Studies on the Production of Insulin Radio-immunoassay Kit

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인슈린 방사면역 측정킷트의 제조에 관한 연구

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요 약

인슈린-^{125}I를 클로라민 -T 법을 써서 30~35%의 표지 수율로 얻었으며 그 비방사능은 대략 100 uCi/ug 이었다.

생성물을 방사면역 측정용으로 표준화하기 위해 최적 표지부분을 전분겔 전기영동, 래디오오토그래피 및 인슈린 항체와의 인큐베이션등으로 선정하였다. 선정된 표지 인슈린으로 표준화한 결과 50 μU/ml 까지의 인슈린농도에 대해 항체와의 결합 인슈린 -^{125}I (B)와 미결합 인슈린 -^{125}I (F)와의 비 (B/F)가 0.2~1.6으로써 매우 가파른 표준 dose-response 곡선을 보여 주었다.

인슈린 방사면역 측정킷트를 만들어 그 사용 가능기간을 측정하였으며 일상 생산방법을 확립하였다.

1. Introduction

Radio-immunoassay (RIA) provides a capability of measuring physiological substances in nanogram (10^{-9}g) and picogram (10^{-12}g) range. The growth of the field is attested to by the number of references used by Skelly et al[1]. In recent years, the quest for the informations by physicians has grown rapidly in this country. Even though some commercial kits of foreign made can be obtained, it is quite costly, and hardly can get in early days. Further, more or less detereoration of the expensive RIA kit is inevitable mainly due to the long term transportation of the labelled biological meterial. Actually, it is a main reason why physicians and researchers cannot popularly use the useful tool. Under the circumstances, the authors have studies on the preparation of insulin RIA kits as the first stage of the RIA development project. The practical data for the preparation of the insulin RIA kit are hardly available.

In present paper, the authors describe the protocol of a micro-scale labelling and purification as well as the standardization of the labelled insulin for RIA use in detail. The trouble-shooting methods for some difficulties often encountered in the assay procedure, and the characteristics of the-prepared kit are also briefly discussed.

2. Experimental

(1) Materials

a) Insulin; porcine, recrystallized, 24.4 USP U/mg, Schwarz/Mann, U.S.A.

b) Anti-porcine insulin-serum; titer; 1 : 500000, kindly donated by Dr. J.H.Kim of Mercy Hospital & Medical Center, Chicago, U.S.A.

c) Sodium iodide-^{125}I; carrier and reducing agent-free for protein iodination, radioactivity concentra-

tion; 100 mCi/ml., Radiochemical Centre, England.

d) Starch powder; partially hydrolyzed for electrophoresis use, Sigma, U.S.A.

e) Buffer solutions;

(i) For gel preparation, 1.55 g of boric acid and 0.4 g of sodium hydroxide were dissolved into double distilled water and made up to 1 liter (final pH 8.6)

(ii) For electrophoresis; 18.55 g boric acid and 3.65 g sodium hydroxide were dissolved into double distilled water, and made up to 1 liter (final pH 8.6)

(iii) For labelling; 0.5 M phosphate buffer, pH 7.4

(iv) For incubation and standardization; 0.02 M veronal buffer, pH 8.6; 4.13 g (0.02 mole) of barbital sodium (C.P., Merck) was dissolved into 500 ml double distilled water., (A), 3.68 g (0.02 mole) of barbital (C.P., Merck) was dissolved into 1,000 ml double distilled water.. (B). Mix (A) with (B) in the volume ratio of 25 : 9.6 and diluted to 50 ml. (twice of the volume of (A)) using double distilled water.

f) Dextran coated charcoal suspension; it was made by mixing the following two solutions in the same volume ratio;

(i) 2.5 g dextran (RIA grade, Schwarz/Mann) was dissolved into 500 ml veronal-buffer, pH 8.6, containing 0.25% bovine serum albumin,

(ii) 25 g charcoal (RIA grade, Schwarz/Mann) was suspended into 500 ml veronal buffer, pH 8.6, containing 0.25% bovine serum albumin.

g) Chloramine-T solution; 52.5 mg chloramine-T (Eastman Kodak) was dissolved in 10 ml phosphate buffer, pH 7.4.

