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≪**Technical Report**≫

Studies on the Quality Control of Insulin Radioimmunoassay Kit (I)

—Pitfalls in Radioimmunoassay of Insulin—

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Abstract

A typical abnormal standard dose response curve of nearly convex form, often encountered in insulin radioimmunoassay (IRIA) has been analyzed by varying incubation conditions.

The cause of such abnormality has been turned out to be the results of an incomplete equilibrium between the two reactants.

By careful control of the temperature of serum sample an immediate cause of sharp deviation of B/F value of the sample tube from the measurable range in the standard curve has also been investigated.

The two main troubles have been proven to be stemed from incubation conditions. Incubation at 4°C for 48 hrs is emphasized for IRIA.

요 약

인슈린방사면역측정(IRIA)에서 흔히 목격하는 비정상적인 표준투여응답곡선(standard dose response curve)을 정온유지(incubation) 조건을 변화시킴으로써 해석하였다.

그와 같은 이상(abnormality)의 원인은 두 반응물질간의 불완전한 평형에 있는 것으로 밝혀졌다.

인슈린측정용 혈청시료의 온도를 조절함으로써 혈청시료에 대한 B/F 값이 표준곡선의 측정가능범위로부터 크게 벗어나는 원인을 조사할 수 있었다.

이들 두 실패요인은 주로 정온유지조건에 관계되었으므로 4°C에서 48시간 실시하는 정온유지조건이 IRIA를 위하여 강조되었다.

1. Introduction

Many kinds of radioimmunoassay (RIA) are nowadays practically applied in the medical diagnosis in-vitro. Among them, insulin radioimmunoassay (IRIA) is popular one for diagnosis of diabetic states.

Since some pitfalls were often encountered during the trial use of IRIA kit prepared in our laboratory the cause of them has thoroughly been studied. Even though a lots of literature on RIA are available[1~8], detailed discussions on the cause of such pitfalls can hardly be

found.

2. Experimental

2.1. Materials

(1) Insulin standard; porcine, recrystallized, 25.5 IU/mg, Schwarz/Mann

(2) Insulin-^{125}I; specific radioactivity 100 uCi /ug, prepared in our lab[9].

(3) Anti-porcine insulin guinea pig serum; titer 1:30,000, Miles Lab.

(4) Dextran; T-80, RIA grade, Schwarz/ Mann

(5) Charcoal; RIA grade, Schwarz/Mann

(6) Dextran coated charcoal (DCC); prepared as previously[9].

2.2. Procedures

Using our prepared kits incubation mixtures were made as shown in Table 1, and incubated them under various conditions.

2.2.1. Incubation time control

Keeping incubation temperature at 4±2°C, the mixtures were incubated for 24 or 48 hrs.

2.2.2. Incubation temperature control

Keeping incubation time in 48 hrs, the mixtures were incubated at 4°C or at 10°C.

2.2.3. Temperature control of serum samples

The temperature of serum sample was also controlled from 1~2°C to 25~30°C before preparing incubation mixtures.

After incubation, 0.3 ml of DCC suspension of about at 4°C was added to each tube, agitated and centrifuged at 3000 rpm for about 15 min. The B/F values were determined as previously reported[9]. The B/F for the test serum was also determined in usual way; the net B value for the test serum was obtained by subtracting the B value of the antibody-blank tube. B values were obtained by subtracting the radioactivity of charcoal layer from the total radioactivity. (Fig. 1~3) (Table 2 & 3).

$$B/F=\frac{B'-S}{F}=\frac{(Ta-F)-S}{F}$$

where

B; (radioactivity of Insulin-^{125}I bound to antibody)$=F'-S$, (S' for test serum)

Table 1. Preparation of Incubation Mixtures

Tube No.	Buffer solution (ml)	Insulin-^{125}I (ml)	Standard solution (ml)	insulin (uU/ml)	Anti-insulin solution (ml)	DCC after incubation (ml)
1*	0.7	7.1	—	—	—	0.3
2**	0.6	0.1	—	—	0.1	0.3
3	0.5	0.1	0.1	2.5	0.1	0.3
4	0.5	0.1	0.1	5.0	0.1	0.3
5	0.5	0.1	0.1	10.0	0.1	0.3
6	0.5	0.1	0.1	20.0	0.1	0.3
7	0.5	0.1	0.1	40.0	0.1	0.3
8	0.5	0.1	0.1	80.0	0.1	0.3
9	0.5	0.1	0.1	160.0	0.1	0.3
10	0.5	0.1	0.1	320.0	0.1	0.3
11***	0.6	0.1	0.1	(?)	—	0.3
12#	0.5	0.1	0.1	(?)	0.1	0.3

