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《**Technical Report**》

Selection of Well Labelled Insulin Fractions for Radioimmunoassay Use

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Abstract

Selection methods of well labelled insulin fractions based on two different criteria were compared to establish an efficient low level RIA of insulin and to elucidate the correlation between the immunoreactivity and the charcoal-adsorptivity of the radioiodine labelled insulin.

The results indicated that the selection of well labelled insulin fractions by means of a charcoal-adsorption test is inappropriate. Generally, the distribution of radioactivity, antibody-bindability, and charcoal-adsorptivity of the labelled insulin was not consistent with each other.

Thus, the selection should be carried out for every labelling batch to get the utmost assay reliability by antibody-bindability but not by charcoal-adsorptivity.

By using the well selected labelled insulin fractions based on antibody-binding, a correct assay for a reference serum was possible, and by extending the incubation time upto 96 hrs, a sharp dose response curve could be obtained even in the range of below 5 μU/ml standard insulin doses.

요 약

효과적인 저농도 인슈린 방사면역 측정법 확립 및 표지인슈린의 면역학적 활성과 탄소분말에 대한 흡착력의 상관관계를 밝히기 위하여 이들 두 다른 기준에 기초를 둔 두가지의 최적표지 인슈린선정 방법을 비교한결과 탄소분말흡착에 의한 방법은 부적당함을 알 수 있었다.

일반적으로 표지인슈린의 방사능, 항체결합능, 탄소분말흡착능등은 서로 일치하지 않았다. 따라서 측정신뢰도를 높이기 위해서는 매 표지때마다 항체결합능으로 최적표지 인슈린을 선정해야 하며 탄소분말 흡착력으로 선정하면 안된다고 본다.

항체결합능에 따라 선정된 표지 인슈린을 써서 대조혈청중의 인슈린량이 정확히 측정되었으며 정온유지시간을 96시간으로 연장함으로써 5 μU/ml이하의 표준 인슈린투여량에 대해서도 매우 예리한 응답곡선을 얻을 수 있었다.

1. Introdution

Original biological or immunological properties of the protein molecules are apt to be changed by introduction of radioiodine.

The cause is mainly attributable to the denaturation or radiolysis of of the molecule

during labelling, purification and storage. It is known that radioiodines are introduced to tyrosine moiety in the protein molecule[1)-4)]. When heavily introduced, tyrosine rings in the molecule are changed to diiodotyrosine rings, and the consequent loss of the intact property is unavoidable [3),5)]. Thus, the structural change by the heavy introduction of radioiodine is also one of the cause of the activity loss.

In the preparation and control of insulin radioimmunoassay kit, the main difficulty was eliminating the discrepancy of the measured hormone levels which is due partly to the defectiveness of the radioinsulin.

B.L.Wajchenberg et al.[6)] has proposed a method of estimating radioinsulin by which a radioinsulin having high adsorptivity to dextran coated charcoal (DCC) is a good fraction for insulin radioimmunoassay (RI A).

DCC is popularly used for separating free unbound radioinsulin (F) from the bound (B) as it selectively adsorbs the free hormone. The good adsorptivity might be a factor in establishing RIA since the B value in the antibody blank tube should better be zero in most RIA.

On the other hand, maintaining intact immunological activity of the radioinsulin would be another factor since RIA is essentially grounded on the immunological reactions.

In present work, the selection methods of well labelled insulin based on two different criteria are compared to establish an efficient low level insulin RIA elucidating the correlation of the immunoreactivity and the charcoal adsorptivity of the radioiodinated insulin.

2. Experimental

2.1. Materials

- Insulin-^{125}I; labelled by means of a lactoperoxidase method, specific activity; about 125 μCi/μg[7)]
- Sephadex G-50;fine, Pharmacia, Sweden
- Dextran coated charcoal (DCC) suspension; prepared as previously reported[8)]
- Insulin antibody;antiporcine insulin guinea pig serum, titer;1:3×10^4, Schwarz/Mann

2.2. Procedure

2.2.1. Sephadex filtration (SF)

Zone **1** or zone **2** in the starch gel electrophoresis plate[7)] was extracted from the gel using eluent buffer (0.02 M phosphate buffer containing 2.5% BSA, pH8.6), and about 0.3 ml aliquot of the extract was charged on the top of the Sephadex G-50 column (1 ×50 cm) which was pre-equilibrated with the eluent buffer. The column was eluted with a flow rate of about 6 ml/hr and 1 ml aliquot was collected in each tube. Radioactivity of each tube was measured using a well type scintillation counter (Aloka Model PC-lOE).