h) Sodium metabisulfite solution: 48 mg-sodium metabisulfite (Wako) was dissolved in 10 ml phosphate buffer, pH 7.4.

i) Blue plasma; small amount of bromophenolblue was added to heparinized-human plasma.

j) Insulin standard solution; 2.1 mg of standard insulin was dissolved in 1 liter double distilled water, 1 ml of which was taken and diluted it to 100 ml with 0.02 M veronal buffer, pH 8.6, containing 0.25% bovine serum albumin. The insulin concentrantion of the final solution is 500 uU/ml. The other standard solutions of 250 uU/ml, 125 uU /ml etc. were made by asuccessive dilution.

(2) Experimental procedure

a) Labelling

Hunter & Greenwood's labelling procedure for human growth hormone[3] was slightly modified for insulin. The following solutions were pipetted by using disposable micropipettes, and set aside in the shelf; (i) 20 ul phosphate buffer, (ii) 280-300 uCi sodium iodide-^{125}I solution (usualy 3 to 10 ul depending on the radioactivity concentration), (iii) 2 ul insulin solution in which 1 ug insulin is dissolved in ca. 10^{-4}M hydrochloric acid solution, pH 4, (iv) 10 ul chloramine-T solution, (vi) 20 ul sodium metabisulfite solution, (vii) 20 ul blue plasma.

These solutions were added in regular ordrer-into a small conical shaped reaction vessel (Lorex glass 1 ml capacity) in 40 sec. over-all.

b) Separation of Labelled Insulin

As soon as the reaction was terminated the whole reaction mixture was applied into the holes of starch gel plate which was preferably prepared in advance of conducting the reaction as following; 30 g of hydrolyzed starch powder was mixed with 250 ml of borate buffer in 500 ml filtering flask, and heated slowly over hot plate with constant stirring with magnetic stirrer bar[4]. As soon as the solution started to boil, heater was turned off, and a vacuum line was attached to the filtering flask, that caused the dissolved air to come out of the gel. Vacuum was removed and the gel was poured into a perspex former (16×8×1 cm) with the sample application hole and the filter paper wicks in position. The gel plate was transfered to refrigerator for about 1 hr to allow it to cool and set. Electrophoresis was carried out in refrigerator for 4 hrs at constant voltage of 300 V. Gel plate was removed, and radioautography was conducted. The gel which comes under the spot on the developed X-ray film was sliced into about 2 mm width in horizontal direction using a razor blade. Each slice was put into test tube separately, and numbered. Then they were-deep frozen. Using veronal buffer,

pH 8.6, containing 0.25% bovine serum albumin as eluate the labelled hormone was eluted from the gel; 1 ml of the buffer was added to each tube, and gently pressed the gel several times with round tipped Lorex glass bar, and then compressed out the eluate. Such manipulation was repeated three times for all of the tubes (usually 8 to 10 tubes) using new 1 ml eluate each time. In some cases the eluate was subsequently centrifuged to remove the trace amount of starch gel came up with the eluate. After-measurement of radioactivities for each-fraction, they were diluted to the radioactivity concentration of 200,000 c/m/ml using the same veronal buffer. The radioactivity in aliquot of electrolyte (anode side) was also counted to check the over-all labelling yield according to the following equation;

$$\text{Labelling yield}(\%)=\frac{\sum ai+\sum bi}{\sum ai+\sum bi+C}\times 100 \cdots\cdots (1)$$

where a, b, and c are radioactivities (c/m) in eluate, in starch gel and in total volume of anode side electrolyte, respectively (Table Ⅰ, Ⅱ).

c) Selection of Well Labelled Fractions

To select the well labelled fractions for insulin RIA, incubation mixture was made as shown in Table Ⅲ. After incubation for 48 hrs at 4°C, the labelled insulin bound to its antibody (B) and the free labelled-insulin (F) was separated by addition of 0.4 ml dextran coated charcoal (DCC) suspension, agitation, and subsequent centrifugation. The radioactivity for the supernatant (antibody bound hormone (B)) and precipitate (free hormone (F)) were separately counted. After background activity subtraction the B/F ratios were calculated for each fraction according to the following equation;

$$B/F=\frac{B-[(B+F)\times D]}{F} \cdots\cdots (2)$$

where D is the ratio between the activity in supernatant (B) and total activity (B+F) in each

