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# Determination of serum thyroxine enantiomers in patients by liquid chromatography with a chiral mobile phase

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**Abstract**

A chromatographic method for the separation and determination of D- and L-thyroxine enantiomers (D-, and L-T4) in human serum with a chiral ligand ion-exchange system using a chiral mobile phase additive and a silica column was established. An aqueous eluent containing L-proline (L-pro) sufficiently complexed copper II ions and triethylamine (TEA) was used. It was monitored with a UV detector. The separation was completed in 12 min. The method has acceptable sensitivity, precision and accuracy for analysis. The limit of detection and the limit of quantitation for both D- and L-T4 were 0.1 μg/ml and 0.8 μg/ml, respectively. Calibration curves were linear within 1–100 μg/ml; the mean correlation coefficients were $r_{\text{D-T4}}=0.9986$ for D-T4 and $r_{\text{L-T4}}=0.9978$ for L-T4. T4 enantiomers were separated on baseline under the optimum condition. L-T4 eluted before D-T4. The concentration of D-T4 and L-T4 in 45 thyroid patients serum (hyperthyroid, hypothyroid, thyroidectomy, goitre or thyroiditis) using HPLC was determined, those results showed that D,L-T4 concentration varied in different thyroid patient. Attention should be paid to this result in treating thyroid disease in the clinic.


*Keywords:* Enantiomer separation; Thyroxine

## 1. Introduction

The thyroid hormone included triiodothyroxine (T3) and thyroxine (T4). To conjugate T3 and T4 in the thyroglobulin (Tgb), it was stored in the gland cavity that was activated by the thyrotropin releasing hormone and the proteolytic enzyme, the T3 and T4 were then separated from the Tgb into the blood. It was found that the thyroid hormone could maintain the normal upgrowth and promote metabolism in vivo. Meantime it sustains the functions of the system of nerve and the cardiovascular effect [1].

Marriq [2] purified the hormonopeptides (containing T3 or T4) and determined T3 and T4 in the hormonopeptides which helped understand the molecular mechanism of thyroid hormonosynthesis. So, thyroid hormonosysthesis and thyroid hormonometabolism were recognized as very important in clinical practice, and had a relationship with T3 and T4. Other papers [3–7] are reported to have determined T3 and T4 by HPLC techniques.

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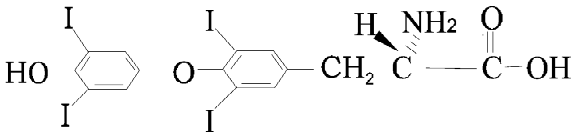

Fig. 1. Structure of 3-[4-(4-hydroxy-3,5-diiodophenoxy)-3,5-diiodophenyl-D-(L)-alanine.

The thyroxine (3-[4-(4-hydroxy-3,5-diiodophenoxy)-3,5-diiodophenyl]-D-(L)-alanine, T4) has two isomeric structures (D-, and L-T4) (Fig. 1), Gorman et al. [8] and Eisdorfer et al. [9] reported that the D- and L-T4 have different biological activity, and considered that it was very important to ascertain whether more L-T4 existed in vivo than D-T4, whether D-T4 is converted in vivo to L-T4, or whether D-T4 and/or its dextro metabolite has true biological activity, but D-T4 and L-T4 were not determined in their papers.

Therefore, Hare et al. [10] reported the separation of D- and L-amino acids by LC using chiral eluents. D-, L-T4 were not determined in this paper, but this paper gave an advice for us to separate D,L-T4 by HPLC using chiral eluents. Oelroch et al. [11], Gübitz et al. [12], Hay and co-workers [13], and Lankmayr and co-workers [14,15] reported to separate the enantiomers of thyroid hormones by high-performance ligand-exchange chromatography using a chiral stationary phase and other methods. Carvalho et al. [16] published the enantioseparation of thyroxine by TLC. The diversified chromatographic methods have been used to separate and determinate the D- or L-T4, but the above papers have the following imperfections: D-T4 and L-T4 could not be well separated on baseline, the analysis time was too long (more 25 min) [11] or the concentration of D-T4 and L-T4 in the thyroid patient serum was not determined by HPLC.

