A Study on Radiolabelling Method in Radioimmunoassay

—Part 1. A New Method for the ^{125}I-Labelling of Human Thyrotropin under Mild Cenditions—

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=국문초록=

放射免疫測定에서 放射性 同位元素 標識法 개발에 관한 硏究*

—第1編. Human Thyrotropin의 온화한 조건하 ^{125}I-표지를 위한 새로운 방법—

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Human thyrotropin(h-TSH)의 ^{125}I-표지를 실온에서 소량의 Chloramine T를 산화제로 사용하여 수행하였다. 이 방법은 다량의 Chloramine T를 사용하는 종래의 방법과 비교하여 균일한 방사능 표지를 용이하게 하고 강력한 산화제인 Chloramine T에 의한 h-TSH의 파손을 줄여 주었다. 새로운 방법에 의해 합성된 h-TSH*는 항체에 대한 친화도와 유효기간에 있어서 Daiichi 회사의 상품에 비교하여 손색이 없었다.

^{125}I-Labelling of human thyrotropin (h-TSH) Was performed using a small amount of chloramine T (CT) as an oxidant at room temperature. When compared with the conventional method that uses a large amount of CT, this method facilitated uniform labelling and reduced the damage of h-TSH by CT, a strong oxidizing reagent. h-TSH* prepared by the new method was comparable to the commercial product of Daiichi Company, in terms of affinity to antibody and effective period.

* 본 연구는 서울대학교병원 연구비 보조로 이루어졌음.

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INTRODUCTION

^{125}I-Labelled human thyrotropin(h-TSH*) is used as the tracer for the radioimmunological estimation of h-TSH in serum.[1~5] The preparation of h-TSH* is carried out either by the direct iodination of the hormone.[6] or by the conjugation method.[7] The direct iodination involves the oxidation of $^{125}I^-$ to $^{125}I^+$ and the subsequent reaction of $^{125}I^+$ with the tyrosyl residues of h-TSH (eq. 1). The radioactive iodide ion is oxidized by

$$^{125}I^- \xrightarrow{\text{oxidant}} {}^{125}I^+ \xrightarrow{\text{h-TSH}-\langle\bigcirc\rangle-\text{OH}} \text{h-TSH}-\langle\bigcirc\rangle(\text{I})-\text{OH} \quad (1)$$

using oxidants such as chloramine T (CT)[8~10] and lactoperoxidase[11~13] or by electrochemical methods.[14,15] The conjugation method employs the chemical modification of h-TSH with a ^{125}I-labelled ester[16].

To date, h-TSH* has been most frequently prepared by the conventional method originally developed by Greenwood and coworkers[17], which uses a large amount of CT and a relatively short reaction period(20—30 sec at 4°C). This method, however, imposes several experimental difficulties for reproducible and dependable labelling. As the reaction completes within a very short period of time, thorough mixing of the reaction mixture to ensure uniform labelling is usually difficult. In addition, the use of a large amount of an oxidant may cause the polymerization of the protein[18,19] and oxidize chemically susceptible functional groups of the hormone, leading to a completely altered immunological behavior[20~22].

We have attempted to label h-TSH in the presence of a small amount of CT in order to overcome these difficulties. The labelling procedure developed in this laboratory and the radioimmuno assay based on the h-TSH* prepared by the new method are reported in this paper.

EXPERIMENTAL SECTION

Materials and Reagents

(1) highly purified h-TSH (6 U/mg): This hormone was the product of Biodata, Italy. When used in the radiolabelling experiments, h-TSH was dissolved in water. The pH and the buffer capacity of the resulting solution was not explicitly specified by the producer.

(2) radioactive iodide: IMS 30 carrier-free $^{125}I^-$ in sodium hydroxide, at a concentration of 100—240 mci/ml was purchased from The Radio Chemical Centre, Amersham, Bucks, U.K.

(3) reagents used for radioimmunoassay: Radioimmunoassay kits including standard h-TSH solution (320 U/ml), anti h-TSH rabbit serum, and anti rabbit β-globulin goat serum were purchased from Daiichi, Japan. Normal rabbit serum is essential for the precipitation of the antibody complexes in the radioimmunoassay of h-TSH. It was found that normal rabbit serum was mixed with h-TSH* in the Daiichi kit. Thus, normal rabbit serum was purchased from Biodata, Italy and used for the examination of the immunological behavior of h-TSH* prepared in this laboratory.

(4) chloramine T and sodium metabisulfite: These were of reagent grade, and the respective aqueous solutions were freshly prepared prior to being used.

