Development of a Validated HPLC Method for the Simultaneous Determination of D- and L-Thyroxine in Human Plasma

Jing Yu Jin, Chae-Sun Baek, and Wonjae Lee*

*College of Pharmacy, Chosun University, Gwangju 501-759, Korea. *E-mail: wlee@chosun.ac.kr*
Received April 4, 2007

Key Words : Thyroxine, Enantiomer separation, Chiral stationary phase

L-Thyroxine (L-T4), the naturally occurring thyroid hormone has been used for the treatment of thyroid dysfunctions, while D-T4 is not active in the thyroid and is used for the treatment of hyperlipidemia.[1,2] Therefore, a convenient and reliable enantiomer separation method has been of great interest in the biological and pharmacological research field. Several groups reported the chromatographic resolution of thyroxine enantiomers by chiral ligand exchange method using cupper solution on achiral column.[3-5] However, only a few of results for direct enantiomer separation on chiral stationary phases (CSPs) have been reported.[2,6-8] Among these reports, the separation of thyroxine enantiomers has been performed on a proline derived CSP (α = 1.90), a protein ovomucoid derived CSP (α = 1.16-1.32) under aqueous buffer solutions and on a CSP derived from (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid (18-C-6-TA) (α = 2.08-3.11), depending upon the used mobile phases.[2,6-8] For resolution of thyroxine enantiomers, the crown ether derived CSP showed better enantioseparation than the proline or ovomucoid derived CSP. However, the validated direct determination of thyroxine enantiomers in plasma on a CSP derived from 18-C-6-TA (CSP 1) has not been performed. In this study, a liquid chromatographic method for the simultaneous determination of D- and L-thyroxine in human plasma using the chiral crown ether derived CSP 1 is developed and validated. To our knowledge, the validated direct simultaneous determination of thyroxine enantiomers in plasma samples using the chiral HPLC column is the first report.

For the optimum chromatographic results of specificity experiment of D- and L-T4 in plasma samples, several mobile phase conditions were used. As shown in Table 1, chromatographic parameters such as separation factors, retention times and resolution factors on CSP 1 are considerably influenced by the nature of mobile phases.[7,9] Although Aboul-Enein and his co-works have shown the chromatographic results using the mobile phases of methanol/H_2O containing H_2SO_4 as an additive,[7] these chromatographic conditions were not effective for the specificity results of D- and L-T4 present in plasma samples. Therefore, the several mobile phases of ethanol/H_2O containing H_2SO_4 as an additive were used and optimized in this study. When the mobile phase of 90% ethanol/H_2O (V/V) containing 10 mM H_2SO_4 was used for the validation study of D- and L-T4 in plasma samples, no matrix peaks interfered with internal standard (IS) of L-phenylglycine, L-T4 and D-T4 at their retention times. Figure 1 shows representative chromato-

Table 1. Effect of mobile phase for the separation of the enantiomers of thyroxine on CSP1

Alcohol/H_2O	Acid Additive	k'_1[a]	k'_2	α[b]	Rs[c]
75% Ethanol/H_2O	10 mM H_2SO_4	1.48	2.69	1.82	3.12
80% Ethanol/H_2O	10 mM H_2SO_4	1.69	3.12	1.85	3.43
85% Ethanol/H_2O	10 mM H_2SO_4	2.47	4.57	1.85	3.68
90% Ethanol/H_2O	10 mM H_2SO_4	6.23	12.00	1.92	4.31
80% Methanol/H_2O	10 mM H_2SO_4	1.00	1.91	1.92	2.67
80% Ethanol/H_2O	5 mM H_2SO_4	1.34	2.64	1.96	2.89
100% Methanol	10 mM H_2SO_4	2.05	4.35	2.12	3.05

Mobile phase: Alcohol/H_2O (V/V) containing acid additive; Flow rate = 1 mL/min; UV = 210 nm. [a]Capacity factor for the first eluted enantiomer. [b]Separation factor. [c]Resolution factor.