Table I. Comparison of the Outline of Labelling for Two Proteic Antigens

	Present Work	In Ref[3].
Reaction medium	0.5 M Phosphate buffer pH 7.5	0.5 M Phosphate buffer pH 7.5
Subject	Insulin 1 ug	HGH 5 ug
^{125}I (uCi)	280−300	2000−4000
Chloramine-T (ug)	52	100
Reaction time (sec)	10	60
Labelling yield (av.%)	33	65
Sp. activity of the product (uCi/ug)	75−100	250−590

Table II. Effect of Reaction Volume on the Specific Activity of the Labelled Insulin#

Run No.	Cold insulin (ug)	^{125}I used (ul)	^{125}I used (uCi)	% ^{125}I utilized	Specific radioactivity (uCi/ug)	% Labelled insulin bound to antibody**
1	1	3	300	30	100 (1)	66
2	1	4	300	36	108 (3)	68
3	1	5	260	20	52 (1)	65
4	1	4	300	35	105 (1)	58
5	1	4	300	38*	114 (2)	40
6	10	4	300	44**	13 (2)	42

Experimental details are given in the text. Percentage of ^{125}I utilized is not corrected for the loss due to the adsorption to glass surface. Specific radioactivities are given as arithmetical means with the number of experiments in parenthesis.

* 80 ug chloramine-T used.

** 80ug chloramine-T used., and the reaction-time elongated to 120 sec.

*** Roughly measured by adsorption to DCC suspension after being incubated with the insulin antibody but without cold insulin.

Table III. Preparation of Incubation Mixture for Selection of well Labelled Insulin

Tube No.	Antiserum* (ml)	Labelled** insulin (ml)	Veronal*** buffer (ml)
1	0.0	0.1	0.9
1′	0.1	0.1	0.8
2	0.0	0.1	0.9
2′	0.1	0.1	0.8
10	0.0	0.1	0.9
10′	0.1	0.1	0.8

* titer 1:500,000

** 200,000 c/m/ml

*** pH 8.6 contains 0.25% bovine serum albumin

Table IV. Preparation of Incubation Mixtures for Standardization and Measurements of insulin Levels in Unknown Serum Samples

Order of addition	1	2		3	4	5
Reagents & q' ty	Veronal buffer (ml)	Insulin standard		Unknown sample (ml)	Antibody** (ml)	Labelled** insulin *
Tube		Volume (ml)	Concetration (mcU/ml)*			
Control	0.9	0	0	—	0	0.1
Control	0.9	0	0	—	0	0.1
1	0.8	0	0	—	0.1	0.1
1′	0.8	0	0	—	0.1	0.1
2	0.7	0.1	3.125	—	0.1	0.1
2′	0.7	0.1	3.125	—	0.1	0.1
3	0.7	0.1	6.250	—	0.1	0.1
3′	0.7	0.1	6.250	—	0.1	0.1
4	0.7	0.1	12.500	—	0.1	0.1
4′	0.7	0.1	12.500	—	0.1	0.1
5	0.7	0.1	25.000	—	0.1	0.1
5′	0.7	0.1	25.000	—	0.1	0.1
6	0.7	0.1	50.000	—	0.1	0.1
6′	0.7	0.1	50.000	—	0.1	0.1
Control for unknown sample	0.8	—	—	0.1	0	0.1
Control for unknown sample	0.8	—	—	0.1	0	0.1
Unknown sample	0.7	—	—	0.1	0.1	0.1
Unknown sample	0.7	—	—	0.1	0.1	0.1

* Cold insulin in mcU in the volume (in 1-ml of incubation mixture)
** Titer; 1:500,000
*** Selected good fraction displaying 200,000 c/m/ml

control tube.