(5) buffers: Buffer A; 0.3M sodium phosphate at pH 7.5, buffer B; 0.03M sodium phosphate at pH 7.5, buffer C; 0.03M sodium phosphate and 0.25% BSA at pH 7.5,

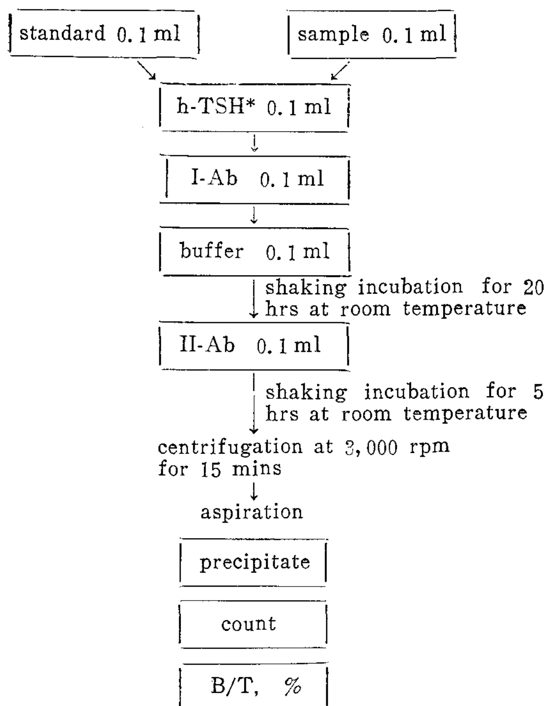
(6) Sephadex G-75: This was the product of Pharmacia, Sweden.

^{125}I-Labelling of h-TSH

(1) using a large amount of CT at 4°C: Labelling of h-TSH by the method of Greenwood and coworkers was carried out by using a large amount of CT as the oxidant. To a mixture of Na ^{125}I (1 mci/10 μl) and h-TSH (15 μg/10 μl water), CT (88 μg/10 μl buffer B) was added and the the resulting solution was stirred at 4°C for 30 sec. The reaction was quenched by the addition of sodium metabisulfite (240 μg/100 μl buffer B) and, subsequently, potassium iodide (1 mg/100 μl buffer B). The product mixture was then separated on a Sephadex G-75 column at 4°C.

(2) using a small amount of CT at 25°C: To a mixture of buffer A (10 μl), Na ^{125}I (0.1-0.7 mci/10 μl) and h-TSH(1-15 μg/10 μl water), CT (0.5-1.8μg/10 μl buffer A) was added. The resulting solution was well stirred for 12-15min at 25°C before being applied to a Sephadex G-75 column. The iodination reaction was sometimes quenched by adding sodium metabisulfite (5 μg/10 μl buffer B) prior to the separation on the Sephadex column. The labelling reaction was performed in a glass tube (4 x 30 mm) which contained a small wire piece as a magnetic stirring bar.

Scheme 1



(3) trichloroacetic acid (TCA) precipitation of h-TSH: During the course of the radiolabelling of h-TSH at 25°C, 1 μl aliquots of the reaction mixture were removed and added to 1 ml solutions of KI (50 μg) in buffer C.

One ml of TCA is added to the resulting mixture, which was then centrifuged at 3,000 rpm for 15 min. From the radioactivity of the whole mixture and the precipitate, the degree of ^{125}I-incorporation to h-TSH was calculated.

Purification of h-TSH*

Separation of h-TSH* was carried out at 4°C with a Sephadex G-75 column (1×60 cm), eluting with buffer B at an elution rate of 0.5 ml/min. Fractions (1 ml) were collected in plastic tubes containing 50 μl of buffer C. The radioactivity of each tube was measured by a counter equipped with a teletype (Packard Model 3320 3JA).

Radioimmunoassay

Two fractions with the largest radioactivity were taken from the protein portion of the Sephadex chromatogram and diluted with buffer B. Portions (10^6 cpm/tube) of the h-TSH* sample were stored at −20°C until they were used in radioimmunoassay. Radioimmunoassay of h-TSH was performed according to the method of the Daiichi kit, which is summarized in Scheme 1.

Results and Discussion

Radiolabelling of h-TSH

The chromatogram for the gel filtration of h-TSH* which was prepared according to the conventional method using a large amount of CT (CT, 88 μg/40 μl ; $[CT]_0/[I^*]_0=800$) is illustrated in Figure 1. The presence of small peaks following the major protein peak may be related to fragmented or damaged proteins. This chromatogram, when compared with that obtained previously in this laboratory[23], reveals that radiolabelling with great excess of CT is hardly reproducible. Oxidation of

