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크라운 에테르에서 유도된 키랄 컬럼을 사용한 레보티록신 나트륨 의약품의 광학순도 모니터링

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Monitoring of the Optical Purity
for Levothyroxine Sodium in Pharmaceuticals
Using Crown Ether Derived Chiral Columns

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이 논문을 약학 석사학위과정 논문으로 제출함.

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Abstract

Monitoring of the Optical Purity for Levothyroxine Sodium in Pharmaceuticals Using Crown Ether Derived Chiral Columns

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L-Thyroxine possessing a chiral center, the naturally occurring thyroid hormone has been used for the treatment of thyroid dysfunctions and marketed as levothyroxine (L-thyroxine) sodium salt. In this study, after extraction of levothyroxine tablet as a pre-treatment process, direct enantiomer separation of thyroxine on crown ether derived chiral columns for determination of optical purity was performed using reversed mobile phase with acid additive. The chromatographic method developed in this study was applied in the determination of optical purity of several current domestic and foreign commercialized levothyroxine tablets. Optical purity values of these commercialized L-thyroxine sodium tablets except one were higher than 99 percents.

Key Words: Enantiomer separation, Thyroxine, Chiral column, Optical purity

국문초록

카이랄 센터를 가진 L-티록신은 갑상선 장애를 위한 치료에 사용되어져 왔으며, 레보티록신 나트륨 염으로 판매되어왔다. 레보티록신 나트륨은 갑상선 기능 장애를 치료하기 위해 상업적으로 이용되지만, 광학순도를 측정하기 위한 몇 가지 직접 분석방법들만이 보고되었다. 본 연구는 크라운 에테르 타입의 카이랄 컬럼에서 직접적 거울상 이성질체 분석을 위한 액체 크로마토그래피 분석 조건을 찾기 위해 시도하였다. 다양한 이동상들 중에서, 10mM H_2SO_4를 포함하는 100% 메탄올 용매가 가장 적절한 것으로 밝혀졌다. 본 연구에서 개발된 크로마토그래피 분석 방법은 여러 가지 현재 국내외 상용화되는 레보티록신 나트륨 정제의 광학 순도 결정에 적용되었다. 상용화되는 레보티록신 나트륨 정제를 크로마토그래피 분석한 결과, 한 가지를 제외한 모든 제약 제품은 거의 99% 이상의 광학 순도를 가지는 것을 보여주고 있다.

PART 1. Direct Enantiomer Separation of Thyroxine in Pharmaceuticals Using Crown Ether Type Chiral Stationary Phase

Ⅰ. INTRODUCTION

In general, the characteristics of two enantiomers of a chiral compound are identical to each other, but they often behave differently in the presence of other chiral compounds and in biological systems (Francotte and Linder, 2006). Many pharmaceuticals are chiral. As each enantiomer may have different physiological activities, achieving high enantiopurity is essential for active compounds in pharmaceutical formulation. Therefore, the development of a convenient and reliable method for enantionmer separation has been a great concern in pharmaceutical industries. Thyroxine is the tyrosine based hormone produced by the thyroid gland. Like most naturally occurring amino acids, thyroxine exists predominantly in L-isomer, called levothyroxine, L-thyroxine or L-T4. Accordingly, synthetic thyroxine, manufactured for the treatment of thyroid dysfunctions specifically hypothyroidism, is synthesized in the form of L-thyroxine sodium salt. D-thyroxine used to be commercially available for its cholesterol lowering effect. Due to its association with high mortality in patients with cardiac disease and the availability of more effective drugs, however, its production was discontinued in 1997 (Bantle et al., 1981; Abou-Basha and Aboul-Enein, 1995; Gika et al., 2004; Thomson Micromedex, 2010).

Of many enantiomer separation techniques, the direct chromatographic analytical method using chiral stationary phases has been widely used due to its convenience and accuracy. However, only a few studies have reported the use of direct separation on a chiral stationary phase for thyroxine. Abou-Basha and Aboul-Enein (1995) firstly attempted the direct separation of thyroxine using the ovomucoid protein based chiral stationary phase with various buffer solutions as mobile phases, and obtained good results (α=1.17-1.32,

Rs=0.95-2.20). They applied the technique to the quantitative analysis of L-thyroxine, but it was not very convenient as it took as long as 50 minutes. Ekborg-Ott et al. (1998) used an antibiotic avoparcin derived chiral stationary phase with methanol buffer solution as mobile phase. They also obtained a very good result (α=3.32, Rs=2.67), but only chromatographic results were reported without providing any relevant information. Aboul-Enein et al. (2002) used a crown ether based chiral stationary phase in the mobile phase of 80% aqueous methanolic solution containing sulfuric acid. They also achieved good results (α =2.08-3.11, Rs=1.00-2.60), but their report was limited to the development of analytical method without demonstrating further application. Recently, a validated analytical method using a crown ether derived chiral stationary phase was developed to separate and determine L- and D-thyroxine in human plasma simultaneously (Jin et al., 2007).

