대한의생명과학회지: 제5권 제1호 1999
Korean J. Biomed. Lab. Sci.
Vol. 5, No. 1, p.11~15, June, 1999

Studies on the Rapid and Competitive Enzyme-linked Immunosorbent Assay for the Detection of Thyroxine (T_4) in Human Sera

Sang-Wook Park† and Jong-Bae Kim

Department of Medical Technology, College of Health Science, Yonsei University, Wonju, 220-710, Republic of Korea

Abstract: Thyroxine (3,5,3',5'-L-tetraiodothyronine; T_4) is the most commonly measured thyroid hormone for the diagnosis of various thyroid disorders. Although radioimmunoassay (RIA) is still considered as the reference technique for the measurement of T_4, it is generally regarded that RIA has its primary disadventages in handling the wastes and controling the human and material resources. Therefore, establishment of enzyme-linked immunosorbent assay (ELISA) has of great significance. To verify the usefulness of our enzyme immunoassay, we have obtained the standard dose response curve of T_4 in patient's sera which is inversely proportional to the amount of horseradish peroxidase (HRP) conjugated monoclonal antibody of T_4 bound to the wells. The correlation coefficient (r) between the ELISA and chemiluminescent assay was 0.444 (n=38). Thus we have investigated the establishment of rapid and sensitive competitive ELISA assay method for detection of T_4 in patient's sera.

Key Words: Thyroxine (T_4), RIA, ELISA

INTRODUCTION

The most commonly used test in the diagnosis of thyroid function is the determination of serum thyroxine (T_4) concentration[1,7]. The current reports describe that RIA method for the quantitative measurement of free T_4 concentration (FT_4) is a rapid, reproducible, and simple in the sera of patients with thyroid dysfunction[5].

Although radioimmunoassay (RIA) is still considered as the reference technique for the measurement of T_4 and is usually the method of choice for T_4 measurements[4] particularly when screening for hypothyroidism in neonates[8]. However, problems associated with radioisotope methods have led to the development of new methodologies which use enzymes[2,3,9] or fluorescent substances[10]. Diagnostic automation introduces a rapid and sensitive non-isotopic method for measuring thyroxine in human sera[9]. Diagnostic T_4 test system is a solid phase competitive binding enzyme immunoassay, in which T_4 in the patient's serum competes with a T_4 enzyme antibody. The amount of T_4 in the patient serum is inversely proportional to the amount of T_4-enzyme conjugate bound to the well, which is determined at the end of the assay.

In this study, we have obtained the standard dose response curve using the competitive ELISA reaction after being competed chemically the T_4 in human sera with horseradish peroxidase (HRP) conjugated T_4. We tried to

*Received : March 29, 1999
Accepted after revision : May 22, 1999
†Corresponding author

examine that usefulness of ELISA in comparison with the chemiluminescent assay in the clinical patients with thyroid disorders and established the assay system for the detection for T_4 in human sera using the ELISA.

MATERIALS AND METHODS

T_4 monoclonal antibody titration

L-Thyroxine (T_4) specific monoclonal antibody (MoAb) was obtained from Hyundae Phamaceutical Company in the development of competitive ELISA.

To testify the effects of the MoAb dilution on the ELISA absorbance values in the wells coated with standard thyroxine (T_4) and control, T_4 monoclonal antibody was diluted with 2-fold series ranging 1/1000 to 1/16,000 and examined in duplicate. The titer of MoAb specific to T_4 has been decided to 1/8,000 available in the batch with control (Data not shown). However we chose 1/4,000 for the end point to confirm.

Preparation of HRP conjugated T_4

Horseradish peroxidase (HRP) was oxidized to establish the optimum concentration of $NaIO_4$ in order to achieve a relatively slow and limited oxidation of HRP[6]. The following conditions were chosen to be constant; solubilized HRP in 0.1 M sodium bicarbonate to which an equal volume of sodium *m*-periodate solution was added was incubated for 2 hours at 20℃ in the dark place. One tenth volume of 0.1 M sodium carbonate was, after the incubation, added to the oxidized HRP. Thyroxine (T_4) in 0.1 M sodium hydroxide was added to the HRP sample and the combined sample was transferred immediately to another tube followed by dry Sephadex G-25 (1/5 of combined weight of HRP and T_4) column chromatography. The tube was closed and left at room temperature for 3 hours or overnight. The conjugate was then eluted from the Sephadex and stabilized by the addition of 1/20 volume of a fresh sodium borohydride solution (5 mg/ml in 0.1 mM NaOH). Elution was repeated after half an hour with an addition of 3/20 vol of another freshly prepared $NaBH_4$ solution and left for another 1 hour.