*Blank of standard insulin and anti-insulin serum
**Blank of standard insulin
***Blank of anti-insulin serum for the test sample
#Test sample with anti-insulin serum

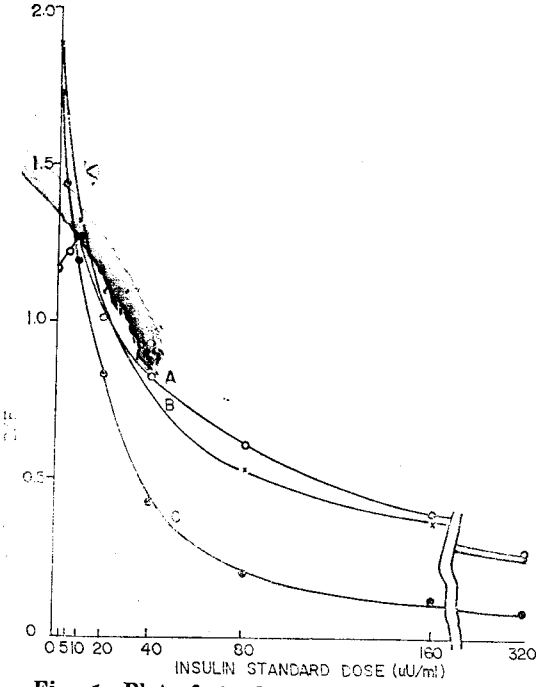

Fig. 1. Plot of standard dose response curves under various incubation conditions; A: 3°C, 24 hrs., B: 6°C 24 hrs., C: 6°C, 48 hrs.

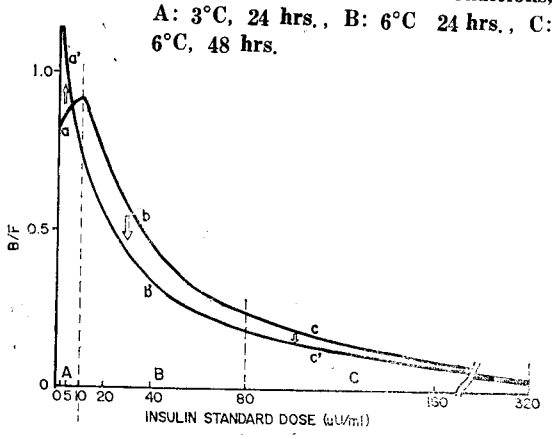

Fig. 2. A diagrammatical expression of the variation of standard dose response curves with incubation conditions; Curve a-b-c changes to curve a'-b'-c' with sufficient incubation time at low temperature (4°C etc)

F; (radioactivity of free insulin^{-125} I)=(radioactivity of charcoal layer (*F'*)−B. G.

B'; *Ta*−*F*

Ta; total radioactivity (*T*)−B. G.

S; *B'* for the Ab blank tube

S'; *B'* for the Ab blank tube for test serum

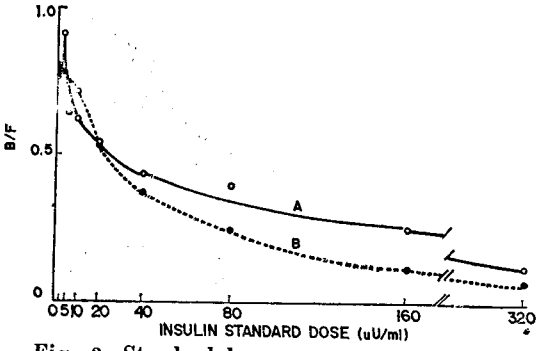

Fig. 3. Standard dose response curves obtained by incubation at 37°C for 3 hrs. A; Insulin-^{125}I fraction No. 6, B; that of No. 5

3. Results and Discussion

3.1. Standard dose response curve(SDRC)

As Fig. 1 shows, a typical abnormal curve (A) was obtained when incubated at 3°C for 24 hrs. The similar curves can be found in some RIA literatures[10~12], however, no discussion was made for them so far.

