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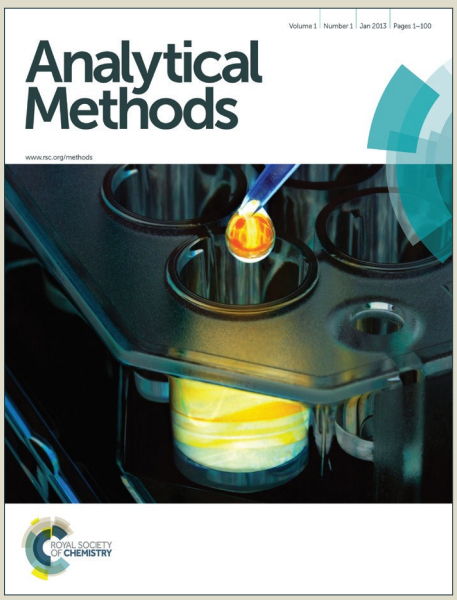

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Determination of tetraiodo-L-thyronine in human serum with competitive indirect chemiluminescence immunoassay

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Based on the monoclonal antibodies against 3, 3', 5, 5'-tetraiodo-L-thyronine (T_4), a competitive indirect chemiluminescence immunoassay (CLEIA) used for quantitative determination of total T_4 in human serum was established. The relevant results indicated that the determined coating antigen concentration was 2.7 μg mL^{-1} and the CLEIA titer of ascites was $1:8.0\times10^4$. The standard curve was $y=-0.495x+1.095$, with coefficient of determination, $R^2=0.988$, and derived half inhibition concentration (IC_{50}) of T_4 was 16.0 ng mL^{-1}. The system detection limit was 9.23 ng mL^{-1}. The mean inter- and intra-assay variations were 5.89 % and 4.02 %, respectively. The detected data of total T_4 in human serum from seven patients with this method exhibited significant correlation with reported clinical data, suggesting this CLEIA method could be applicable to clinical diagnosis of thyroid gland disease.

Yilin Gao and Zhirui Deng contributed equally to this work.

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1 Introduction

Thyroid dysfunction is common in general population and quite prevalent among people aged over 60 years.[1] The concentration of 3, 3', 5, 5'-tetraiodo-L-thyronine (T_4) is a useful parameter for thyroid function diagnosis in clinical. Under normal physiological conditions, approximately 99.95% of T_4, binding to some specific proteins (mainly thyroxine binding globulin), exist in compound form, the rest remain free.[2] Since free T_4 level is always susceptible to the amount of bound T_4, and its detection requires complicated procedure,[3] the concentration of total T_4 in human serum is generally accepted as an index of thyroid dysfunction. When the concentration of total T_4 in human serum is out of the reference range, 46.62 ng mL^{-1} to 140.64 ng mL^{-1}, thyroid gland is in abnormal state.

Currently, several immunoassay technologies for total T_4 detection are available. These include radioimmuno-assay (RIA), enzyme linked immunosorbent assay (ELISA), time resolved fluoroimmunoassay (TRFIA), high-performance liquid chromatography (HPLC), and electro-chemiluminescence immunoassay (ECLI). Although, RIA gives sensitive detection limit (5.0 ng mL^{-1}),[4] the demand for isotopes restricts its application inevitably. Both ELISA and HPLC methods, using safer agents, yet exhibit poor sensitivity (about 13.83 ng mL^{-1}, and 0.10 μg mL^{-1}, respectively).[5, 6] TRFIA and ECLI can separately offer sensitivity at 5.36 mg mL^{-1} and lower than 0.30 pmol L^{-1} level,[7, 8] but sophisticated operations are only managed by specialized technicians.

A promising alternative to above-mentioned assays is chemiluminescence enzyme immunoassay (CLEIA). It owns safety, sensitivity, convenience and broad applicability.[9-12] The quantitative analysis is achieved through substance binding to special antibody, further

triggering photons release. So far, CLEIA test has been mainly applied in diagnosis of small cell lung carcinoma,[9] squamous cell carcinoma,[13] hepatocellular carcinoma,[14, 15] etc., but satisfactory CLEIA detection for thyroid dysfunction has not been reported. Hence, a sensitive and convenient method, based on CLEIA, is urgent to the clinical diagnosis and life science research.