The chiral mobile phases (CMPs) HPLC has become a powerful and widely applicable analytical tool for this purpose [10], which is a convenient method to separate the underivatized enantiomer compounds and the reagent used is cheaper than chiral solid-phase HPLC. Since no adequately sensitive method was availably applied to determine the D- and L-forms in both endogenous and exogenous. In previous paper, we reported on separation of racemic thyroxine with chiral additive as mobile-phase by normal-phase ion-exchange HPLC technique [17]. We will use the CMPs HPLC method for the clinical purpose to separate and determine the thyroxine enantiomers in vitro and in vivo human serum. We considered to establish accuracy determining the D,L-T4 method that is very necessary for monitor and cure the thyroid diseases. Thereby the method of enantioseparation thyroxine is studied and the determination method of the D,L-T4 concentration in the thyroid patients' serum is established by HPLC in this paper.

This paper mainly investigated the determination methods of the D-T4 and L-T4 isomers in thyroid patient serum using the separation conditions mentioned in previous paper [17]. It was the first time to determine the concentration of the T4 enantiomer in serum from about 45 patients (who were known to be euthyroid, hyperthyroid, hypothyroid, thyroidectomy, goitre and thyroiditis) by HPLC with a chiral additive and monitored with a UV detector. The result could be very useful in providing advice in the treatment of different thyroid diseases and symptoms.

## 2. Experimental

### *2.1. Materials*

L-proline (L-pro), D-thyroxine (D-T4) and L-thyroxine (L-T4) were purchased from Sigma (St. Louis, MO, USA). HPLC-grade methanol and acetonitrile, AR-grade cupric acetate and ethyl acetate, all solvent were purchased from SiYou (Tianjing, China). Sep-Pak silica cartridges were supplied by Kaide (Tianjing, China). The water was de-ionized.

Whole blood precipitation reagent was purchased from Abbott Laboratories (USA) that was used to remove the protein in the whole blood, and more than 90% protein was removed when the equimultiple whole blood precipitation reagent and blood sample was added.

D-, and L-T4 stock solution: 5.01 mg D-T4 and 5.37 mg L-T4 were dissolved in an appropriate amount of methanol, respectively. The standard solution: those stock solutions were diluted with methanol. All solutions were stored at 4 °C.

### 2.2. Chromatographic condition

An HPLC system (Shimadzu, Kyoto, Japan) was used that included: LC-6A pump, SPD-6AV detector, CTO-6A column oven, SIL-6B system controller, 7125 injector value with a 20-μl loop (Rheodyne, USA). Data analysis was performed using a C-R3A Chromatopac integrator (Shimadzu). A Hypersil $SiO_2$ column (5 μm, 250×4.6 mm I.D.; Elite, Dalian, China), the constant temperature of 40 °C, a flow-rate of 1.0 ml/min, a 225-nm UV detector wavelength, and a 20-μl injection volume.

The mobile phase consisted of an acetonitrile–water (35:65, v/v), the water contained 0.1 m*M* of cupric acetate, 0.2 m*M* of L-proline and 0.5 m*M* of TEA. Acetonitrile and water mixed and then the pH of mobile phase was then adjusted with acetate acid to 5.41. In order to improve the chromatogram well, the TEA was added. Prior to chromatographic analysis, the mobile phase mixture was degassed for 10 min.

### 2.3. Calibration curve

Blank human sera without drugs were provided by healthy volunteers. Serum was separated from the whole blood by centrifugation, the upper layer as the serums that was immediately transferred into a collection tube and stored at −25 °C. The serum was thawed at room temperature before using, and it kept at 4 °C.

Standard serum solutions were prepared by spiking a serial dilution of standard D-T4 and L-T4 to blank human serum to obtain T4 enantiomer standard serum solutions of 1, 5, 25, 45, 65, 85 and 100 μg/ml. The spiked standard serum solutions were then extracted as described below.