Competitive ELISA reaction

To coat the microplate wells, we pipetted 200 μl of the anti-T_4 IgG monoclonal antibodies, diluted 1:4000 in 50 mM carbonate buffer (pH 9.6) and let the plates stand at 4℃ for 16 hours and after the plates were washed 3 times in 300 μl PBS (phosphate buffered saline) containing 0.05% Tween 80 and dried by tapping the inverted plate on paper towel. Uncoated active sites were saturated with 300 μl of 2% BSA (bovine serum albumin) in PBS for 1 hour at 37℃. The plates were washed 3 times as above. The plates were dried for 20 min at 37℃. Meanwhile, HRP conjugated T_4 were diluted 1:500 in the assay buffer (0.04% 8-anilino-1-naphthalene sulfonic acid; ANS, 2% bovine serum albumin; BSA, 0.1 M barbital buffer, 0.02% thimerosal, pH 8.6). Powdered standard T_4 was prepared with 2-fold serial dilutions from 600 ng/ml to 37.5 ng/ml and 0 ng/ml dissolved in PBS. Competitive reaction was run with HRP conjugated T_4 and standard T_4 for 1 hour at 37℃ and were washed 3 times as above. They were dried for 20 min at 37℃. For the color reaction, we used 200 μl of a solution of 10 μg of *o*-phenylenediamine dihydrochloride in 12.5 ml of a citrate-phosphate buffer (pH 5.0) to which 10 μl of 30% hydrogen peroxide was added. The reaction was stopped after 30 min with 50 μl of 2.5 M sulfuric acid and read at 490 nm using a microtiter plate reader (SOFTmax, U.S.A.). All assays were carried out in duplicate.

RESULTS

Optimal coupling ratio

To establish the optimal coupling ratio between HRP and T_4, we examined what dilutions

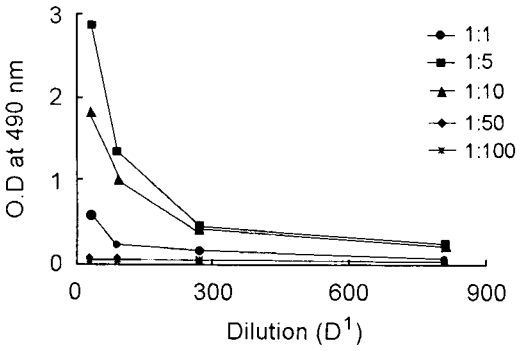

Fig. 1. Influence of molar ratio between T_4 and HRP on the absorbance of ELISA was determined on its molarity of various T_4 and HRP with Sephadex G-25 column chromatography. T_4-HRP ratio of 1:5 (■) showed highest optical density (O.D.).

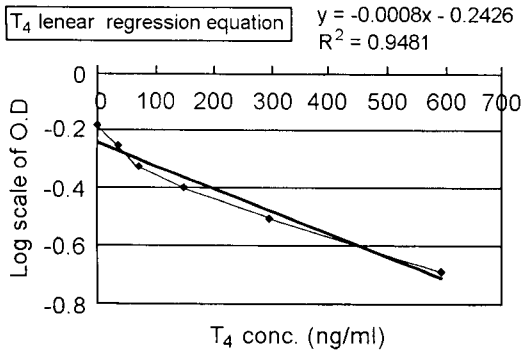

Fig. 2. Dose response curve for the competitive enzyme immunoassay of standard T_4. Linear regression line was fitted to the concentrations of standard T_4.

of HRP and T_4 yielded optimal molar relevance. When HRP and T_4 was diluted 1:1, 1:5, 1:10, 1:50 and 1:100 based on its own molarity, dilution of 1:5 conjugate solution was found to be the highest absorbance in ELISA. We chose to use a 1:5 coupling ratio of HRP and T_4 conjugate solution in all subsequent assays (Fig. 1).