As mentioned above, synthetic thyroxine is available in the form of L-thyroxine sodium salt, but its direct optical purity analysis has not been actively performed. There is one study appeared in literature; Gika et al. (2004) performed the separation of thyroxine enantiomers using a quinine derived chiral stationary phase in aqueous acetonitrile solution (α=1.28, Rs=2.32), and applied the technique into the quantitative analysis and optical purity analysis for L-thyroxine. In order to assure the quality of L-thyroxine and produce optically pure products for maximum benefits, it is necessary to develop a convenient and reliable analytical method for enantiomer separation. The purpose of this study was to find an effective condition for direct enantiomer separation of thyroxine on a crown ether based chiral stationary phase, and to analyze the optical purity of commercially available D-and L-thyroxine reagents and pharmaceutical products in Korea using the chromatographic condition.

Ⅱ. MATERIALS AND METHODS

MATERIALS

D- and/or L-thyroxine were purchased from Fulka (Switzerland), Sigma (USA) and Acros (Belgium). DL-thyroxine and all acids used in this study were purchased from TCI (Japan). All L-T4 tablets marketed as L-thyroxine sodium salt in domestic pharmaceutical company (Samnam, Bukwang, Dalim Biotec) were purchased. All chromatographic experiments 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). The HPLC grade methanol was obtained from J.T. Baker (PA, USA). Water was purified using a milli-Q water purification system (Bedford, MA, USA). The chiral stationary phase derived from (-)-(18-crown-6)-2,3,11,12-tetracarboxylic acid (250 mm L × 4.6 mm I.D.) was obtained from RS Technologies (Daejon, Korea).

HPLC ANALYSIS AND METHOD VALIDATION

The chromatographic condition was methanol in H_2O containing acid additive as the mobile phase at 1.0 mL/min of flow rate with UV detection at 230 nm. For HPLC analyte preparation, the tablet of each drug formulation was pulverized and the powder was suspended in methanol containing 10mM H_2SO_4, ultrasonicated at room temperature for 5min and finally undissolved materials were removed by filtration. The filtrate was directly used for HPLC injection and was stored for 1 month at -4°C. The intra-day precision and accuracy of the method were evaluated by analyzing samples in five replicates, performed by one operator within a day at three different optical purities of L-thyroxine, respectively. The inter-day precision and accuracy were assessed

by replicating the analysis of samples on 5 days at three different optical purities of L-thyroxine, respectively. Precision was expressed as the intra-day and inter-day percent relative standard deviation.

III. RESULTS AND DISCUSSION

The separation of thyroxine enantiomers was performed using various mobile phases to find an appropriate analytic condition for the optical purity. Table I shows the effect of different methanol contents in mobile solution on the chromatographic parameters of thyroxine enantiomers; as the methanol content of mobile solution decreases, the separation factor increases but the capacity factor decreases. Thes sepsults are similar to thos sof previous studypacit amino acids (Hyun, M.H., et al, 1998). Table I also shows the effect of acidity; as the concentration of sulfuric acid or the acidity of various acids increases ($HClO_4$ > HCl > CH_3SO_3H > $C_2H_5SO_3H$), the capacity factor increases acitthe separation factor decreases. Thes sepsults also are conacitent acit previous epsults (Hyun et al, 1998; Hyun et al, 1999). The amino group of thyroxine becomes protonized y facidic mobile phase condition; the mo). Tcidic the mobile phase is, the stronger hydrogen bonding acit oxygen atomhe cocrown ether of chiral septionary phase is formed, epsulting y fincrease of capacity factor acitdecrease of separation factor (Lee et al., 2005; Bacg et al., 2001). As a mobile phase, 90% methanol/H_2O(V/V) containing 10mM H_2SO_4 was u > C in the nt irat> C2nalytical ethanolto avoid overlapping plasma matrix peaks (Jin et al., 2007), but pharmaceutical levothyroxine products u > Cin this studyp containpcolors acitother aiditives. Therefo).,factifferent mobile phase, 100% methanol containing 10mM H_2SO_4, was sacect> Cfor mo). Tppropriate analytical condition. When condureaon deparation of thyroxine enantiomers, either (+)-18-C-6-TA or (-)-18-C-6-TA derived chiral septionary phase is u > C2H5a HPLCpcolumn. The elution order changes depending on the column:f vThyroxine get elut> Cfirst on the former column, while D-thyroxine does on the latter column (Jin et al., 2006). The (-)-18-C-6-TA derived chiral stationary phase was used in this study to avoid overlapping matrix peaks with the thyroxine products. The chromatographic analytical method to determine optical