2.2.2. Measurement of Immunoreactivity

Each fraction of SF was incubated with a definite titer of the antiporcine insulin rabbit serum. After incubation for 24 hrs at 4°C, the labelled insulin bound to antibody (B) was separated from the free unbound labelled hormone (F) by adsorbing the F to DCC suspension as reported previously[8)]. (Fig. 1-Fig. 4)

2.2.3. Measurement of Adsorptivity to DCC

According to the method reported by Wajchenberg et al.[6)], the adsorptivity to DCC was checked as following; in the abse-

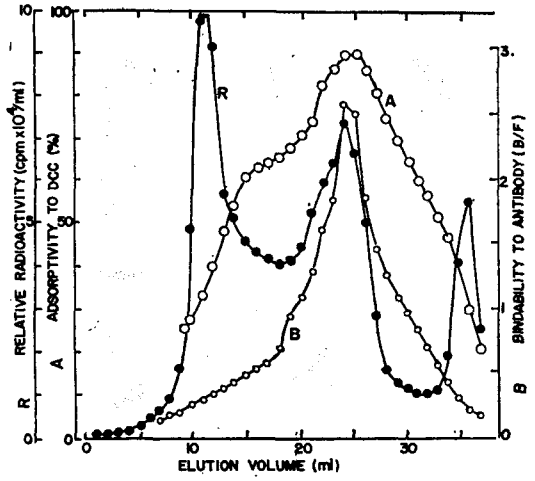


Fig. 1. Distribution of Radioactivity, Adsorptivity and Bindability of the Extract of Zone 1 in the Starch Gel Electrophoresis (mainly LPO-^{125}I)

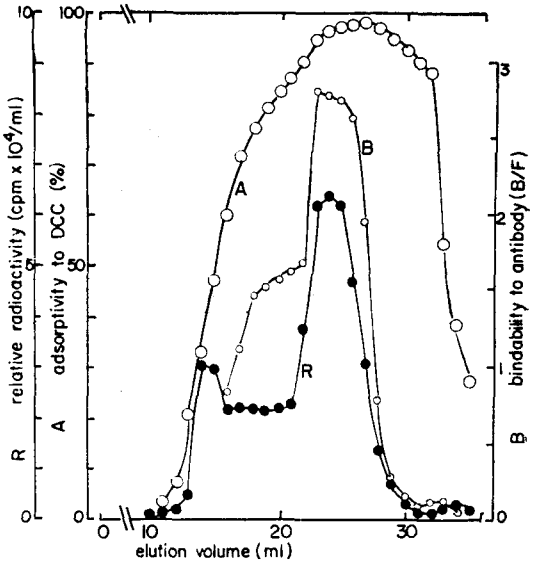


Fig. 2. Distribution of Radioactivity, Adsorptivity and Bindability of the Extract of Zone 2 in the Starch Gel Electrophoresis (mainly insulin-^{125}I) (A case of the three peaks are nearly superimposed)

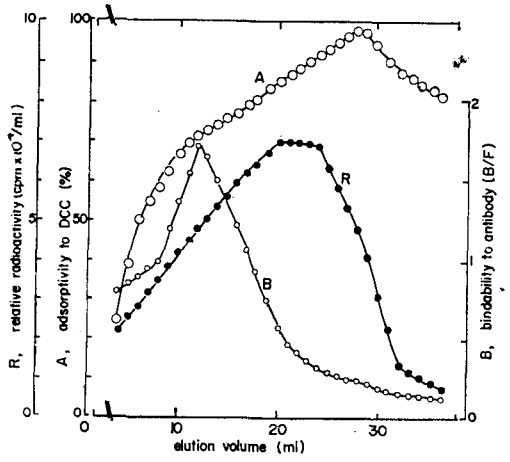


Fig. 3. Distribution of Radioactivity, Adsorptivity and Bindability of the Extract of Zone 2 in the Starch Gel Electrophoresis (mainly insulin-^{125}I) (A case of bindability peak is before the adsorptivity peak)

