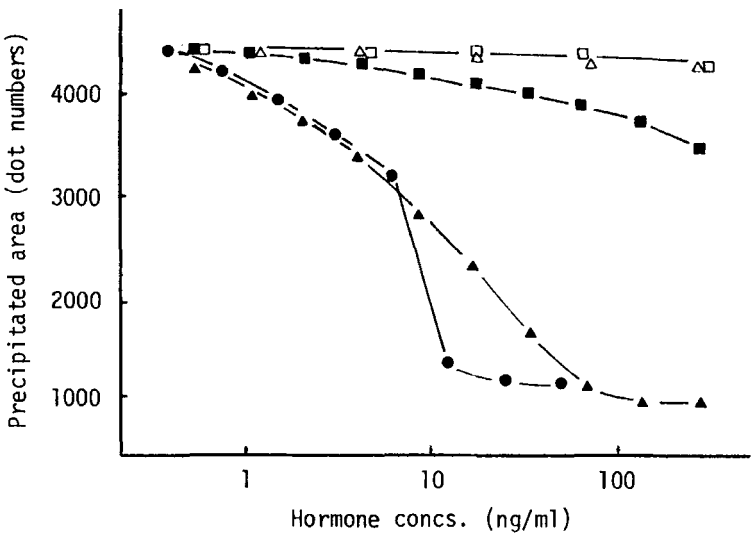

FIGURE 4. Decreasing PMIA curves obtained by delayed-addition technique using an anti-β-hCG McAb. A mean of two measurements is plotted for each point for each hormone. The symbols used are the same as those in Fig. 3.

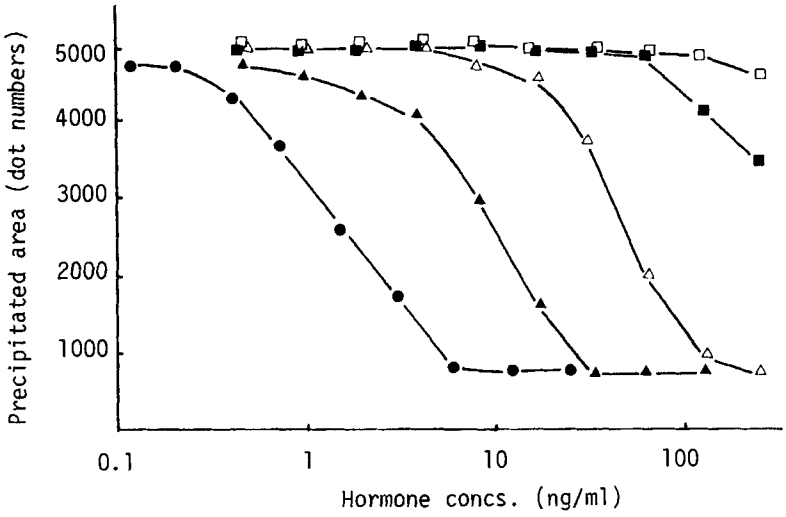

FIGURE 5. Decreasing PMIA curves obtained by competitive technique using an anti-β-hCG PcAb. A mean of two measurements is plotted for each point for each hormone. The same symbols are used as in Fig. 3.

An immunoassay by volume distribution measurement of agglutinated and precipitated LP (11) was not suitable for the LP for this study, because collection of the LP by aspiration was made difficult by the tendency of LP to adhere to surface of the well. Futhermore, unstable LP reagents caused nonspecific agglutination, and some inappropriate LP reagents produced precipitates with irregular edges because of the falling of adhering LP, which resulted in poor reproducibility.

As shown in Table 2, the sensitivity of the PMIA technique was superior to, or at least comparable to, those of ELISA by the avidin-biotin technique and of double-antibody RIA. The

TABLE 2

Comparison of PMIA, ELISA and RIA for HCG Quantitation

Assay method	Quantifiable range (IU/l)	Reproducibility: C. V. (%)		Consumption[*5] of anti-hCG McAb (ul)	Requisite time for quantitation(hours)
		Intra-assay[*3]	Inter-assay[*4]		
PMIA (c[*1])	8 - 435	4.1	3.2	0.0125	3
PMIA (d[*2])	4 - 62	2.5	3.1	0.0125	4
ELISA(c[*1])	60 - 500	2.5	11.8	0.01	24
RIA (d[*2])	10 - 100[*6]	-	-	0.01[*6]	24