Thus, the percent of antibody bound fraction of labelled insulin for-each fraction, or each batch were assessed and selected the well labelled fraction (Table Ⅱ).

d) Standardization

To standardize the selected well labelled fractions for RIA, incubation mixtures were-made as shown in Table Ⅳ. The well labelled fractions were used after asceptic-filtration. After incubation for 48 hrs at 4°C, B/F ratios were determined in the same way as described in previous section, and the standard dose-response curve was plotted (Fig. 2). In case of patient's serum the control for unknown sample should be assumed as control for standard. Thus, the insulin levels in-serum samples could be determined.

e) Preparation of Kits, and Determination of Shelf-life

The following materials of definite quantities required for duplicate measuring insulin levels of 5 individuals were freeze dried, and stocked in refrigerator (kit components);

(i) Labelled insulin, millipore filtered, 200,000 c/m/ml, 3 ml/btl·····1 btl
(ii) Cold insulin standard; 31.25, 62.50, 125.00, 250.00, and 500.00 uU/ml/btl·····each 1 btl
(iii) Insulin antiserum. titer 1:500,000, 3 ml/btl·····1 btl
(iv) Barbital 0.368 g/btl, barbital sodium 0.826 g/btl·····each 1 btl
(v) Dextran 0.125 g/btl, charcoal 1.25 g/btl·····each 1 btl
(vi) Bovine serum albumin; i) 0.12 g/btl, 0.05 g/btl·····each 1 btl

Weekly plotting of the standard dose-response-curve was carried out to determine the shelf life of the prepared kits.

3. Results and Discussion

(1) Labelling, Purification, and Selection of Well Labelled Fractions

The most important thing in labelling of antigen is to obtain a highly purified product having a high specific radioactivity without sacrificing any of its immunoreactive properties. The probable immunoreactivity change of the antigen due to the labelling and purification procedure should always be checked. Labelled antigen of a high specific activity having the intact immunological behavior is most useful since the quantity of labelled antigen in an assay should be kept at a minimum. In the earlier literatures[5],[6],[7] the labelling carried out using 10 to 20 mCi of ^{125}I with 0.1 to 1 mg antigen obtaining the specific radioactivity of the product of upto 5 uCi/ug. Greenwood and Hunter[3] had modified the early known method of labelling human growth hormone by means of a chloramine-T procedure, and obtained the product of which spcific radioactivity was 250-590 uCi/ug. Since less than 1-1.2 atom of I should-be introduced per protein molecule[8], the upper limit of the specific radioactivity is 2-3 Ci/mg depending on the protein-molecule. If more than 1-1.2 atom of ^{125}I is introduced per protein molecule, the change of immunological behavior will-occur. When less than 1 atom had been introduced and thus the specific radioactivity was less than 300 uCi/ug, the preparations were reported to be quantitatively identical with the unlabelled hormone as measured by an immuno-electrophoretic method[9]. Since insulin has 4 tyrosine rings and each ring can accommodate two ^{125}I atoms, the total accommodable number of ^{125}I atom is 8. When total of 8 atoms are substituted, the specific radioactivity will be 3 mC/ug insulin. Thus, the needful radioactivity for insulin labelling will be 370 uCi for only one ^{125}I atom substitution. In this consideration the reactants ratio in present work is quite adequate (Table 1). However, the specific activity of the product was slightly-lower due to the low labelling yield. The low labelling yield may attributable to the slight detereoration of chloramine-T. In general the low specific activity product gives a poor sensitivity in the assay[10]. Even though the poor sensitivity was found in some cases the preparations were often satisfactory. As Table II shows, the effect of-reaction volume on the specific activity of the product is remarkable in case of increasing the amount of-choloramine-T or elongating reaction time the yields were slightly increased, while the the product's poor binding to the antibody was noticeable in such cases. Izzo et al[8] reported that the ^{131}I labelling yield for insulin is about 60% in case of using iodine monochloride. Thus, the specific activity was 100-600 uCi/ug. However, it is wondered how much of the labelled insulin could combine with its antibody. Furthermore, the commercial, so called 'carrier free' ^{131}I is never, in strict sense, free of stable or carrier iodine. Since ^{131}I decays on storage, while the other two possible isotopes of ^{127}I and ^{129}I ($t1/2$; $1.6\times10^{7}y$) do not, the ratio of stable to radioactive iodine increases progressively. Therefore, when more than 1 atom of ^{131}I was introduced per molecule of insulin[8], and thus far from the intact, the specific activity is not only important in such case but also the binding ratio of the labelled insulin to the antibody is quite important. The generally low labelling yields in present work may also be attributable to the short reaction time of nearly 10 sec from the chloramine-T addition to the sodiummetabisulfite addition.