Table I. Effect of mobile phase for the separation of the enantiomers of thyroxine

Alcohol/H_2O	Acid Additive	k'_1[a]	k'_2[b]	α[c]
100% Methanol	10mM H_2SO_4	1.15	2.46	2.15
95% Methanol/H_2O(V/V)	10mM H_2SO_4	1.04	2.26	2.17
90% Methanol/H_2O(V/V)	10mM H_2SO_4	0.64	1.52	2.38
85% Methanol/H_2O(V/V)	10mM H_2SO_4	0.46	1.11	2.40
80% Methanol/H_2O(V/V)	10mM H_2SO_4	0.42	1.07	2.54
100% Methanol	5mM H_2SO_4	0.54	1.32	2.44
100% Methanol	10mM H_2SO_4	1.15	2.46	2.15
100% Methanol	15mM H_2SO_4	1.28	2.67	2.09
100% Methanol	10mM $C_2H_5SO_3H$	0.57	1.44	2.53
100% Methanol	10mM CH_3SO_3H	0.83	1.99	2.38
100% Methanol	10mM HCl	1.08	2.40	2.22
100% Methanol	10mM $HClO_4$	4.86	9.61	1.98

Mobile phase: Alcohol/H_2O (V/V) containing acid additive; Flow rate = 1mL/min; Detection UV 230nm. a,b Capacity factor for the first and second eluted enantiomer. c Separation factor.

purities of thyroxine was validated and applied for commercially available D- and L-thyroxine reagents and pharmaceutical L-thyroxine products.

The accuracy for both intra- and inter-day as well as the precision for the analytical method at three optical purities of L-thyroxine are listed in Table II.

Table II. Intra-day and inter-day precision and accuracy of the analytical method for L-thyroxine

Optical purity of L-thyroxine(%)	Intra-day(n=5)		Inter-day(n=5)	
	Accuracy(%)	Precision(%)	Accuracy(%)	Precision(%)
99.70	100.13	0.54	100.11	0.85
99.30	100.16	0.66	100.16	0.79
95.10	100.98	1.03	100.99	1.15

The accuracy for intra- and inter-day assay was determined to be 100.13-100.98% and 100.11-100.99%, respectively. The precision for intra- and inter-day assay expressed in % RSD was determined to be 0.54-1.03% and 0.79-1.15%, respectively. The results for accuracy and precision indicate that this validated method is highly suitable.

Table III. Determination of the optical purity of commercially available D- and L-thyroxine analytes

Reagent	Company	D:L ratio[a]	RSD(%)[b]	Specific rotation data[c]
D-Thyroxine	Fluka	94.47 : 5.53	1.52	-18.00 (c=2, 1M HCl: EtOH = 1:4)
L-Thyroxine	Fluka	0.24 : 99.76	0.50	-5.3±0.5 (c=1, 1M NaOH: EtOH = 1:2)
L-Thyroxine	Sigma	5.12 : 94.88	1.26	+21 (c=1, 1M HCl: EtOH = 1:2)
L-Thyroxine	Acros	0.23 : 99.77	1.30	-4.2 (c=3, 0.1M NaOH in 70% EtOH)

[a] Average value of more than three times determined. [b] Relative standard deviation. [c] Specific rotation data indicated on reagent label of commercially available D- and L-thyroxine.