### 2.4. Extraction procedure

Serums were separated from the whole blood by centrifugation after the whole blood samples of the thyroid patient were collected. Three ml whole blood precipitation reagent was added into 3 ml serum samples. The tubes were vigorously vortexed to allow complete mixing of whole blood precipitation reagent for precipitation serum proteins. After centrifuging for 5 min at 10 000 rpm, the upper layer was transferred into a 5-ml collection tube, which was then transferred to a Sep-Pak silica cartridge with a glass transfer pipette and equilibrated with 5 ml of ethyl acetate. After washing with 8 ml of ethyl acetate, the extract was eluted with 4 ml of methanol–ammonium hydroxide (90:10, v/v), the eluent was then reduced to dryness under nitrogen to get the extraction. The sample extraction was dissolved in 600 μl of methanol before it was injected into the column.

### 2.5. Assay validation

Five sets of serum standards were prepared and analyzed on the same day to establish the intra-day variation. The assay was repeated over a 3-day period to establish the inter-day variation. Accuracy of the analytical methods was determined by comparing actual concentrations to the predicted concentrations calculated from equations obtained by linear regression of calibration curves. Recoveries of D-, L-T4 from the standard serum solution were evaluated by spiking a series of known amounts T4 enantiomers into the blank samples and extracting as previously described. The recovery was determined by comparing the concentration of D- and L-T4 from the extracted serum standard with those from the non-extracted standard of the same concentration.

## 3. Results and discussion

### 3.1. Chromatogram

Under the optimum separation condition, the T4 enantiomers were separated on the baseline. On evaluating 20 consecutive chromatograms, it was possible to obtain the retention times of 8.25±0.05 min for standard L-T4 and 11.820±0.05 min for standard D-T4. Typical chromatograms of standard solution containing 5 μg/ml D-T4 and 25 μg/ml L-T4, blank serum and a spiked serum containing 9 μg/ml D-T4 and 45 μg/ml L-T4 are shown in Fig. 2A–C.

In these circumstances, the chromatogram of the blank serum showed no matrix interference peaks (see Fig. 2B). Fig. 2C showed the HPLC pattern of a serum solution spiked with the standard D-T4 and