Using developed monoclonal antibodies against T_4 (T_4 McAb)[5] and specific polyclonal antibodies conjugated with horseradish peroxidase (HRP), we established a competitive indirect CLEIA. Methodology parameters of CLEIA were evaluated and optimized. It is demonstrated that the optimized method satisfies clinical detection criteria, as the examined concentrations of total T_4 in human serum samples are in accordance with the clinical data.

2 Experimental

2.1 Chemicals and reagents

3,3',5,5'-tetraiodo-L-thyronine (T_4) and horseradish peroxidase (HRP), coupled with goat anti-mouse IgG were purchased from Sigma-Aldrich, USA. Dimethylsulfoxide (DMSO), hydrogen peroxide (H_2O_2) and trihydroxy methyl aminomethane (Tris) were obtained from Sinopharm Chemical Reagent Co., Ltd (SCRC). Luminol and *p*-lodophenol were purchased from Alfa Aesar, USA.

T_4-BSA, T_4 McAb,[5] CLEIA washing buffer, CLEIA coating buffer, blocking buffer (1×PBST buffer containing 1% gelatin) and chemiluminescence substrate buffer were prepared in our laboratory.

2.2 Serum samples

Patients' venous blood samples were supplied by Central Hospital of Nanhui District in Shanghai.

2.3 Instrumentation

Synergy 2 SL Luminescence Microplate Reader (10 amol ATP in flash analysis system and 100 amol ATP in glow system), with Gen 5 Data Analysis Software, was employed for photon signals capturing, multi-analyzing and data-processing. 75004261 centrifuge was from Thermo Scientific, USA. 96-well chemiluminescence white microplates were purchased from Greiner Bio-One, Germany.

2.4 Establishment of T_4 CLEIA

2.4.1 Working concentration determination for coating antigen and T_4 McAb. The working concentrations of coating antigen and antibody are critical to sensitivity of immunoassay**.** According to checkboard assay,[16] with coating buffer, sequentially dilute T_4-BSA (8.0 mg mL^{-1}) solution into 4.0 μg mL^{-1}, 2.7 μg mL^{-1}, 2.0 μg mL^{-1}, 1.6 μg mL^{-1}, 1.3 μg mL^{-1} and 1.1 μg mL^{-1}, and then transfer 100 μL solution into 96-well chemiluminescence microplate per well, and incubate for 4 h at 37 ºC. Wash the microplate three times with 1×PBST buffer and add 200 μL 1% gelatin 1×PBST into each well to block. Then wash three times again, and orderly add 100 μL T_4 McAb in dilution ratios (1:4.0×10^4, 1:6.0×10^4, 1:8.0×10^4, 1:1.0×10^5, 1:1.2×10^5, 1:1.4×10^5 and 1:1.6×10^5) into the corresponding blocked wells. Use 1×PBST buffer containing 1% gelatin as negative control. Here, we use 1×PBST

buffer that contains 5 % fetal bovine serum (FBS) and 0.05 % Tween-20 as antibody dilution solution. FBS can restrain antibody from nonspecific absorption, and Tween-20 can remove nonspecifically binding antibody and enhance washing effect. Incubate the microplate for 1.5 h at 37 ºC. Then, wash the plate three times and add 100 μL goat anti-mouse IgG-HRP ($1{:}4.0\times10^3$ dilution) to each well. After that, add 1006.225 μL reaction solution (1.0 mL chemiluminescence substrate buffer, 0.625 μL *p*-Iodophenol, 4.0 μL Luminol and 1.6 μL H_2O_2) per well, and detect instantaneous chemiluminescence signals, defined as counting photon per second (CPS), within 5 minutes.