Table III shows that the optical purities of thyroxine enantiomers were different from reagent to reagent, ranging from about 95% (Fluka D-thyroxine and Sigma L-thyroxine) to 99.8% (Fluka L-thyroxine and Acros L-thyroxine). Fig. 1 shows typical chromatograms of determination of the optical purity of commercially D- and L-thyroxine reagents. Commercially available chiral reagents often provide optical rotation values but hardly offer optical purity information. In fact, specific rotation values were provided with every thyroxine reagent used in this study; for example, Fluka D-thyroxine: -18.00 (c=2, 1M HCl:EtOH=1:4), Fluka L-thyroxine: -5.3 ± 0.5 (c=1, 1M NaOH: EtOH = 1:2), Sigma L-thyroxine: +21 (c = 1, 1M HCl: EtOH = 1:2), and Acros L-thyroxine: -4.2 (c=3, 0.1M NaOH in 70% EtOH), but no information on optical purity were available. To our knowledge, this study is the first report of determination of the optical purity of thyroxine reagents. Enantiomer impurity of about 5-6% with two of four reagents calls attention of pharmaceutical companies and researchers handling chiral reagents.

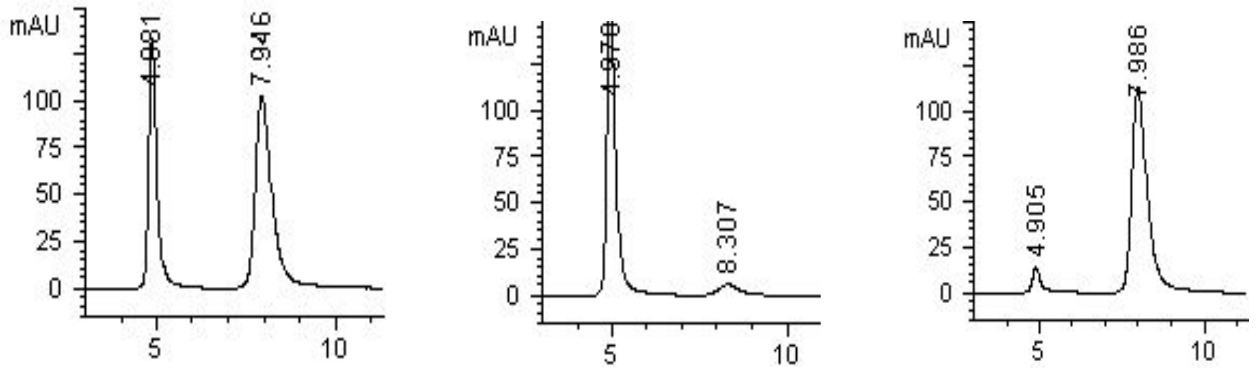

Fig. 1. Typical chromatograms of determination of the optical purity of commercially available commercially available D- and L-thyroxine analytes: racemic thyroxine (the left), D-thyroxine (Fluka reagent) (the middle, D:L = 94.47 : 5.53) and L-thyroxine (Sigma reagent) (the right, D:L = 5.12 : 94.88). Mobile phase: 100% methanol containing 10mM H_2SO_4. Flow rate: 1 mL/min. Detection UV 230nm.

There are six commercially available thyroxine products in Korea, manufactured from three pharmaceutical companies in the form of levothyroxine (L-T4) sodium tablets. All of them were obtained and their optical purities were analyzed using the optimal analytical condition described above. The tablets were pre-treated in methanol containing 10mM H_2SO_4, and their extracts were directly injected into HPLC without any derivatization process. For mobile phase, 100% methanol containing 10mM H_2SO_4 was used. Chromatographic analyses were conducted more than three times for each product, and the average values were presented in Table IV. As shown in Table IV, the optical purity was found to be high with every product, ranging from 97.61% to 99.76% (D-T4 impurities 0.24 - 2.39%), and all but F product showed higher than 99% purity. These results are superior to those of previous research conducted in other countries (purity: 92.7-99.9%, D-T4 impurity 0.1-7.3%) (Gika et al., 2004). The relative standard deviation (RSD) of optical purity was 0.59-5.86%, demonstrating the results are fairly reliable. Typical chromatograms of enantiomer impurity in pharmaceutical formulation of L-T4 sodium tablet (F sample) are shown in Fig. 2.