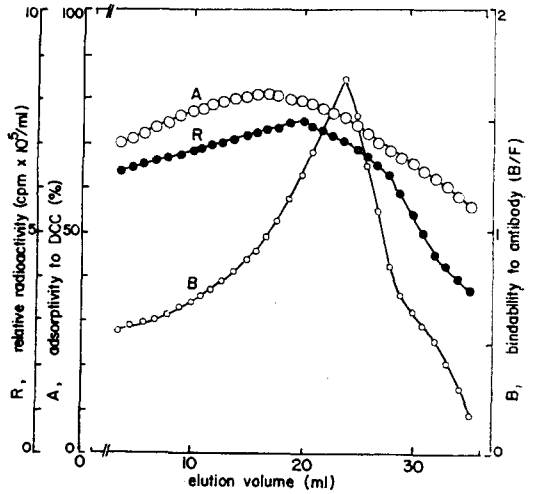


Fig. 4. Distribution of Radioactivity, Adsorptivity and Bindability of the Extract of Zone 2 in the Starch Gel Electrophoresis (mainly insulin-^{125}I) (A case of bindability peak is after the adsorptivity peak)

nce of antiserum and without incubation, the labelled insulin was just allowed to contact with 0.3 ml DCC suspension for about 2 min. with occational shaking at room temperature. B and F was separated by a centrifugation and the radioactivity of charcoal layer was counted. (Fig. 1-Fig. 4).

2.2.4. Insulin RIA

To check the validity of the selection of the well labelled insulin fractions, standard

dose response curves were drawn using labelled insulin fractions which were selected on the bases of either adsorptivity to DCC or bindability to antibody. The protocol was just same as previously reported[8]. (Fig. 5).

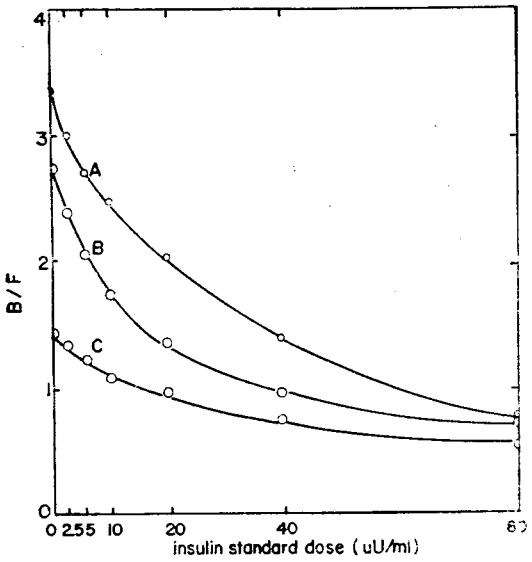


Fig. 5. Plots of Standard Dose Response Curves Using Different Radioiodine Labelled Insulin (A: selected by bindability to antibody after Sephadex filtration, B: selected by adsorptivity to DCC after Sephadex filtration, C:not selected and not Sephadex filtered)

The insulin level in the serum (a reference serum, level 1, Lot. RAB-1-110, DADE Div., American Hospital Supply Corp) was also measured. (Fig. 6).

By extending incubation time to 96 hrs, a standard dose response curve at very low insulin doses was also drawn using the labelled insulin selected by bindability to antibody. (Fig. 7).

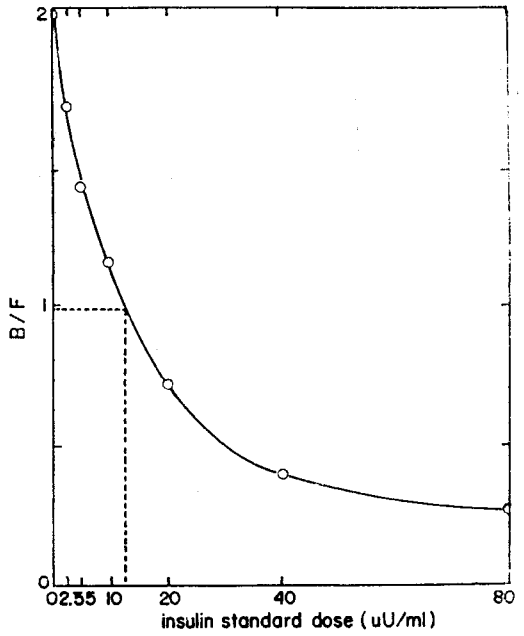
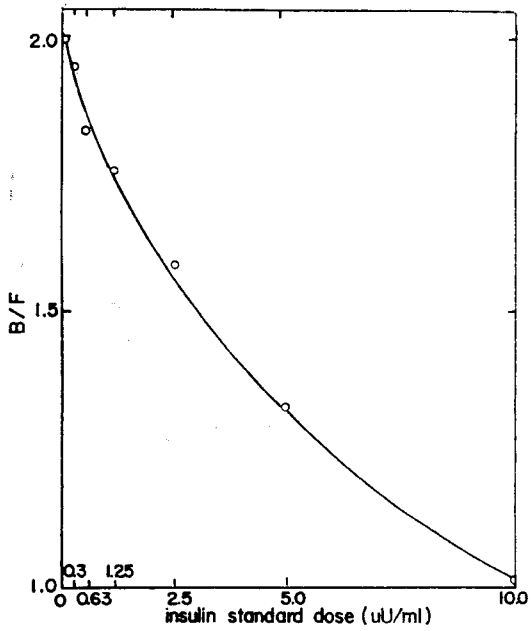