Such a phenomenon, the convex nature of the SDRC or a low dose gradient curve is well explained with an incomplete equilibrium between the two reactants such as antigen (Ag) and antibody (Ab). In the region of lower [Ag] (upto 10 uU/ml) it takes longer time to reach equilibrium since the reaction rate is directly related to [Ag] as following equations[13].

$$[Ag]^*+[Ag]=[AG] \quad (2)$$

$$[AG]+[Ab]\underset{k2}{\overset{k1}{\rightleftharpoons}}[AG\cdot Ab] \quad (3)$$

$$\text{Rate}(R)=k1[AG][Ab] \quad (4)$$

Equilibrium constant

$$K=\frac{k1}{k2}=\frac{[AG\cdot Ab]}{[AG][Ab]} \quad (5)$$

Thus, the B/F values in the region of lower [Ag] (upto 10 uU/ml) are lower than those in the region of moderately higher [Ag] (nearby 10 uU/ml). However, the B/F values in the region of moderately higher [Ag] (10−80 uU/ml) are higher than those in the region of the

higher [Ag] (80—320 uU/ml) since there was already at near equilibrium between the reactants, and the principle of RIA was adopted in the region. As Fig 1 shows, the convex curve changes to a higher dose gradient curve as increasing incubation time. The B/F values in the region of more than 10 uU/ml were decreased while those in the region of less than 10 uU/ml were increased. It means that reactants arrive at equilibrium very slowly especially in the lower [Ag] region. B/F decrease is attributable to the incomplete mixing, minute thermal discrepancies or higher temperature before starting incubation at 4°C. It suggests that a SDRC of higher dose gradient can only be obtained when the incubation time is sufficient to bring about equilibrium even in the lower [Ag] region as illustrated in Fig. 2.

Incubation at 37°C for 3 hrs was also carried out as reported[14] (Fig. 3). In the experiments 0.3 ml buffer was added to each tube after incubation to maintain higher [Ag] during incubation. However, the B/F values were generally low (below 1.0), and dose gradients were smaller than those shown in Fig. 1. This is because the incubation time is insufficient due to the slow rising of temperature from 1~2°C to 37°C in the incubator. It took about 2.5 hrs. Thus the actual incubation time would be only 30 min. Incubation at ambient temperature is also undesirable because it is difficult to keep exact incubation time at the temperature; i.e., incubation mixtures are usually cold, and it takes long time to arrive at the ambient temperature. It is considered that incubation at lower temperature for longer time is prefered to those at higher temperature for shorter time. Incubation at 4°C would be adequate considering both the activation energy and inhibition of bacterial growth.

3.2. Determination of Insulin Levels

As Table 3-1 shows, when the serum sample was directly added to the incubation mixture without equilibrating the temperature with other materials and incubated at 10°C, the B/F value of the sample tube was so high that the true insulin level in uU/ml could not be measured from the SDRC which was obtained by a pa-

Table 2-1. Typical IRIA data obtained by incubation at 10°C for 24 hrs with thermal pre-equilibration between the serum sample and other materials in preparing incubation mixtures (average of duplicate runs using insulin-^{125}I batch 13-7-78)

Tube No.	Total radioacty (cpm)	radioact'y of charcoal (cpm)	F	T-F	B	B/F
1*	15965	14476		1489		
2	15668	3659	2939	12009	10520	3.57
3	15673	3795	3075	11860	10371	3.37
4	15419	3825	3105	11594	10105	3.25
5	15943	4302	3582	11631	10142	2.83
6	15950	5100	4380	10850	9361	2.14
7	16137	6488	5768	9648	8159	1.41
8	16621	9196	8476	7425	5936	0.70
9**	15962	3471	2751	12491	10508	3.82
10***	16829	14846		1983		

*Ab blank for standard dose tubes
**serum sample tube
***Ab blank for the serum sample tube

Table 2-2. Typical IRIA data obtained by incubation at 2°C for 48 hrs with thermal pre-equilibration between the serum sample and other materials in preparing incubation mixtures (average of duplicate runs, using insulin-^{125}I batch 13-7-78)

Tube No.	Total radioact'y (cpm)	Radioact'y of charcoal (cpm)	F	T-F	B	B/F
1*	12603	9497		3106		
2	12618	3436	2749	9182	6076	2.21
3	12900	3695	3008	9205	6099	2.03
4	11687	3386	2699	8301	5195	1.92
5	12673	3883	3196	8956	5850	1.83
6	12553	4255	3568	8298	5192	1.45
7	12613	5412	4725	7210	4099	0.86
8	12904	6600	5913	6204	3198	0.54
9**	13181	5257	4570	7294	3934	0.86
100***	13375	9385		3990		