Table Ⅳ. Determination of the optical purity data in pharmaceutical formulations of commercially available levothyroxine sodium

Commercial sample	D : L ratio[a]	RSD(%)[b]
A	0.42 : 99.58	5.86
B	0.44 : 99.56	5.51
C	0.24 : 99.76	0.59
D	0.36 : 99.64	3.21
E	0.57 : 99.43	3.46
F	2.39 : 97.61	1.53

[a]Average value of more than three times determined. [b]Relative standard deviation.

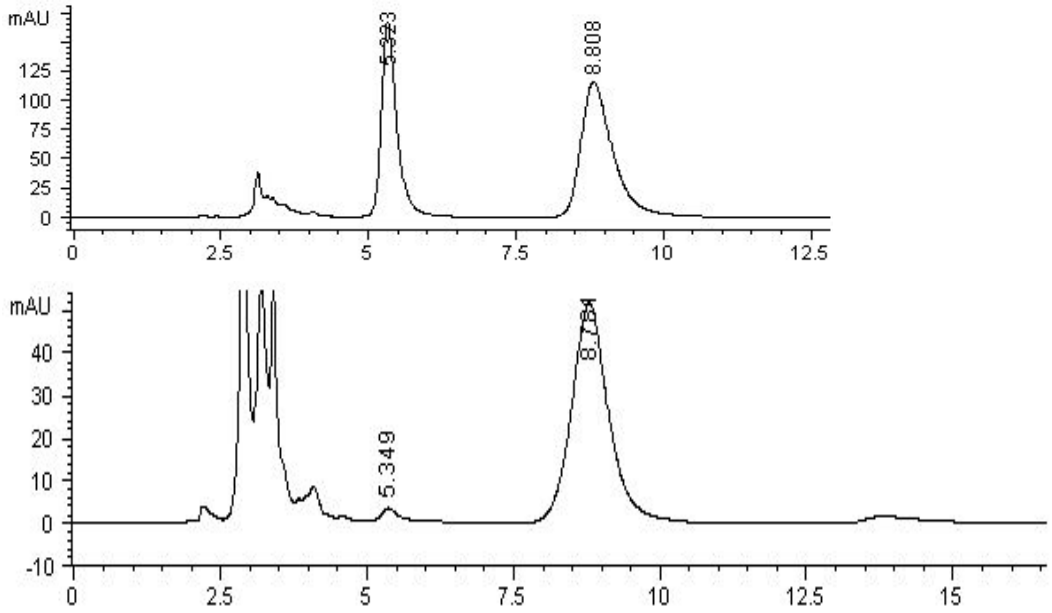

Fig. 2. Typical chromatograms of enantiomer separation of thyroxine (the top) and L-T4 sodium tablet (F sample) (the bottom). Mobile phase: 100% methanol containing 10mM H_2SO_4. Flow rate: 1 mL/min. Detection UV 230 nm.

Ⅳ. conclusion

In conclusion, this study established an optimized analytical condition for direct separation of thyroxine enantiomers using crown ether derived chiral stationary phase with methanol containing acid additive as a mobile phase. This chromatographic method was validated for optical purity analysis and the results for accuracy and precision indicate that this validated method is highly suitable. When four D- and L-T4 reagents and six domestically available L-T4 sodium tablets were analyzed under this condition, all products except one showed higher than 99% optical purity with L-T4 sodium tablets, and revealed relatively higher eanantiomer impurity with two of four thyroxine reagents. It is anticipated that the developed chromatographic analytical method and subsequent findings would contribute for pharmaceutical companies to manufacture better quality controlled products.

V. REFERENCES

1. Francotte, E. and W. Lindner, (Ed.) (2006) *Chirality in Drug Research*, Wiely-VCH, Weinheim.
2. Bantle, J., J. Oppenheimer, H. Schwartz, D. Hunninghake, J. Probstfield and R. Hanson (1981), TSH response to TRH in euthyroid, hypercholesterolemic patients treated with graded doses of dextrothyroxine, *Metabolism*, **30**, 63-66.
3. Abou-Basha, L. I. and H. Y. Aboul-Enein (1995), Enantiomeric separation and optical purity determination of thyroxine enantiomers in bulk and pharmaceutical formulations, *Pharm. Acta Helv.* **70**, 237-240.
4. Gika, H., M. Lämmerhofer, I. Papadoyannis and W. Lindner (2004), Direct separation and quantitative analysis of thyroxine and triiodothyronine enantiomers in pharmaceuticals by high-performance liquid chromatography, *J. Chromatogr.*, **800**, 193-201.
5. Aboul-Enein, H. Y., I. Ali, M. H. Hyun, Y. J. Cho and J. S. Jin (2002), Effect of acidity on the enantiomeric resolution of thyroxine and tocainide by HPLC on a (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid column, *J. Biochem. Bioph. Methods.* **54**, 407-413.
6. Jin, J. Y., C.-S. Baek, and W. Lee (2007), Development of a validated HPLC method for the simultaneous determination of D- and L-thyroxine in human plasma, *Bull. Kor. Chem.* Soc. **28**, 1070-1072.
7. Hyun, M.H., J. S. Jin and W. Lee (1998), Liquid chromatographic resolution of racemic amino acids and their derivatives on a new chiral stationary phase based on crown ether, *J. Chromatogr. A*,