As Fig. 1 shows, the labelled insulin is about 4 cm migrated from the origin of the autoradiogram. The fractions showing higher binding to-antibody were mostly located at one fifth from the anode of the spot. However, sometimes it was one fifth from the cathode side of the spot with unknown reason. About 80% of the labelled insulin could be extracted from the gel using 3 ml veronal buffer. Among the 8 to 10 fractions, the well labelled were usualy 2 to 3, and consequently the other fractions were discarded. Anyway, the selection of well labelled fractions is essential for obtaining a steep dose-

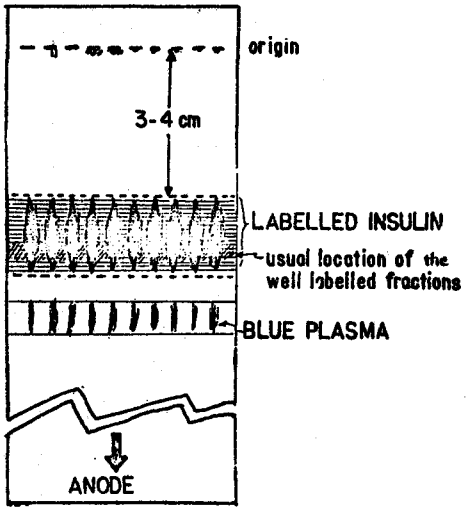

Fig. 1. A sketch of the autoradiogram for a starch gel plate.

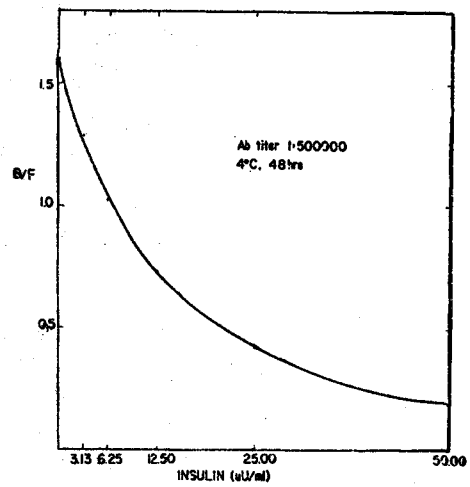

Fig. 2. A typical-plot of a standard dose-response curve.

response curve.

(2) Standardization of Labelled Insulin

Taking a sequential saturation into account, the incubation mixture was made by adding the reagents in the following order; a) veronal buffer, b) insulin standard (or unknown sample), c) anti-insulin serum, and d) labelled insulin. According to Zettner and Dully[11] a sequential saturation technique is useful for steepening the dose-response curve. They also noticed that the sequential saturation technique gives a lower precision than that in equilibrium saturation technique, if the incubation temperature is high. In the sequential saturation technique, the mixture not containing labelled antigen is incubated to proceed an equilibrium, and then the labelled antigen is added. In present work, however, the order of the addition of reagents was based on this technique, and incubated it only once. Keeping such order of addition of reagents was also useful to avoid a probable radioactivity contamination.

As Fig. 2 shows, the dose-response curve is steep ranging the B/F-ratio from 0.2 to 1.6 which is better than that reported by Higa et al[10]. As Fig. 3s hows,

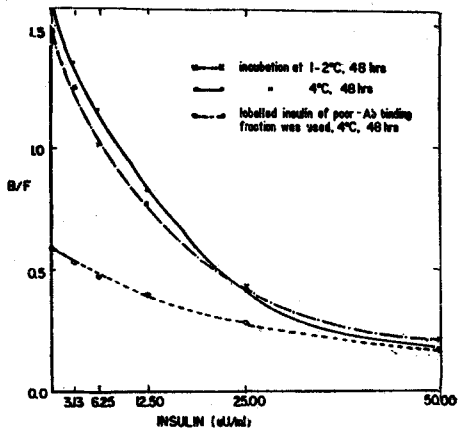

Fig. 3. The variation of the dose-response curve with the incubation temperature, and the labelled insulin fracion.