Standard dose response curve

Based on the concentration of standard T_4 and constant T_4-HRP, a typical representative standard curve is shown in Fig. 2. The coefficient of variation (CV) for each point ranged

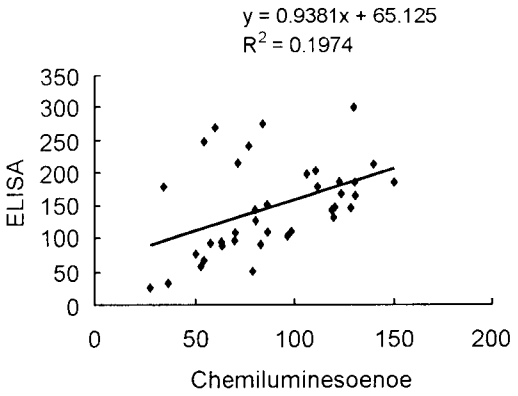

Fig. 3. Correlation between T_4 serum concentrations determined by competitive ELISA and chemiluminescence. Regression gives following relationship: y (ELISA) = 0.9381 x + 65.125, with r=0.444 ($p<0.005$).

Table 1. Intra- and inter-assay coefficients of variation (CV) for T_4 measurements

	T_4 (ng/ml)					
	0	37.5	75	150	300	600
Intra-assay	0.4	4.7	4.3	2.9	7.1	1.3
Inter-assay	24.4	20.7	21.2	17.7	4.1	25.1

from 12% to 17%, indicating that the precision of the standard curve is satisfactory. The lower limit of detection for T_4 (response distinguishable from that seen in the absence of T_4) was 26 ng/ml. The standard curve gave a useful assay range of 26~300 ng/ml.

Precision studies

Intra- and inter-assay precision (CV) were evaluated by measuring T_4 concentrations in blood samples. The results are shown in Table 1.

Comparison between ELISA and chemiluminescent assay

The correlation between the proposed ELISA method and the chemiluminescent assay is shown in Fig. 3, using patient's sera with thyroid disorders. The overall correlation coefficient (r) was 0.444 (n=38) and the slope of the regression line for the results was 0.9381

and the y-intercept was 65.125.

DISCUSSION

Measurement of thyroxine (T_4) in blood has been long recognized as providing a valuable aid in the clinical diagnosis of various thyroid disorders. Although radioimmunoassay (RIA) is still considered as the most sensitive technique for the measurement of T_4, numerous non-isotopic methods have been published or introduced in the last decade.

In this study, we have established the competitive reaction between standard T_4 and patient's sera in which anti-T_4 monoclonal antibody was coated in the wells. The typical standard curve was obtained (r=0.9737) and the coefficient of variation (CV) value ranged from 12% to 17%. Intra-assay precision (CV) was 1% to 7%, while inter-assay precision was highly variable (CV=10%～20%) comparing with those in inter-test. We have set up the standard optimal coupling ratio of 1:5 between thyroxine (T_4) and horseradish peroxidase (HRP). Finally, we have verified the usefulness of detecting T_4 in patient's sera using ELISA we have established. The regression equation resulted in the following data: y=0.9381x+65.125, r=0.444 where y and x are the ELISA and chemiluminescence results, respectively. The Pearson's coefficient of correlation between ELISA and chemiluminescence assay was of significant ($p<0.005$). The enzyme immunoassay presented in this paper permits specific and accurate determination of total T_4 using standard laboratory equipments and avoids the limitations associated with radioisotopes. This method might be extended to the measurement of free T_4, provided that the T_4-HRP conjugate does not interact with transport proteins.

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=국문초록=

경쟁적 효소면역측정법을 이용한 환자 혈청 내 Thyroxine (T_4)의 검출

연세대학교 보건과학대학 임상병리학과

박 상 욱† · 김 종 배

갑상선 질환의 진단에 필요한 갑상선 호르몬의 측정시 thyroxine (T_4) 농도 측정방법이 가장 흔히 이용되고 있다. 과거에 많이 활용되어 온 방사면역측정법 (RIA)이 폐기물 처리 및 관리인원 등의 인적, 물적인 문제가 크게 대두되고 있는 점을 감안하면 효소결합면역측정법의 확립은 큰 의미가 있다고 하겠다. 본 연구에서는 thyroxine (T_4)에 대한 단클론 항체 (monoclonal antibody)를 이용하여 T_4에 horseradish-peroxidase를 화학적으로 결합시킨 microtiter plate에 HRP-conjugate T_4와 혈청 내의 T_4간의 서로 경쟁적인 반응을 이용한 표준곡선을 얻었다. 그리고 이렇게 확립한 기법을 임상적인 갑상선 질환 환자의 혈청을 기존의 chemiluminescence 방법을 이용하여 얻은 결과와 비교하여 본 실험에서 확립한 ELISA 검사 수치의 유용성을 검토하였으며 이 방법의 임상이용 가능성을 검토하였다.

[대한의생명과학회지 5(1): 11-15, 1999년 6월]

†별책요청 저자