822, 155-161.

8. Lee, W., J. Y. Jin and C.-S. Baek (2005), Comparison of enantiomer separation on two chiral stationary phases derived from (+)-18-crown-6-2,3,11,12-tetracarboxylic acid of the same chiral selector. *Microchemical Journal*, 80, 213-217.
9. Jin, J. Y., W. Lee and M. H. Hyun (2006), Development of the antipode of the covalently-bonded crown ether type chiral stationary phase for the advantage of the reversal of elution order, *J. Liq. Chrom. & Rel. Tech.* 29, 841-848.
10. Jin, J. Y. and W. Lee (2007), Liquid chromatographic enantiomer resolution of *N*-hydrazide derivatives of 2-aryloxypropionic acids on a crown ether derived chiral stationary phase, *Chirality*, 19, 120-123.
11. Lee, T., W. Lee, M. H. Hyun and J. H. Park (2010), Enantioseparation of native α-amino acids on an 18-crown-6-tetracarboxylic acid-bonded silica by capillary electrochromatography, *J. Chromatogr. A*, 1217, 1425-1428.
12. Perry, J. A., J. D. Rateike and T. J. Szczerba (1987), Eluting trace components before major constituents : I. Sensitivity enhancement in analytical determinations of optical purity, *J. Chromatogr.,* 389, 57-64.
13. Jin, D., M. Zhang, S. Jin, M. Lee, G. Song, G. Back and Y. Lee (2007), Enantioselective resolution of thyroxine hormone by high-performance liquid chromatography utilizing a highly fluorescent chiral tagging reagent, *Chirality*, 19, 625-631.

PART 2. Monitoring of the Optical Purity for Levothyroxine Sodium in Pharmaceuticals Using Crown Ether Derived Chiral Columns

Ⅰ. Introduction

One of the two enantiomers comprising of chiral compounds mainly represents a desired physiological activation, and the other indicates other types of activation. Hence, it has become a major field of interest not only in physiological or medication research but also in pharmaceutical companies to develop convenient and reliable methods of optical separation for chiral medical supplies. L-thyroxine(L-T4), a thyroid hormone present in a living body, is used to treat functionally disordered thyroids, while D-T4 is reported to have worked as physiological activation to keep thyroid-stimulating hormones (TSH) from being released and also known to have been in use for the treatment of hyperlipidemia. Of many methods for optical separation, an analysis using the chiral stationary phase (CSP) is most widely used as it is very convenient and accurate, but so far only a few results have been reported as to studies on direct methods for optical separation of thyroxines using the chiral stationary phase (CSP).

In the first research report on direct methods for optical separation, this analysis was applied for a quantitative analysis into L-T4 sodium medical supplies by returning good results (α=1.32, Rs=2.20) in which thyroxines were optically separated in the experimental conditions where several buffer solutions were used in the chiral stationary phase (CSP) based on ovomucoid proteins. For this, a long period of analysis time of about 50 minutes were required. Positive results for optical separation of thyroxines with the use of the chiral stationary phase (CSP) in the form of crown ether were released

(α=2.08-3.11, Rs=1.00-2.60). But this study was limited to the development of an analysis, and though an analysis simultaneously into D- and L-thyroxine was developed recently, it did not work on the actual study on its applications. The use of the chiral stationary phase (CSP) induced from Quinine brought in good results (α=1.28, Rs=2.32) for optical separation from the reversed-phase aqueous solutions, and research findings with its applications to a quantitative analysis into levothyroxine sodium medicine and optical purity were once reported. Thyroxines in the form of amino acids are hormones present as L-isomer in a living body and drugs are for sale after produced in the form of L-T4 sodium.