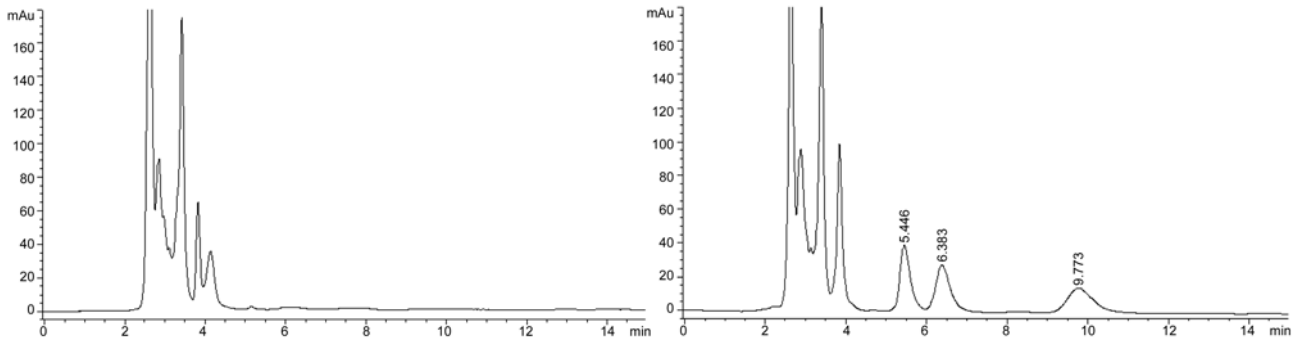

Figure 1. Chromatograms of a blank human plasma sample (the left) and a typical plasma sample spiked with IS (50 μg/mL, 5.446 min), L-T4 (20 μg/mL, 6.383 min) and D-T4 (20 μg/mL, 9.773 min) (the right). See experimental for chromatographic conditions.

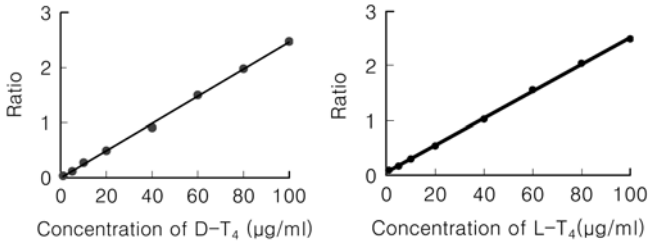

Figure 2. Calibration curve of D-T4 (the left) and L-T4 (the right) spiked to blank plasma. The coefficients of correlation (r) were 0.9987 and 0.9996 for D-T4 and L-T4, respectively.

grams of a blank human plasma sample and a typical plasma sample spiked with IS, L-T4 and D-T4 after pretreatment of samples. The calibration curves were constructed for demonstrated the linear relationship between the relative peak area and the concentration of D- and L-T4 standard serum solutions. Each eight standard solution (1, 5, 10, 20, 40, 60, 80, 100 μg/mL) of D- and L-T4 was run for the calibration curve, respectively. As shown in Figure 2, the coefficients of correlation (r) were 0.9987 and 0.9996 for D-T4 and L-T4 over the concentration ranges of 1-100 μg/mL, respectively. The equations were $Y_{D\text{-}T4} = 0.0247X_{D\text{-}T4} - 0.0067$ and $Y_{L\text{-}T4} = 0.0246X_{L\text{-}T4} + 0.054$ [Y for peak area ratio to IS and X for concentration (μg/mL)]. These results are better than those by the other group of the analytical method using a chiral mobile phase additive on a silica column.[5]

Tables 2 and 3 show a summary of intraday and interday precision and accuracy of measurement of D-T4 and L-T4 in plasma, respectively. The intraday accuracy was determined to be 97.6-103.4% (D-T4) and 97.1-102.0% (L-T4) by performing five replicate analyses at each concentration level (10, 20, 40, 100 μg/mL), respectively. The intraday precision expressed in % RSD was determined to be 1.68-8.27% (D-T4) and 1.96-6.54% (L-T4) at the same four concentration levels, respectively. Similarly, the interday accuracy was determined to be 97.8-108.4% (D-T4) and 98.9-100.2% (L-T4) by performing five replicate analyses at each concentration level (10, 20, 40, 100 μg/mL), respectively. The interday precision expressed in % RSD was determined to be 1.52-7.07% (D-T4) and 6.49-8.32% (L-T4) at the same four concentration levels, respectively. The limit of detection was defined as the peak signal of D-T4 and L-T4 equal to three times the average noise level. The limit of detection for both D- and L-T4 in plasma sample were 1.8 ng and 3.2 ng per injected sample, respectively.

Table 2. Intraday precision and accuracy of D- and L-T4 in standard plasma samples

Added Concentration (μg/mL)	Intra-day (n = 5)		
	Mean (μg/mL)	Accuracy (%)	Precision (%)
D-T4			
10	10.34	103.4	6.67
20	19.53	97.7	8.27
40	39.02	97.6	1.68
100	99.63	99.6	5.67
L-T4			
10	10.20	102.0	6.54
20	19.41	97.1	4.63
40	39.77	99.4	1.96
100	98.10	98.1	6.41

Table 3. Interday precision and accuracy of D- and L- T4 in standard plasma samples

Added Concentration (μg/mL)	Inter-day (n = 5)		
	Mean (μg/mL)	Accuracy (%)	Precision (%)
D-T4			
10	10.84	108.4	7.07
20	20.63	103.2	4.42
40	39.13	97.8	1.52
100	101.87	101.9	3.35
L-T4			
10	9.96	99.6	7.99
20	20.04	100.2	8.32
40	39.54	98.9	6.49
100	99.93	99.9	8.25