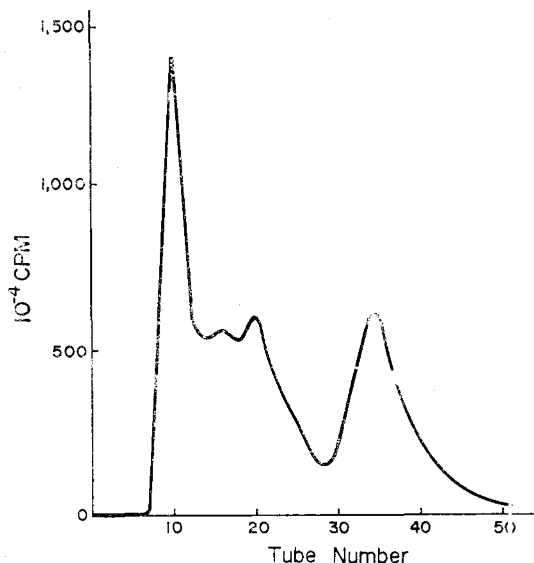


Fig. 1. Chromatogram for the Sephadex G-75 separation of h-TSH* prepared by the conventional method.

iodide ion under this condition is very fast, the overall labelling being complete within 20—30 sec. Thus, the method of mixing the reactants would be crucial for obtaining reproducible results. As the degree of exposure of h-TSH to the large amount of the strong oxidant cannot be controlled strictly, the immunological activity of h-TSH* would be reproduced by this method only with great difficulties.

In order to overcome these complications, radiolabelling of h-TSH was attempted using small amounts of CT (CT, 0.5−1.8 μg/40 μl; $[CT]_0/[I^*]_0=10-150$). The reaction was carried out at 25°C, and the incorporation of radioactivity into h-TSH approaches plateau 10 min after the initiation of the reaction as illustrated in Figure 2. Uniform radiolabelling of h-TSH is ensured when the reaction poceeds this slowly because efficient mixing of the reactants can be achieved quite easily. A typical chromatogram for the Sephadex G-75 separation of h-TSH* prepared by this method is illustrated in Figure 3. Six labelling experiments have been performed using various amounts of the reactants, all of which manifested chromatograms similar to that of Figure 3. The absence of small peaks after the major protein peak suggests that the damage of h-TSH during the radiolabelling reaction is minimal. Thus, it appears that the direct reaction of h-TSH with CT is not appreciable under the condition that iodide ion is oxidized very mildly.

The portion of radioactivty incorporated into h-TSH, (I* bound to h-TSH)/(total radioactivity), was not affected appreciably when the amount of CT was fixed at 1.6×10^{-4}M and those of ^{125}I and h-TSH were varied over $1.1\times10^{-6}-7.9\times10^{-6}$M and $0.2\times10^{-6}-1.8\times10^{-6}$M respectively. However, (I* bound to h-TSH)/(total radioactivity) increased significantly, although not linearly, when $[I^*]_0$ was fixed at 0.3 mCi and $[CT]_0/[I^*]_0$ was raised from 10 to 40. Thus, the overall labelling efficiency appears to be affected directly by the initial concentration of CT instead of the molar ratios of CT, ^{125}I, and h-TSH, under the conditions of the present study.

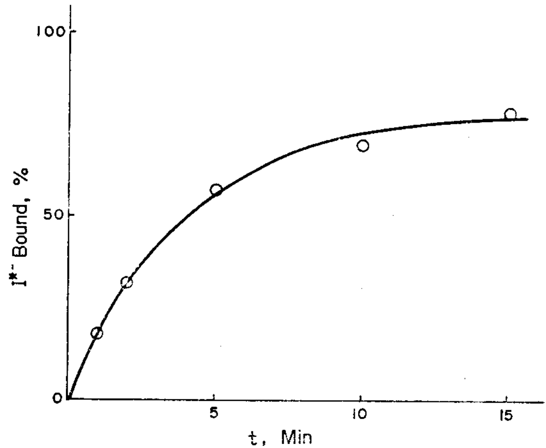


Fig. 2. Degree of the incorporation of ^{125}I into h-TSH as the labelling reaction proceeds in the presence of a small amount of CT.

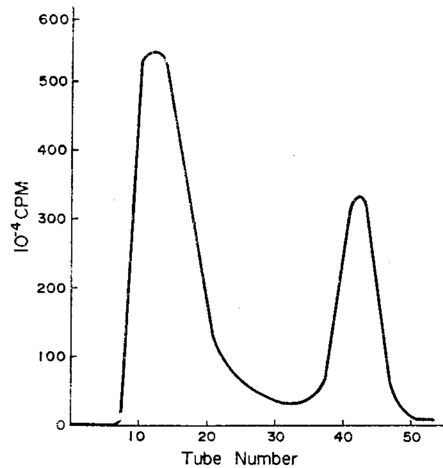


Fig. 3. Atypical chromatogram for the Sephadex G-75 separation of h-TSH* prepared in the presence of a small amount of CT.

Although bovine TSH has been ^{125}I-labelled with a small amount of CT at room temperature[24,25], the present results represent the first successful prepration of immunologically dependable h-TSH* under such conditions. In the following sections, the characterization of the immunological behavior of h-TSH* prepared at room temperature is discussed.