Direct methods for optical separation using the chiral stationary phase (CSP) are very convenient and high in accuracy, which is why they are applied most comprehensively. Only one single research group issued a report, until now, on research findings of measurements of optical purity of levothyroxine sodium in the market by means of such a direct analysis into optical separation. So, to lead a production of optically-pure and quality medical supplies by identifying the quality of L-T4 available in the market, it is necessary to develop an analysis for measuring prompt and accurate optical purity of those medical supplies, followed by a subsequent monitoring process. This study is involved in newly developing and applying direct analytical methods and the pre-treatment process regarding the optical separation of thyroxine in the chiral stationary phase (CSP) taking on several useful forms of crown ether which is useful for the optical separation of amino acids, so as to measure the optical purity of levothyroxine sodium tablets for sale at home and abroad.

Ⅱ. Materials and Methods

Instruments and Reagents

A liquid chromatography test, allowing the use of HPLC composed of the following apparatus, was conducted at a room temperature. The apparatus consisting of HPLC includes a Waters model 1525 binary pump, a Rheodyne model 7125 injector with a 20 μL loop, and a dual absorbance detector (Waters 2487 detector).

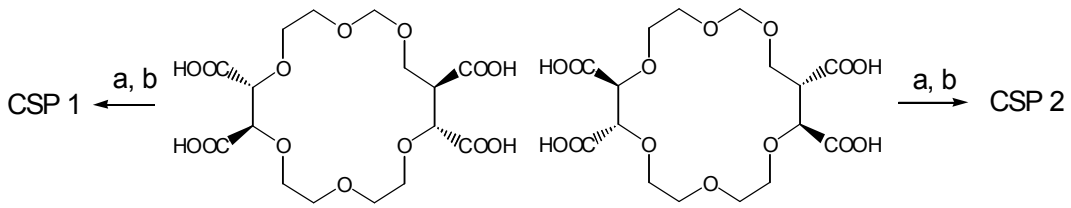

Fig. 3. Covalently-bonded CSP 1 and CSP 2 derived from (+)-and (-)-18-C-6-TA, respectively; (a) acetyl chloride (b) aminopropyl silica gel, triethylamine.

As shown in Fig. 1, the following columns were used for HPLC : CSP 1 induced from the (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid (18-C-6-TA) and the CSP 2 (250mm L x 4.6mm I.D. Daejeon, RS Technologies) from (-)-18-C-6-TA (7-9). Solvents used for HPLC was supplied by J. T Baker (Phillipsburg, NJ), D- and L-thyroxine by Fulka (Switzerland) and Acros (Belgium) respectively, and DL-thyroxine and sulfuric acids by TCI (Japan). With levothyroxine sodium as a main ingredient, tablets of many drug companies in the market of Korea, the U.S., and China were purchased. The pre-treatment process for an analysis by HPLC included breaking each of the tablets into pieces in a mortar, applying methanol containing 10mM H_2SO_4, and then doing the extraction process. A centrifuge (VS-15000, Vision

Scientific Co., Korea) was used to inject directly in HPLC the liquid obtained through separating the extracted liquid from the upper layer and then filtering it.

Ⅲ. Result and Discussions

Tests for optical separation of racemic thyroxines were performed under a few mobile phase conditions, in order to search for a suitable analysis condition for measuring optical purity of distributed levothyroxine sodium tablets from many drug companies. In the event of the optical separation which simultaneously analyzes D- and L-thyroxines in the plasma of the human body, an analysis condition where it is not overlapped with matrix impurities present in the plasma was selected and a 90% ethanol/H_2O (V/V) reversed mobile phase including 10mM H_2SO_4 was used accordingly. However, as levothyroxine sodium tablets in this study contain pigments and additives, a mobile phase - different from the one in the previous test to avoid them being overlapped with peaks - using a 100% methanol solution including 10mM H_2SO_4 was selected as the most appropriate analysis condition.

