USE OF GEL FILTRATION FOR INSULIN IMMUNOASSAY

YOSHIHIKO HORIUCHI, MITSUAKI KAKINUMA
AND ROKURO IRIYAMA

*The Second Department of Internal Medicine, Hokkaido University
School of Medicine, Sapporo, Japan*

INTRODUCTION

Since radio-immunoassay was introduced by Yalow and Berson (1960) in the determination of plasma insulin, the method has been applied for the assay of other hormones of protein or peptide nature. Yalow's method consists briefly in that, tracer amounts of ^{131}I -insulin and a proper dilution of anti-insulin serum are added to insulin solutions to be tested, which are incubated at 4°C for 3 to 5 days, and then the insulin content in the sample solutions are calculated from the distribution curves of the radioactivities both in the antibody-bound fraction and in the residual. Yalow used chromato-electrophoresis for the fractionation. However, since chromato-electrophoresis is a delicate technic and ^{131}I -insulin of high specific radioactivity is indispensable in the procedure, attempts have been made for modification in this laboratory.

MATERIALS AND METHODS

Materials: Use was made of commercially available crystalline pork insulin and ultra-lente insulin (Novo Industri, A/S, Copenhagen, Denmark), bovine serum albumin (Dai-ichi Kagaku Co., Tokyo, Japan) and pork ^{131}I -insulin which was kindly supplied by the courtesy of Abbott Laboratories, Oak Ridge, U.S.A. The specific radioactivity of the pork ^{131}I -insulin ranged from 270 to 315 mc per mg insulin. It was purified prior to use according to the method described by Banerjee and Gibson (1962).

Standard insulin solution: Crystalline pork insulin was dissolved in 0.15 *M* phosphate buffer saline (pH 7.4) containing 1 per cent bovine serum albumin. Its conventional activity was 24 units per mg insulin.

Antiserum: Male albino guinea pigs weighing from 400 to 700 g were immunized with beef ultra-lente insulin. The insulin (10 mg/ml) was emulsified in an equal volume of complete Freund's adjuvant (liquid paraffine: 8.5 ml arlacel A: 1.5 ml and killed tubercle bacilli: 5 mg) and in total 1 ml of the emulsion was injected in several sites intradermally, twice a week for 5 weeks. The animals were bled 2 weeks after the last injection, and the sera obtained were used for the test.

Reaction of insulin and antiserum: First, 1 ml of the standard insulin solution or the same volume of human plasma was added to 0.1 ml of ^{131}I -insulin, then 0.1 ml of diluted antiserum was added to the mixture. The mixture was allowed to react at 4°C . (The incubation time will be described in the text.)

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Gel filtration: Use was made of dextran gels (Sephadex G 100 and Sephadex G 75, Pharmacia Co. Sweden). The gels were settled into 1.4×13 cm columns, which were then saturated with 0.9 per cent saline. Portions of the above mixtures were charged on the top of the columns thus prepared, and followed by the filtration with saline. Filtrates were fractionated by 2 ml amounts. The time required for the completion of the filtration ranged from 40 to 100 mins. Six or 8 columns were used in one experiment.

Radioactivity: One minute countings were done with a well type scintillation counter (Kobe Kogyo, Kobe, Japan).

RESULTS

Selection of Sephadex gels

Sephadex G 100 and Sephadex G 75 were compared for their separating

Fig. 1. Gel filtration patterns
Distribution of antibody-bound fractions (black areas)
and free insulin fractions (blank area) were shown.

abilities. The results were presented in Fig. 1. In this experiment, 0.5 ml of ^{131}I -insulin and 0.2 ml of 3,000-fold antiserum were added to 0.5 ml of 1 per cent bovine serum albumin solution. After having been incubated at 4°C for 19 hrs., the mixtures were filtered through Sephadex columns. The amount of the antibody-bound insulin (B) was shown in the black area and that of the free insulin (F) in the blank area. Recovery percentage of B as against the total insulin amount (B+F) was 62.8 for Sephadex G 100 and 64.1 for Sephadex G 75, respectively. The maximum error for filtrate collection was 2.0 per cent for Sephadex G 100 and 3.6 per cent for Sephadex G 75, respectively. On the basis of these results, Sephadex G 100 was used throughout the experiment.

Relation between B/F ratio and antiserum dilution

Relation between B/F ratio and antiserum dilution was studied of the reaction mixtures which had been incubated at 4°C for 92 hrs. At zero point 1 ml of ^{131}I -insulin identical to 7.5 μu and the same dose of serial antiserum dilutions were

Fig. 2. Relation of B/F ratio and antiserum dilution titers

added to 1.0 ml of 1 per cent bovine serum albumin and allowed to react at 4°C. Then the reaction mixtures were submitted to gel filtration. In Figure 2, B/F ratio were plotted against antiserum dilution titers. An approximately linear relationship was found to exist between the two.