2.4.2 Establishment of T_4 competitive indirect CLEIA. In order to avoid the interference of endogenous T_4 in FBS, we use 1×PBST complemented with 1% gelatin instead of 5% FBS to dilute the antibody. With 1% DMSO 1×PBS, prepare 2.0 ng mL^{-1}, 10 ng mL^{-1}, 20 ng mL^{-1}, 40 ng mL^{-1}, 80 ng mL^{-1}, 120 ng mL^{-1}, 160 ng mL^{-1} and 200 ng mL^{-1} T_4 standard solution. According to the procedure in 2.4.1, use coating antigen and T_4 McAb at working concentration, respectively, and replace 100 μL T_4 McAb solution with the mixture of 50 μL T_4 standard solution and 50 μL T_4 McAb solution. The inhibitory effect of different T_4 standard concentrations is expressed as percent inhibition (B/B_0) according to the formula:

$$(B/B_0)\% = (B/B_0)\times 100\%$$

Where, B_0 = CPS in well containing no T_4, B = CPS in well containing T_4. Accordingly, establish plot T_4 standard curve, where *x* axis represents $\log_{10}$ of standard T_4 concentration while *y* axis stands for relative luminous intensity (B/B_0). Calculate IC_{50} value, according

to the equation of the standard curve.[17]

2.4.3 System detection limit. The limit value of EIA, which represents detecting sensitivity, has to be as low as possible. The CPS values of 16 blank controls, where, physiological saline instead of T_4 standard solution, are measured by CLEIA method. The mean value ($\bar{X}$) and standard deviation (SD) are statistically analyzed. On this basis, calculate the value of "$\bar{X}$-3SD". By introducing the calculated values into the established equation of the standard curve, the corresponding T_4 concentration will be obtained, which is defined as the detectable limit that indicates sensitivity of immunoassay.[18]

2.4.4 Precision and accuracy. Precision is expressed as inter- and intra- coefficient of variation (CV%), which are required to be lower than 10% and 5.0% separately in immunoassay. Intra- and inter-assay variations of competitive CLEIA assay for T_4 standard solution in 2.4.2 are analyzed with five replicates on the strength of the formula:

$$CV\% = SD / \left[1.414 \times \left(B / B_0\right)\right] \times 100\%$$

2.5 Sample volume determination

To determine the suitable serum volume for T_4 detection, different volumes (20 μL, 30 μL, 35 μL, 40 μL) are used in CLEIA method. Then, the relative difference between examined data and clinical data is assessed.

2.6 Applicability of T_4 CLEIA

With optimized sample volume, T_4 concentrations in serum samples from seven patients are detected by the established method in 2.4.2 to evaluate analytical accuracy. The correlation between tested data and clinical data is determined through the plotted graph, where *x* axis represents reported data clinically and *y* axis indicates detected data.

2.7 Statistical analysis

The quantitative data of three to five repetitions are expressed as averages ± SD. Statistical significance is assessed with Student's *t*-test, when $P > 0.05$, suggesting no significant difference.

3 Results and Discussion

3.1 Establishment of T_4 CLEIA

3.1.1 Working concentration determination for coating antigen and T_4 McAb. The determined working concentrations of coating antigen (T_4-BSA) and T_4 McAb were shown in Table 1. When the dilution ratio of T_4 McAb kept constant, CPS obviously reduced with decreasing concentration of T_4-BSA; On the other hand, when the concentration of T_4-BSA kept fixed, the CPS diminished with the increasing dilution ratio of T_4 McAb. When the 96-well microtiter plate, coated with 2.7 μg mL^{-1} T_4-BSA, was applied with $1:8.0\times10^4$ dilution of T_4 McAb, the CPS value was 101924, most approximate to 1.0×10^5. Therefore, the appropriate coating antigen concentration in CLEIA was determined as 2.7 μg mL^{-1}, and the working dilution of T_4 McAb, $1:8.0\times10^4$.

Table 1 Working concentration determination for coating antigen and T_4 McAb

3.1.2 Confirmation of T_4 CLEIA. Based on the optimized CLEIA assay and data acquired from 2.4.2, a standard curve for T_4 was obtained, showed in Fig. 1. The regression equation was $y = -0.495x + 1.095$ with coefficient of determination, R^2 = 0.988. The derived IC_{50} for T_4 was 16.0 ng mL^{-1}, slightly lower than that reported with time-resolved immunofluorometric assay by Tan Yuhua[7] and 54.4% lower than that with competitive indirect ELISA (ciELISA) we established.[5] Excellent linear correlation existed between the relative luminous intensity (B/B_0) and $\log_{10}$ of standard T_4 concentration. The working range of this assay was determined from 2.0 ng mL^{-1} to 200 ng mL^{-1}.