PMIA and ELISA were performed with the same anti-hCG McAb.
*1) c = competitive technique
*2) d = delayed-addition technique
*3) Mean value of C.V. calculated from 4 assays for each of 5 dilutions, in which the hCG concs. were in or near the quantifiable ranges
*4) Calculated from results of 4 assays for each of 5 dilutions performed 6 times in a week
*5) Indicated by converted quantities of an undiluted anti-hCG McAb for one test with one specimen dilution
*6) Values shown by the manufacturer of this McAb

sensitivity and reproducibility of PMIA by the delayed-addition technique was better than that of the competitive technique. The intra-assay coefficient of variation (C.V.) of the delayed-addition technique was similar to that of ELISA by the competitive technique. The inter-assay C.V. of PMIA, by either the standard or the delayed-addition technique, was low compared with that of ELISA by the competitive technique. Our experiment showed that the detection limit of hCG by PMIA was lower than those obtained

by ELISA by the avidin-biotin and standard competitive techniques. Both of these assays were performed with the same anti-β-hCG McAb whose hCG binding capacity was lower than some batches of PcAb. We presume that this type of McAb is unsuitable for ELISA but not for PMIA. As shown in Figs. 6 and 7, the results of PMIA were correlated to those obtained by ELISA by the avidin-biotin technique and double-antibody RIA. The correlation coefficient of PMIA with ELISA and RIA, calculated from the results of 20 and 24 specimens, were 0.941 and 0.990, respectively.

DISCUSSION

A potentially useful planimetric immunoassay was made possible by two technical improvements. The first was the development of heavy, highly sensitive latex particles. The hCG-coated LP reagent prepared from this type of particle was agglutinated even by small quantities of antibodies, such as McAb, with very weak agglutinating avidities. Thus, potentiation of agglutinating properties by latex antiglobulin procedures (10) was unnecessary. The other improvement was an automated system for measuring the precipitated area, which allowed quantitative results to be obtained.

In PMIA by the competitive technique, agglutination of LP may not occur consistently owing to insufficient mixing of hCG, McAb and hCG-coated LP. PMIA by the delayed-addition technique

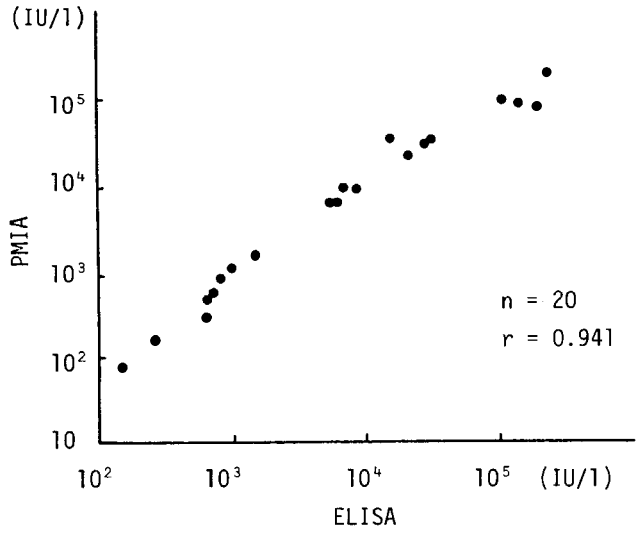

FIGURE 6. Correlation of hCG levels in urine specimens, estimated by ELISA and PMIA techniques.

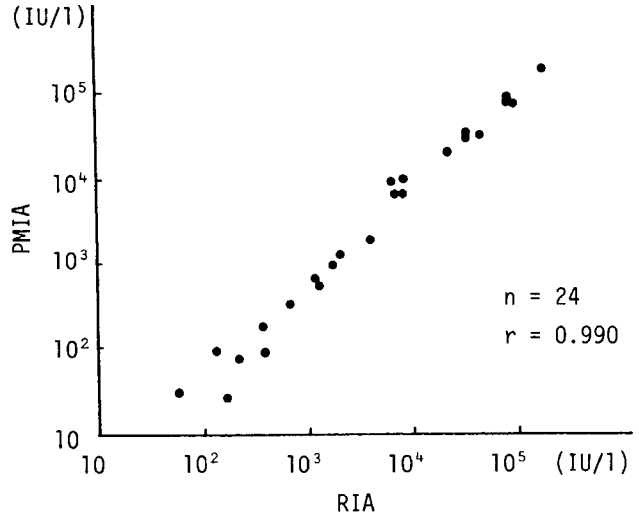

FIGURE 7. Correlation of hCG levels in urine specimens, estimated by RIA and by PMIA.

resulted in better reproducibility and sensitivity than that by the competitive technique.

ELISA by the sandwich technique for hCG has been considered to be very sensitive, and detection limits are reported from 2.2 IU/l by Longhi et al. (20) to 10 to 20 IU/l by Cartwright et al. (21). The detection limits of ELISA by the sandwich technique with commercial reagents (21) and of ELISA by the competitive technique that we performed were comparable to those by PMIA using the competitive and delayed-addition techniques. The procedure of PMIA was simple. Results were obtained within 3 to 4 hours, and the measuring time for each precipitation pattern was about 20 seconds. Measurement time could be further reduced, since the numbers to be planimetrically measured could be narrowed by previous visual inspection. The equipment for PMIA was inexpensive compared with that for ELISA or for RIA. The amount of McAb (anti-hCG) required for PMIA was comparable to that needed for ELISA and RIA. Radiolabelled reagents and a second antibody are not required.

PMIA using McAb should be capable of broad applicability for the quantitation of various antigens and haptens.

ACKNOWLEDGEMENTS

This work was supported by grant No. 61870032 from the Japanese Ministry of Education.

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