Affinity to Antibody

The affinity of h-TSH* to antibody was examined by measuring the radioactivity bound to the antibody in the absence of added standard h-TSH (B_0). The ratio of B_0 and the total radioactivity, B_0/T, was compared with the number of ^{125}I atoms incorporated into a molecule of h-TSH*, #I*/h-TSH*, as illustrated in Fig. 4. The value of #I*/h-TSH* was calculated from the total radioactivity of the protein fractions, that of the non-protein fractions, the initially added molar amount of $^{125}I^-$, and the initially added molar amount of h-TSH. The molar amount of h-TSH is calculated from the weight of h-TSH used in the labelling experiment and the molecular weight of h-TSH, but it was assumed that the commercial h-TSH is only 20% pure. This is because the highest activity of h-TS H reported in the literature is 30 U/mg while the activity of the commercial h-TSH used in the present investigation is 6 U/mg.

The value of B_0/T reaches the maximum when #I*/h-TSH* is 1—2. Incorporation of less than one ^{125}I atom leaves a significant portion of h-TSH unmodified, which competes with the modified hormone for binding to the antibody. This is reflected in the small values of B_0/T for #I*/h-TSH* <1, as illustrated in Figure 4. When several ^{125}I atoms are contained in a h-TSH* molecule, the immunological behavior of the labelled hormone deviates greatly from that of the normal hormone. Much smaller B_0/T values for #I*/h-TSH*> 3 (Figure 4) indicate the lower affinity of multiply iodinated h-TSH.

The h-TSH* sample provided in Daiichi kits for h-TSH radioimmunoassay revealed B_0/T of about 40% when tested immediately after arrival. The antibody affinity of h-TSH* prepared by the present method, thus, can be brought above that of the commercial product by controlling the amounts of the reactants appropriately.

Radioimmunoassay

As h-TSH* prepared by the new method manifested satisfactory B_0/T values, its application to radioimmunoassay was tested according to the procedure described in the experimental section. A typical standard curve of B/T against the logarithm of the amount of added standard h-TSH, log [h-TSH], is illustrated in Figure 5. This demonstrates that h-TSH* prepared in the present study is suitable for the radioimmunological estimation of h-TSH.

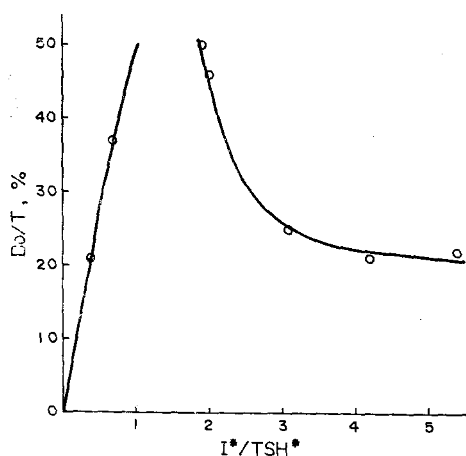


Fig. 4. A plot of B_0/T against the number of ^{125}I atoms incorporated into a molecule of h-TSH* (#I*/h-TSH*) for h-TSH* prepared by the new method.

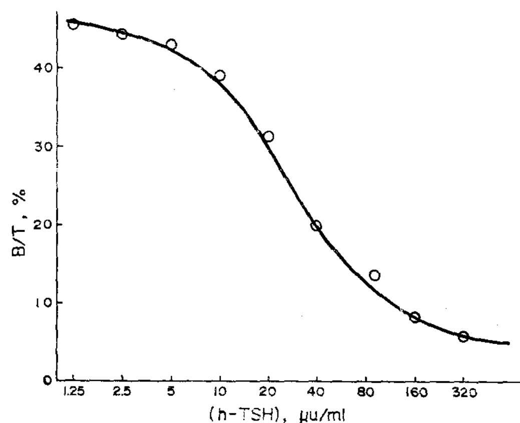


Fig. 5. A typical standard curve of B/T against the amount of standard h-TSH for radioimmunoassay with h-TSH* prepared by the new method.

Radiolalled proteins gradually lose their immunological activity due to radiation damage[26] as well as radioactivity loss caused by the decay of the radioactive nuclide. When tested at an interval of one week, satisfactory radioimmunoassay results were obtained with h-TSH* prepared by the new method until at least 6—7 weeks after preparation. This is comparable to the effective period (5—6 weeks) of h-TSH* of the Daiichi Kit. Therefore, h-TSH* prepared by the new method appears to be compaable to that of the commercial product in terms of B_0/T and the effective life.

Clinical examinatination of h-TSH* prepared by the new method will be the subject of the following paper.

Acknowledgment

This investigation was supported by grant from Seoul National University Hospital. The authors appreciate the expert technical assistance of Mr. Il-Tack Seo and Mr. Chul Soo Chang in the department of Nuclear Medicine, Seoul National University Hospital.

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