In summary, we demonstrated the simultaneous determination of D-T4 and L-T4 on a chiral crown ether derived CSP 1 in human serum with base-line separation. This new analytical method for the determination of D-T4 and L-T4 using internal standard has been developed and validated. Validation experiments have shown that this method has good precision and accuracy as well as high linearity of 0.9987 and 0.9996 for D-T4 and L-T4 over the concentration ranges of 1-100 μg/mL, respectively. This new analytical results using the chiral HPLC column may be used to investigate not only the simultaneous determination of D-T4 and L-T4 for biological samples but also the degree of conversion of each T4 enantiomer *in vivo*.

Experimental Section

Reagents and Apparatus. L-Thyroxine and L-phenylglycine were purchased from Aldrich (WI, USA) and DL-thyroxine was purchased from TCI (Japan). Human plasma was obtained from Red Cross Blood Center (Gwangju, Korea). HPLC-grade alcohols and acetonitrile were purchased from J. T. Baker (PA, USA). All chromatographic measurements were performed using an Agilent 1100 series combinatorial LC instrument equipped with isocratic pump, auto-sampler and diode array detector from Agilent Technologies (Wilmington, DE, USA). Water was purified using a milli-Q water purification system (Bedford, MA, USA). CSP 1 derived from (+)-18-C-6-TA (250 mm L × 4.6 mm I.D.) was obtained from RS Technologies (Daejon, Korea). Chromatographic conditions are as follows: flow rate = 1 mL/min; detection UV 210 nm; Temperature ambient (about

25 °C); 20 μL injection volume. The mobile phase used for validation data is 90% ethanol/H_2O (V/V) containing 10 mM H_2SO_4.

Sample Preparation. A stock solution of DL-thyroxine (2.4 mg/mL) and L-phenylglycine (internal standard, 0.6 mg/mL) dissolved with the mobile phase were prepared. From the stock solution, DL-thyroxine working standard solutions of 0.024, 0.12, 0.24, 0.48, 0.96, 1.44, 1.92, 2.40 mg/mL were prepared by dilution with the mobile phase. Plasma samples (0.2 mL) were pipetted into glass tubes and spiked with 20 μL of each thyroxine working standard solution and internal standard (IS) solution, respectively. Then standards in plasma at eight different concentration levels (1, 5, 10, 20, 40, 60, 80, 100 μg/mL) of D- and L-thyroxine were prepared for calibration curves, respectively. After adding 0.5 mL of acetonitrile to glass tube, the plasma samples were shaken for 1 min. After 10 min of centrifugation at 3000 g, the supernatant (0.5 mL) was transferred into another glass tube and completely evaporated under a stream of nitrogen. The dry residue was reconstituted with 400 μL mobile phase and centrifuged for 2 min at 15000 g. And the upper layer (20 μL) wad injected directly onto the HPLC system. Five replicate analyses were performed on plasma standards at four different concentration levels (10, 20, 40, 100 μg/mL) of D-and L-thyroxine, respectively, to assess both interday and intraday precision and accuracy of the method.

Acknowledgment. This study was supported by research funds from Chosun University, 2006.

References

1. Gika, H.; Lämmerhofer, M.; Papadoyannis, I.; Lindner, W. *J. Chromatogr. B* **2004**, *800*, 193.
2. Abou-Basha, L. I.; Aboul-Enein, H. Y. *Pharm. Acta Helv.* **1995**, *70*, 237.
3. Oelrich, E.; Preusch, H.; Wilhelm, E. *J. High Resol. Chromato*. **1980**, *3*, 269.
4. Hay, I. D.; Annesley, T. M.; Jiang, N. S.; Gorman, C. A. *J. Chromatogr. B: Biomed.* **1981**, *226*, 383.
5. Wang, R.; Jia, Z.-P.; Hu, X.-L.; Xu, L.-T.; Li, Y.-M.; Chen, L.-R. *J. Chromatogr. B* **2003**, *785*, 353.
6. Ekborg-Ott, K. H.; Kullman, J. P.; Wang, X.; Gahm, K.; He, L.; Armstrong, D. W. *Chirality* **1998**, *10*, 627.
7. Aboul-Enein, H. Y.; Ali, I.; Hyun, M. H.; Cho, Y. J.; Jin, J. S. *J. Biochem. Bioph. Methods* **2002**, *54*, 407.
8. Jin, J. Y.; Lee, W.; Hyun, M. H. *J. Liq. Chrom. & Rel. Tech.* **2006**, *29*, 841.
9. Hyun, M. H. *Bull. Korean Chem. Soc.* **2005**, *26*, 1153.