Fig.1 The standard curve for T_4 obtained with the established CLEIA (Triplications per point).

3.1.3 System detection limit. According to T_4 CLEIA standard curve, the CPS values of 16 blank samples (Table S1) were used to calculate the value of $\bar{X}$-3SD. The assay sensitivity of system detection limit was shown to be 9.23 ng mL^{-1}, generally similar to those reported so far.[4, 19]

Table S1 CPS values of 16 blank standard samples with CLEIA method

3.1.4 Precision and accuracy. As shown in Table 2, the mean intra- and inter- assay variations were 4.02 % (< 5.0 %) and 5.89 % (< 10 %), respectively, demonstrating this CLEIA possessed high accuracy.

Table 2 The mean intra- and inter- assay variations of CLEIA

3.2 Sample Volume determination.

As listed in Table 3, when 30 μL sample volume was taken, the relative difference between detected data and reported clinical data of human serum samples exhibited minimum, suggesting that 30 μL was the most appropriate volume for detection.

Furthermore, significant difference among different sample volumes of human sera was analyzed with Least Significant Difference (LSD) method (Table S2), and mean coefficient variation of 30 μL was quite significantly different from those of 35 μL and 40 μL, but not from those of 20 μL. Consequently, sample volume from 20 μL to 30 μL could be used in CLEIA.

Table 3 Total T_4 concentrations in serum samples from three patients with CLEIA method

Table S2 Significant difference among four mean coefficient of variations in Table 3 using Least Significant Difference (LSD) (Letter notation)

3.3 Applicability of T_4 CLEIA.

The correlation between T_4 concentrations detected with CLEIA and those clinically reported in the same seven patients' sera was appraised in Fig. 2. The regression equation was $y = 1.003x - 1.357$, with coefficient of determination, $R^2 = 0.918$, indicating good linear correlation. Hence, this CLEIA method could satisfy clinical detection criteria.

Besides, according to data examined with CLEIA, T_4 concentration in one of human serum samples, was 37.28 ng mL^{-1}, out of the normal reference value range (46.62 ng mL^{-1} to 140.64 ng mL^{-1}), suggesting the serum-owner's thyroid disfunctioned.

Fig.2 Correlation between detected data with CLEIA and reported clinical data (Triplications per point of detected data).

4 Conclusions

In this study, we established a competitive indirect CLEIA method to detect total T_4 in human serum. Meanwhile, we also confirmed methodology parameters of this method, including IC_{50}, linear detecting range, system detection limit, inter-assay and intra-assay variation. Concretely, IC_{50} of this CLEIA was 16.0 ng mL^{-1}. The linear detecting range was 2.0 ng mL^{-1} to 200 ng mL^{-1}, and the limit value was 9.23 ng mL^{-1}. The method we established gave low inter-assay variation (5.89%) and intra-assay variation (4.02%). All results above suggested that we have established a highly-specific and excellently-sensitive T_4 examination system.