Incubation time and standard curves

The reaction between insulin and antiserum was studied kinetically. One half ml of ^{131}I -insulin and 0.5 ml of a 3,000-fold antiserum were added to 5 ml of pork insulin solution ($15\ \mu\text{u/ml}$), and incubated at 4°C. After having been allowed to react for 6, 24, 48, 72, 96, and 168 hrs., respectively, 0.7 ml amounts were drawn from the reaction mixture and submitted to gel filtration. As seen in Figure 3, the reaction between insulin and antiserum was found to reach its equilibrium after 96 hrs. incubation.

The standard curves were obtained by incubating the reaction mixture at 4°C for 19, 69, and 92 hrs. respectively, and presented in Figure 4. In this experiment, $7.5\ \mu\text{u}$ to $120\ \mu\text{u}$ per ml of serial dilutions of the standard insulin mixed with 0.1 ml of tracer insulin and the same dose of a 3,000-fold antiserum were allowed to react, then the mixtures were submitted to gel filtration. In the figure, the standard curves expressed as B/F ratio were found to be slightly higher for

Fig. 3. Kinetic study of the reaction between insulin and antiserum

Fig. 4. Standard curves expressed as B/F ratio, which were obtained after various incubation times

92 hrs. incubation than 69 hrs. incubation, however the two curves were identical to each other in their configurations. From these results, the following experimental conditions were adopted.

Tracer insulin:	0.1 ml containing 7 to 10 μ u
Antiserum:	0.1 ml of a 3,000-fold dilution
Sample solution:	1 ml
Incubation:	almost 4 days at 4°C
Column volume:	1.4 $\times$ 13 cm

Recovery of pork insulin added to human plasma

In order to evaluate the recovery of pork insulin added to human plasma, two different serial dilutions of pork insulin were prepared: one was made in 1 ml of 1 per cent bovine serum albumin, the other was in 1 ml of 50 per cent human plasma. They were then mixed with tracer insulin and antiserum, and incubated. In Figure 5, B/F ratio were plotted against the pork insulin amounts. The two curves obtained were found to parallel to each other with a difference of 13 μ u pork insulin, suggesting a good recovery of the pork insulin added.

Fig. 5. Recovery of pork insulin added to human plasma. Two curves were found to parallel to each with a difference of 13 μ u pork insulin amounts.

Assay of human plasma insulin

As the results of the above experiments suggested the possibility of human plasma insulin being determined in the same manner, the method was applied to assay of human specimen. Thus mean fasting insulin level as obtained in 9 non-diabetic persons was calculated to be $25 \pm 7.8 \mu$ u per 1 ml plasma on crystalline pork insulin reference. No unfavourable effect was found in assaying 2-fold dilutions of plasma insulin.

DISCUSSION

As is well known, the radio-immunoassay of plasma insulin is generally carried out on the basis of the immune reaction between plasma to which tracer amounts of radioactive insulin was added and the anti-insulin antibody. In this method of the separation of the antibody-bound insulin from the residual free insulin plays

the most important role. Yalow *et al.* (1960) used chromato-electrophoresis for this purpose. However, because of its technical difficulties, simplified modifications have been proposed by some investigators. (Grodsky and Forsham, 1961; Morgan and Lazalow, 1963)

In the present study, gel filtration was attempted for the separation. Since gel filtration is harmless for differential fractionation of materials of different molecular weights, the present method is thought to be suitable for the assay of peptide hormones of relatively low molecular weights.

Recently, gel filtration has been used by Manipol *et al.* (1962) for the demonstration of anti-insulin antibody. For this purpose, only Sephadex G 75 has been by far used. In this case, columns of a large volume have been required to obtain satisfactory results. In the present study, the use of Sephadex G 100 instead of Sephadex G 75 has been found to be more suitable for separating the antibody-bound fraction from the free fraction with less column volume.

The results of the present basic experiments on insulin immunoassay quite agreed with the investigations of other authors who used immune precipitation and/or chromato-electrophoresis (Yalow and Barson, 1960; Morgan and Lazalow, 1963; Samols and Bilkus, 1964).

However, one minor disadvantage of the present method is that regarding the so called "radiation damage". When human plasma is added with ^{131}I -insulin the radiation damage has to do with the serum protein filtrable prior to use by removing the albumin concerned.

SUMMARY

In the present study, gel filtration was tested for radio-immunoassay of plasma insulin, as a mean for separating antibody-bound insulin from the residual free insulin.

Using Sephadex G 100 columns, the curves of the two fractions, bound and free were found to be dissociated within an error of 2.0 per cent for eluate collection. The results of the basic experiments showed that this procedure is suitable for immunoassay of insulin as well as for that of other peptide hormones of relatively low molecular weight.

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