Fig. 6. Determination of Insulin Level in a Reference Serum by a RIA



7. Plots of a Standard Dose Response Curve at Very Low Insulin Doses

3. Results and Discussion

3.1. Sephadex Filtration

As shown in Fig. 1, the zone 1 in the starch gel electrophoresis[7] plate was separated into three distinct peaks by the Sephadex filtration. As previously reported[7],

the first peak is assigned as LPO-^{125}I, the second peak is assigned as insulin-^{125}I, and the third peak is assigned as degraded insulin-^{125} I. The main component of the eluate of the zone **1** in the starch gel electrophoresis is thus LPO-^{125}I. However, the main component of the eluate of the zone **2** is insulin-^{125}I, as shown in Fig.2.

3.2. Immunoreactivity v.s. Adsorptivity to DCC

As shown in Fig. 1, both of the immunoreactivity and adsorptivity of the first and the second fraction are quite low. This fact supports the assignments made as above described. The similar tendency can also be seen in Fig. 2. The second radioactivity peak, assigned as insulin- ^{125}I, is well consistent with those of adsorptivity and bindability (Fig. 1). However, as shown in Fig. 2, 3, and 4, the peak of adsorptivity is generally not well superimposed to the peak of bindability. It means that there is not always close correlation between the two properties.

3.3 Insulin RIA

As shown in Fig. 5, the dose gradient in the standard dose response curve obtained by using the labelled insulin selected on the basis of bindability is larger than that obtained by using the labelled insulin selected on the basis of adsorptivity to DCC. It means that the selection of well labelled insulin fractions by means of a DCC adsorption is not efficient enough. In RIA, the free labelled insulin should better be adsorbed to DCC as far as DCC is used as a separation matrix of F (free unbound insulin) from B (bound insulin), and with this criterion, the labelled insulin showing high adsorptivity to DCC would certainly be a good fraction for RIA. On the other hand, the tracer antigen should be well bound to its antibody, on which base the RIA principle can come into being. The results of the present study indicate that the criterion of the latter overcomes that of the former. Thus, to maintain high accuracy and sensitivity of RIA, the well labelled fraction of the tracter antigen should efficiently be selected by the bindability to its antibody opposing to the insist in the literature[6].

The measured insulin level (12.4 ± 2 μU/ml) for the reference serum was well consistent with the value indicated on the label (12.0 ± 1.2 μU/ml) (Fig. 6). The intra-assay and inter-assay deviation for the reference serum was not more than $\pm16\%$ of the mean value. It suggests that the RIA system is reliable. It is our experience, however, that when using the labelled insulin prepared by a chloramine-T method and purified by a starch gel electrophoresis only, the reliable value could hardly be obtained. The cause of which was attributable to the use of an inadequate tracer.

To confirm the assay sensitivity using the selected fraction (bindability based) of the labelled insulin, a standard dose response curve at extremely low standard insulin doses was also plotted by extending the incubation time upto 96 hrs. As shown in Fig. 7, a curve having sufficiently large dose gradient was obtained in the region of upto 5 μU/ml, which indicates the insulin is well labelled and well selected to use it for RIA of exteremely low insulin level.

4. Conclusion

Since there is only a rough proportionality but not always close correlation between the adsorptivity of the labelled insulin to DCC and the bindability of the labelled

insulin to its antibody, the well labelled insulin fraction for RIA use should better be selected by the test of bindability but not by the test of adsorptivity to keep a good assay reliability. By using a well labelled insulin fraction (labelled by LPO method, and selected by the bindability to antibody), consistent assay data in a reference serum assay as well as a large dose gradient curve at low standard dose level could be obtained.

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