Acknowledgements

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References

1. K. Boelaert, *Nature reviews. Endocrinology*, 2013, **9**, 194-204.
2. J. Bernal, A. Guadano-Ferraz and B. Morte, *Nature reviews. Endocrinology*, 2015, **11**, 406-417.
3. L. F. Maciej Adamczyk, Jeffrey R. Fishpaugh, Donald D. Johnson, Phillip G. Mattingly, *Bioconjugate Chem.*, 1994, **5**, 459-462.
4. Y. C. Tyan, S. B. Jong, M. H. Yang, T. L. Wu, T. W. Chung, P. C. Liao, C. Y. Lin and Y. F. Huang, *Clinica chimica acta; international journal of clinical chemistry*, 2013, **420**, 99-103.
5. Y. L. Gao, J. C. Wei, Z. R. Deng, W. J. Zhang, Y. C. Liu, W. Jiang, Y. J. Chen, J. L. Shi, Q. Chen, *Animal Husbandry & Veterinary Medicine*, 2013, **45**, 54-58.
6. H. Gika, M. Lämmerhofer, I. Papadoyannis and W. Lindner, *Journal of Chromatography B*, 2004, **800**, 193-201.
7. H. H. Tan Yuhua, Lu Shunduo, Shen Jian, *Chinese Journal of Clinical Laboratory Science*, 2008, **26**, 33-35.
8. M. A. Marta S C, Alfayate R., *Clin. Biochem.* , 1999, **32**, 395-403.
9. X. Fu, M. Meng, Y. Zhang, Y. Yin, X. Zhang and R. Xi, *Analytica chimica acta*, 2012, **722**, 114-118.
10. J.-B. Dai, Z.-L. Xu, F.-Y. Liu, J.-Y. Yang, Y.-M. Sun, H. Wang, H.-T. Lei and Y.-D. Shen, *Chinese Journal of Analytical Chemistry*, 2015, **43**, 871-875.

11. Z.-S. L. Chao Lin, Dong-Xu Wang, Hong-Lin Ren, Yan-Song Li, Pan Hu, Yu Zhou, Yi-Ping Guo, Xian-Mei Meng, Shi-Ying Lu, *Analytical Methods*, 2014, **6**, 7142-7148.
12. X. L. Feng, H. L. Ren, Y. S. Li, P. Hu, Y. Zhou, Z. S. Liu, D. M. Yan, Q. Hui, D. Liu, C. Lin, N. N. Liu, Y. Y. Liu and S. Y. Lu, *Analytical biochemistry*, 2014, **459**, 12-17.
13. H. Zhang and S. Qi, *Clinica chimica acta; international journal of clinical chemistry*, 2011, **412**, 1572-1577.
14. Q.-Y. Zhang, H. Chen, Z. Lin and J.-M. Lin, *Journal of Pharmaceutical Analysis*, 2011, **1**, 166-174.
15. X. Liu, X. Wan, S. Lu, L. Zhang, S. Yu and X. Lu, *Clinica chimica acta; international journal of clinical chemistry*, 2015, **445**, 54-59.
16. H.-m. Niu, X.-m. Huang, K.-k. Han, Y.-z. Liu, D.-m. Zhao, J.-f. Zhang, F. Liu, T.-t. Li, X.-b. Zhou, X.-r. Li and Y. Li, *Journal of Integrative Agriculture*, 2013, **12**, 1638-1643.
17. C. Wyns, L. Derycke, B. Soenen, S. Bolca, D. Deforce, M. Bracke and A. Heyerick, *Talanta*, 2011, **85**, 197-205.
18. X. Wang, Q. Y. Zhang, Z. J. Li, X. T. Ying and J. M. Lin, *Clinica chimica acta; international journal of clinical chemistry*, 2008, **393**, 90-94.
19. Y. Huang, S. Zhao, M. Shi and Y. M. Liu, *Analytical biochemistry*, 2010, **399**, 72-77.

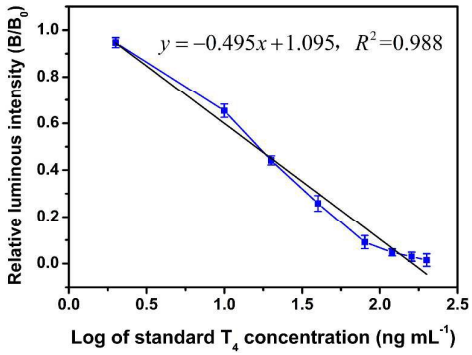


Fig.1 The standard curve for T_4 obtained with the established CLEIA (Triplications per point).

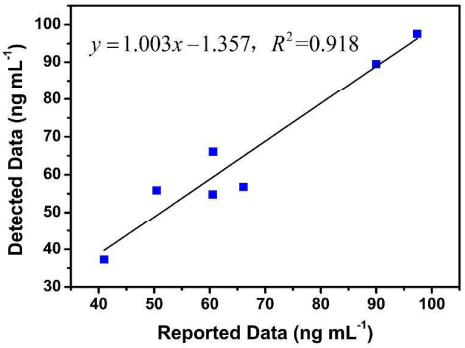


Fig.2 Correlation between detected data with CLEIA and reported clinical data (Triplications per point of detected data).