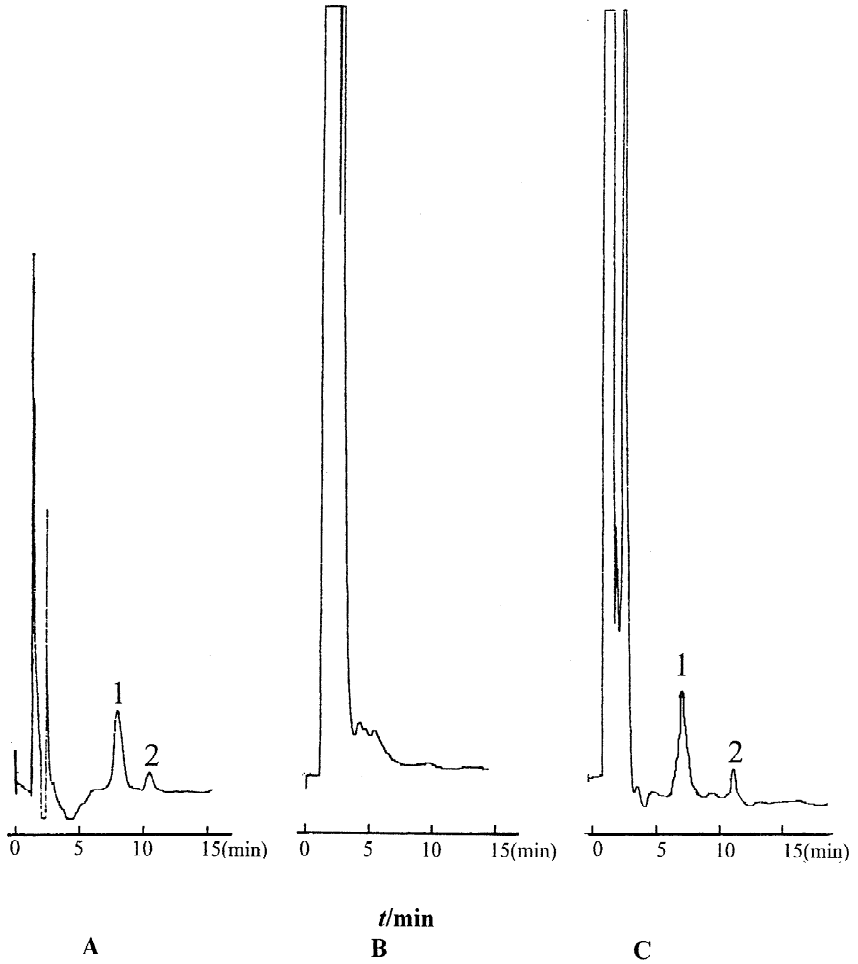


Fig. 2. Chromatogram of a thyroxine sample. Column: Hypersil $SiO_2$ (250×4.0 mm I.D., 5 μm). Flow-rate: 1 ml/min. Column temperature; 40 °C. Detection wavelength: 225 nm. Mobile phase: acetonitrile–water (35:65, v/v). For other conditions, see Section 2. (A) Standard solution: (1) 25 μg/ml L-thyroxine, (2) 5 μg/ml D-thyroxine; (B) blank serum; (C) serum spiked with D- and L-T4: (1) 45 μg/ml L-thyroxine, (2) 9 μg/ml D-thyroxine.

L-T4, the T4 enantiomers peaks can be ascertained with the retention of standard D-T4 and L-T4. This chromatographic system provided a good separation condition of D-T4 and L-T4 from the ectogenesis components.

The HPLC method for the separation and determination of D-, L-T4 in human serum was established with a chrial ligand ion-exchange system using a chiral mobile phase additive and a silica column. We found the retention time of T4 enantiomers that would increase while increasing the volume of acetonitrile of the mobile phase under the condition of silica as column particulate and acetonitrile–water as mobile phase in our experiment. So this separation chromatography process as ''normal-phase'' on the silica column was considered, the separation mechanism of T4 enantiomers was that the different stable T4 isomer complexes ([(L-pro)Cu(L-T4)] and [(L-pro)Cu(D-T4)]) were formed by the mobile phase containing L-pro–copper complex with T4 enantiomers and this reaction was processed in the above mobile phase. Furthermore,

the complex of D-T4 with L-pro–copper was more stable than the [(L-pro)Cu(L-T4)] complex; therefore, L-T4 eluted quicker than D-T4 on the silica column.

Sample processing was successful by a protein-precipitation procedure using an equal volume of the whole blood precipitation reagent and followed by a solid-phase extraction in a silica microcolumn that prevented the analysis column from clogging, and increased the column lifetime.

### 3.2. Linear relation

Calibration graphs were constructed for demonstration the linear relationship between the peak-area and the concentration of the D-, L-T4 standard serum solution. Under the established chromatographic conditions, 20 μl injection volume, the peak areas plotted against the D-, L-T4 standard serum solution to give the calibration curves: $Y_{\text{D-T4}} = 16601C_{\text{D-T4}} - 2790.1$, $r = 0.9986$ for D-T4 and $Y_{\text{L-T4}} = 37960C_{\text{L-T4}} + 2389.1$, $r = 0.9978$ for L-T4. $Y_{\text{D,L-T4}}$ were the peak area of D-, L-T4 and $C_{\text{D,L-T4}}$ were the concentration of D-T4 or L-T4, respectively. The concentration ranges of D-T4 and L-T4 are 1–100 μg/ml. According to the calibration curve of D,L-T4 form, the concentration of D-, L-T4 of the thyroid patient serum was calculated with calibration curves.

### 3.3. Precision and accuracy

According to the calibration curve, the spiked D-, L-T4 standard serum solutions were injected. Then, the precision and accuracy were calculated and shown in Table 1. The average with-day precision, between-day precision and accuracy were, respectively, 4.11, 4.20 and 3.24% for D-T4, and 3.89, 2.41 and 3.97% for L-T4. The limit of detection and the limit of quantitation for both D- and L-T4 were 0.1 μg/ml and 0.8 μg/ml, respectively. The detection limit was defined as the peak signal of D-T4 and L-T4 equal to three times the average noise level.

### 3.4. Recovery

The recoveries of D-T4 and L-T4 were calculated by comparing the concentration of D-T4 and L-T4 obtained from the spiked D-, L-T4 standard serum solution after extraction with the same concentration of D-, L-T4 in the standard solution.