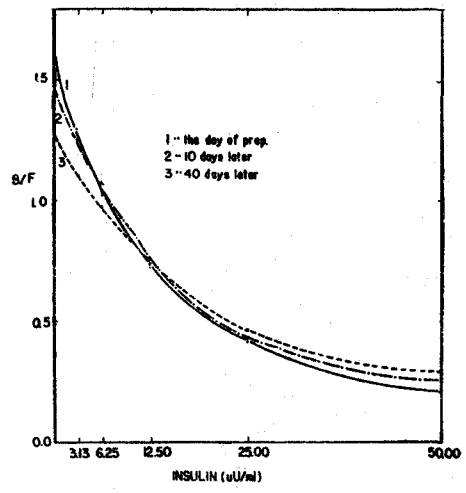

Fig. 4. The variation of dose-response curve with the elapse of the kit preservation time.

the slope is getting lower as the kit storage time was elongated. Five weeks later the slope was still moderate as shown in the figure. When the labelled insulin of lower antibody binding fractions were used for the standard curve plot, the slope was quite low. In case of incubation at 2°C for 48 hrs-using selected good labelled fractions, the slope was also lower than that obtained after incubation at 4°C, for 48 hrs (Fig. 4). It may be due to the insufficient equilibrium between the antigen and antibody.

(3) Preparation of Kits and Measurement of Shelf Life

When the selected well labelled fractions were diluted to 200,000 c/m/ml, the total volume was usually 20 to 25 ml. In case of duplicate running, the labelled insulin solution of 1.4 ml is needed for standard curve-plot, and 0.4 ml for a test of one unknown sample. Therefore, about 3.5 ml of labelled insulin solution is needed for the test of 5 unknown samples. For convenience, definite amount of barbital sodium, bovine serum albunin, dextran, and charcoal etc. were separately packed as the kit components. One hr is sufficient for the preparation of incubation mixture. The procedure is simple and convenient. In the assay the followings are important-for trouble shooting; a) to use double distilled water throughout the assay, b) to-make temperature equilibrium before mixing the reagents for incubation, c) to maintain definite ionic strength in the mixture, d) to avoid repeated freezing and thawing, e) to keep the exact temperature and time of incubation, f) to keep away from the contaminants such as chemicals and bacteria, and g) to keep your hands away from the incubation tube bottom etc.

As a summary, the labelled insulin obtained according to the established procedure in present work can effectively be used for the insulin RIA. The selection of the well labelled fractions and the standardization procedure are all efficient enough for a kit preparation.

4. Conclusions

(1) ^{125}I labelling of insulin and the standardization of the labelled insulin for RIA use are well established.

(2) A protocol of routine kit preparation is established.

(3) Maximum two 100 tube kits can-be prepared from one batch.

(4) The kits show steep dose-response curve. The expire date is usualy 40 days after preparation.

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REFERENCES

(1) Skelly, D.S. et al., *Clin. Chem.* ***19***, *146 (1973)*

(2) V. Herbert et al., *J. Clin. Endocrinol. & Meta.* ***25***(*10*), *1375 (1965)*

(3) R.C. Greenwood, and W.M. Hunter, *Biochem. J.* ***89***, (*123*), *114 (1963)*

(4) Plummer, Introd. Prac. Biochem., *McGraw-Hill, p.63 (1971)*

(5) Samles, R. and William, H.S., *Nature, London,* ***190***, *1211 (1961)*

(6) Staub, A. et al., *Int. J. Appl. Radiat. Isotopes,* ***2***, *59 (1957)*

(7) Yalow, R.S., and Berson, S.A., *J. Clin. Invest.,* ***39***, *1157 (1960)*

(8) Joseph L. Izzo, et al., *J. Biol. Chem.,* ***239*** (*11*), *3743 (1964)*

(9) Hunter, W. M., and Greenwood, F.C., *Biochem. J.* ***85***, *39 (1962)*

(10) O.H. Higa et al., *Report, Proc. Symp., Istanbul, Sept., 1973 IAEA, Vienna (1974), IAEA-SM-177/84*

(11) A. Zettner and P.E. Duly, *Clin. Chem.,* ***20***, (*1*), *5(1974)*