Table 1 Working concentration determination for coating antigen and T_4 McAb

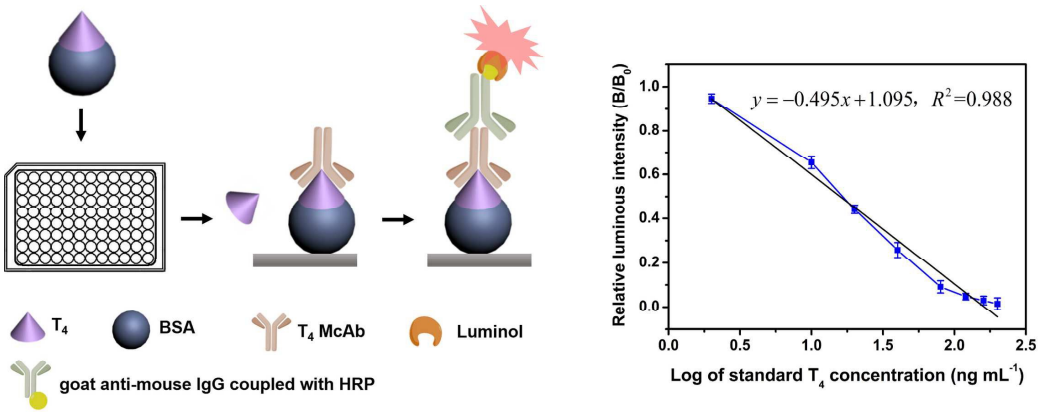
Coating antingen concentration (μg mL^{-1})	T_4 McAb dilution (×10^4)							
	1:4.0	1:6.0	1:8.0	1:10	1:12	1:14	1:16	0
4.0	214997	175135	154967	140972	118175	114054	84432	449
2.7	156973	125830	101924	93210	73698	75280	49545	302
2.0	127560	104857	72649	78028	57100	59605	38530	127
1.6	67306	59155	35763	36122	25819	28450	18888	213
1.3	67943	51360	44246	37774	29363	24726	18878	418
1.1	42680	34183	24667	21981	16281	16170	11503	628

Table 2 The mean intra- and inter- assay variations of CLEIA

Standard T_4 concentration (ng mL^{-1})	Intra-replication	Intra-(B/B0) ±SD	Inter-replication	Inter-(B/B0) ±SD	CV%	
					Intra-	Inter-
200	5	0.0088±0.0000	5	0.0161±0.0000	0.00	0.00
160	5	0.0311±0.0000	5	0.0427±0.0050	0.00	8.28
120	5	0.1450±0.0141	5	0.1462±0.0250	6.88	12.1
80	5	0.3658±0.0312	5	0.3387±0.0477	6.03	9.96
40	5	0.6587±0.0304	5	0.7217±0.0915	3.26	8.97
20	5	0.8314±0.0529	5	0.9250±0.0335	4.50	2.56
10	5	0.9036±0.0194	5	0.9869±0.0255	1.52	1.83
2	5	0.9531±0.0085	5	0.9954±0.0172	0.89	1.73
mean		0.4872±0.0196		0.5216±0.0307	4.02	5.89

Table 3 Total T_4 concentrations in serum samples from three patients with CLEIA method

Sample Volume (μL)	Sample 1			Sample 2			Sample 3			
	Detected data	Reported data	Relative difference	Detected data	Reported data	Relative difference	Detected data	Reported data	Relative difference	Mean relative difference
20	91.97	106.68	13.79%	82.25	77.08	6.71%	46.42	57.07	18.67%	13.06%
30	108.23	106.68	1.45%	86.97	77.08	12.83%	53.37	57.07	6.48%	6.92%
35	132.19	106.68	23.91%	98.61	77.08	27.93%	65.79	57.07	15.28%	22.37%
40	127.37	106.68	19.39%	95.45	77.08	23.83%	65.18	57.07	14.21%	19.14%



Detection of T_4 by the established CLEIA method.