The average recoveries of the T4 enantiomers were 69.44±10.68 for D-T4 and 74.57±12.55 for L-T4. The extractions of D-T4 and L-T4 were not complete but those were sufficient to determine D-T4 and L-T4 at lower levels in patient serum.

Table 1
Precision and accuracy of analysis of D- and L-T4 in standard serum samples

| Added concentration (μg/ml) | Intra-day ($n=6$) | | Inter-day ($n=6$) | | Error (%) |
|---|---|---|---|---|---|
| | Mean measured concentration (μg/ml) | RSD (%) | Mean measured concentration (μg/ml) | RSD (%) | |
| D-T4 | | | | | |
| 5 | 5.43 | 3.84 | 5.20 | 9.40 | 4.00 |
| 10 | 11.09 | 8.73 | 10.28 | 3.44 | 2.80 |
| 20 | 20.02 | 3.48 | 21.43 | 3.63 | 7.15 |
| 50 | 50.27 | 3.21 | 49.76 | 3.86 | 0.48 |
| 100 | 99.97 | 1.29 | 101.77 | 0.65 | 1.77 |
| L-T4 | | | | | |
| 5 | 5.23 | 4.95 | 5.19 | 3.30 | 3.80 |
| 10 | 11.40 | 9.63 | 10.87 | 3.99 | 8.70 |
| 20 | 20.19 | 1.69 | 21.08 | 2.13 | 5.40 |
| 50 | 50.39 | 2.60 | 49.95 | 2.16 | 0.10 |
| 100 | 101.75 | 0.56 | 101.84 | 0.47 | 1.84 |

RSD (%): relative standard deviation = (standard deviation/mean) × 100%.
Error = [Measure value − true value]/true value × 100%.

### 3.5. Quantitative analysis of T4 enantiomers in patient serum samples

After we investigated the validations, this analysis technique was applied to determine the concentration of T4 enantiomeric in serum of about 45 patients who were known to be euthyroid, hyperthyroid, hypothyroid, or have thyroidectomy, goitre and thyroiditis. The determined result of HPLC was compared with the result of the radio-immunity technology (Table 2). The radio-immunity technology is a standard method of the clinical diagnosis and biochemical assessment for the thyroid disease. The result of the radio-immunity technology (see Table 2) only provided T4 concentration of the thyroid patient serum. In the paper, we mentioned the CMPs technology that could determine the concentration of D-T4 and L-T4, and found that L-T4 was mainly in hyperthyroid patient serum, and D-T4 was mainly in the hypothyroid, thyroidectomy, goitre and thyroiditis.

Fig. 3 showed the patient serums chromatograms of euthyroid, hyperthyroid and hypothyroid; the appearance of peaks 1 and 2 and a retention time were confirmed from the standard solution of D-T4 and L-T4.

Finally, an euthyroid volunteer was orally dosed 10 mg of D-T4 and his blood sampled 5 h after ingestion (blood was processed as described in Section 2.4), to determine the concentration of D-T4 and L-T4. The HPLC chromatogram is shown in Fig. 4. The chromatogram had surprising differences comparing the dosed D-T4 euthyroid volunteer serum (Fig. 4) with the non-dosed D-T4 euthyroid serum

Table 2
Results of chromatography and the radio-immunity technology

| Patient type | Radio-immunity (mean±SD; μg/ml) | Chromatographic method (μg/ml) | |
|---|---|---|---|
| | | D- | L- |
| Euthyroid | 131.20±5.89 | – | – |
| Hypothyroid | 30.40±7.32 | 25.63 | 0.21[a] |
| Hyperthyroid | 307.60±8.98 | 0.13[a] | 244.56 |
| Thyroidectomy | 291.60±7.98 | 1.26 | 235.86 |
| Goitre | 115.06±6.32 | 1.03 | 82.19 |
| Thyroiditis | 79.30±8.58 | 8.93 | 61.00 |

[a] The data is less than 1 μg/ml which exceeded the concentration range but it was determined in this experiment.