Table 3-1. Typical IRIA data obtained by incubation at 10°C for 48 hrs without thermal pre-equilibration between the serum sample and other materials in preparing incubation mixtures (average of duplicate runs, using insulin-^{125}I batch 21-6-78)

Tube No.	Total radioact'y (cpm)	Radioact'y of charcoal (cpm)	F	T-F	B	B/F
1*	14226	12403		1823		
2	14475	3114	2408	11361	9538	3.96
3	14087	3200	2494	11593	9770	3.91
4	14714	3310	2604	11404	9581	3.68
5	14867	3576	2870	11291	9468	3.30
6	14943	4008	3302	10935	9113	2.76
7	14532	5445	4739	9087	7264	1.53
8	14569	7278	6572	7291	5468	0.83
9**	15067	2906	2200	12161	9698	4.41
10***	14662	12199		2463		

*Ab blank for standard dose tube
**Serum sample tube
***Ab blank for the serum sample tube

rallel running with the serum samples. The cause of such a trend can be explained with temperature discrepancies between the incubation tubes. According to the Arrhenius theory, the rate of the complex formation would be increased with increasing temperature upto 37°C in a definite incubation time. Thus, the increased B/F value for the serum sample is attributable to the higher incubation temperature of the serum sample tube comparing with those of normal standard insulin dose tubes. In case of using common refrigerator for incubation, it has been found that maintaining a definite temperature is difficult especially in mid-summer or mid-winter time. However, even when a definite temperature was maintained the temperature of the serum sample tube would be higher than those of standard dose tubes as far as the serum

Table 3-2. Typical IRIA data obtained by incubation at 2-4°C for 48 hrs without thermal pre-equilibration between the serum sample and other materials in preparing incubation mixture (average of duplicate runs, using insulin-^{125}I batch 21-6-78)

Tube No.	Total radioact'y (cpm)	Radioact'y of charcoal (cpm)	F	T-F	B	B/F
1*	11601	9094		2507		
2	12325	3552	2810	8773	6266	2.24
3	11691	3599	2857	8705	6198	2.17
4	12219	3619	2877	8600	6093	2.11
5	11788	3582	2840	8206	5699	2.01
6	11781	4075	3333	7706	5199	1.56
7	11706	4837	3095	6869	4362	1.07
8	12060	6230	5488	5830	3323	0.61
9**	11995	3643	2901	8352	5519	1.90
10***	11916	9083	2833			

at room temperature is used instead of using cold standard insulin solution for the standard dose tubes. As Table 2-1 shows, the extent of the over-ranging was decreased when thermal equilibrium was achieved between the serum sample tube and standard dose tubes before preparing incubation mixtures but incubated at 10°C for 24 hrs. The cause of it is attributable to the probable temperature discrepancy still persisting due to the latent heat of the serum sample under such insufficient incubation conditions.

Even when incubated at 2-4°C for 48 hrs, the B/F value of the same serum sample was slightly higher (Table 3-2) if obtained without thermal equilibrium before preparing incubation mixture comparing with that obtained with thermal equilibrium (Table 2-2). As Table 2-2 shows, the over ranging tendency was disappeared when incubated at 4°C for 48 hrs and the temperature of the serum sample was equilibrated with others before preparing incubation mixtures.

The discrepancy of protein content can also partly affect the over ranging tendency because B/F is increased with protein content. In strict sense, the protein content in the serum sample tube is larger than those of the standard dose tubes. However, it is our experience that the effects of temperature and protein content discrepancies are apparently decreased when the labelled insulin has its intactness under a given specific radioactivity to show smooth binding to its antibody.

4. Conclusion

(1) The originating of an abnormal SDRC of convex form or low dose gradient curve is explained as a result of an insufficient equilibrium between two reactants. It has been proven by extending the incubation time.

(2) A sharp deviation of B/F values for the serum sample tubes from the measurable range in the SDRC often encountered in IRIA has been turned out to be originated from the temperature discrepancy between the serum sample tube and the standard dose tubes.

(3) Incubation for 48 hrs at 4°C for IRIA is emphasized as a good practice.

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