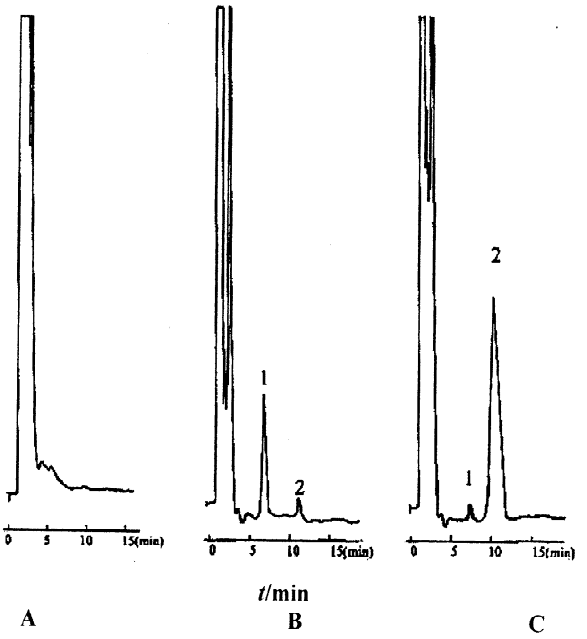


Fig. 3. Chromatogram of serum of patients with thyroid disease. Column: Hypersil $SiO_2$ (250×4.0 mm I.D, 5 μm). Flow-rate: 1 ml/min. Column temperature: 40 °C. Detection wavelength: 225 nm. Mobile phase: acetonitrile–water (35:65, v/v). For other conditions, see Section 2. (A) Euthyroid patient serum; (B) hyperthyroid patient serum: (1) 27.63 μg/ml L-T4, (2) 0.21 μg/ml D-T4; (C) hypothyroid patient serum: (1) 0.13 μg/ml L-T4, (2) 244.56 μg/ml D-T4.

(Fig. 3A), which showed the concentration of D-T4 and L-T4 was not determined in non-dosed D-T4 euthyroid serum (Fig. 3A), but the concentration of D-T4 and L-T4 could be determined in dosed D-T4 euthyroid serum (Fig. 4). The more exciting possibility was found that ingestion D-T4 might be converted to the more bioactive L-T4 in vivo of euthyroid patients. This result was the same as that hypothesised by Hay et al. [13]. The reason will require further investigation.

The technique was first applied to determine the concentration of T4 enantiomers in patients' serum who were known to be the euthyroid, hypothyroid, hyperthyroid, or have thyroidectomy, goitre or thyroiditis. From Fig. 3 and Table 2, it is shown that the hyperthyroid patient serum possessed a lot of L-T4 but the reverse result was found in hypothyroid patient serum. D-T4 and L-T4 were not determined in euthyroid patient serum by HPLC. It could be

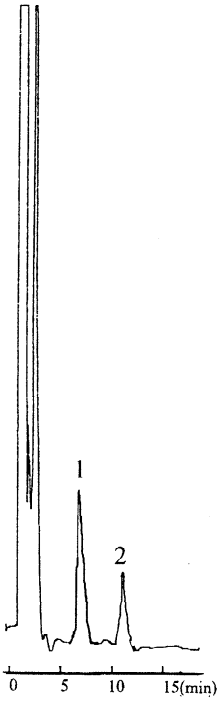


Fig. 4. Chromatogram of the euthyroid patient given 10 mg D-T4 5 h prior to venipuncture. Column: Hypersil $SiO_2$ (250×4.0 mm I.D., 5 μm). Flow-rate: 1 ml/min. Column temperature: 40 °C. Detection wavelength: 225 nm. Mobile phase: acetonitrile–water (35:65, v/v). For other conditions, see Section 2. (1) L-Thyroxine, (2) D-thyroxine.

conjectured that in the hyperthyroid patient serum the result of the radio-immunoassay technology was mainly L-T4, and that in the hypothyroid patient it was D-T4. We believed that the determination of the concentration of both D-T4 and L-T4 could provide more information for treating thyroid gland patients than T4 alone.

The present technique provided a methodology to determine D-, L-T4 in vivo. This method may be used to investigate whether D-T4 is converted in vivo to L-T4 or vice versa, by HPLC.

The paper provided the separate determination method of T4 enantiomers in human serum, the D-, L-T4 was separated on baseline. The method is better than previous reports [10–16].

The concentration of T4 enantiomers determined in patient serum could provide the basis for clinicians to alter their treatment and prescriptions for thyroid gland diseases.

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