CSP1 induced from (+)-18-C-6-TA and CSP2 from (-)-18-C-6-TA were used in this study respectively for HPLC columns. L-thyroxine gets eluted first in CSP1 when separating thyroxines optically, while CSP2 has an advantage of reversing the elution order by D-thyroxine getting eluted first. Reversing the elution order enables a useful and effective analysis when working on the analysis of a tiny bit of optical purity or unwanted impurity and the peak to measure are overlapped in the tablet including matrix. For the study, all commercially available medical tablets formulated mainly with levothyroxine sodium were purchased along with ones for sale in a foreign market, all of which were used for an optimal analysis conditions to measure their optical purity. For a pre-treatment process, the solution obtained after extracting thyroxines by adding 100% methanol containing 10mM H_2SO_4 to the levothyroxine sodium tablets available on the market was injected directly in the HPLC injector for use without any derivatization process.

To ensure an effective pre-treatment process, a stability test was fulfilled on thyroxine samples in 100% methanol including 10mM H_2SO_4 of extracted solvent used for the pre-treatment process. Table 1 shows results of the measurement test for optical purity depending on a storage period, at room temperature, of D- and L-thyroxine samples melted in the extracted solvent of the above pre-treatment process. Even in case a sample is newly produced out, ensure that the optical purity of L-thyroxine (Fluka Inc.) agent on the market is 99.8%, and D-thyroxine(Acros Inc.) agent presents 94.5% of optical purity. When D- and L-thyroxine samples melted in 100% methanol including 10mM H_2SO_4 kept stored at room temperature, lacemization developed over time at a very slow pace, while lacemization was never observed though stored under refrigerated conditions at 4 ℃ for over one month. Amid the conventional studies without any specific indication as to the stability of thyroxine sample solvents, these findings are the first detailed study to prove it is not recommended to store thyroxine samples at room temperature. Compared to the research results presented previously where solvents were used for pre-treatment purposes as 2.0% (670mM) HCl/ ethanol or 10mM NaOH: methanol = 1:1 or 1N NaOH: methanol = 1:4, the pre-treatment experimental conditions in this study are far milder which is therefore regarded to be a much more effective lacemization-free pre-treatment analysis process.

Table 5. Determination of the optical purity data of D-thyroxine (Fluka reagent: left) and L-thyroxine (Acros reagent: right) dissolved in 100% methanol containing 10mM H_2SO_4 on CSP 2 as a stability test at room temperature

Storage period	D : L ratio[a]	RSD[b]	Storage period	D : L ratio[a]	RSD[b]
0 Day	94.5 : 5.5	1.5%	0 Day	0.2 : 99.8	1.3%
1 Day	94.2 : 5.8	0.8%	1 Day	0.3 : 99.7	0.5%
2 Day	94.1 : 5.9	0.5%	2 Day	0.4 : 99.6	0.6%
3 Day	94.0 : 6.0	0.6%	3 Day	0.5 : 99.4	0.6%
4 Day	93.9 : 6.1	0.2%	4 Day	0.6 : 99.4	1.0%

Mobile phase: 100% methanol containing 10mM H_2SO_4. Flow rate: 1 mL/min. Detection UV 210 nm. [a]Average value of three times determined. [b]Relative standard deviation.

Table 6. Optical purity data of commercially available levothyroxine (L-thyroxine) sodium on CSP 1 and CSP 2

Sample	CSP 1		CSP 2	
	D:L ratio[a]	RSD[b]	D:L ratio[a]	RSD[b]
A	0.2 : 99.8	1.6%	0.5 : 99.5	1.0%
B	0.3 : 99.7	1.7%	0.5 : 99.5	1.5%
C	0.2 : 99.8	1.5%	0.3 : 99.7	0.6%
D	0.2 : 99.8	2.1%	0.4 : 99.6	0.6%
E	0.3 : 99.7	1.5%	0.6 : 99.4	1.0%
F	2.2 : 97.8	5.0%	2.4 : 97.6	1.7%
G	0.2 : 99.8	1.1%	0.3 : 99.7	0.8%
H	0.5 : 99.5	0.8%	0.7 : 99.3	0.5%
I	0.2 : 99.8	0.7%	0.5 : 99.5	0.5%

Mobile phase; 100% methanol(V/V) containing 10mM H_2SO_4 Flow rate=1mL/min ; Detector 210 nm; [a]Average value of more than three times determined. [b]Relative standard deviation.

Each sample obtained following the pre-treatment process stated earlier for several levothyroxine sodium medicines for sale at home and abroad was used over three times respectively for a chromatographic analysis, through which average values came out and Table2 shows their analysis findings. All of the levothyroxine sodium on the market at home and abroad, except for one sample, generally showed over 99% of high optical purity. But, the sample F came out with a much lower level of optical purity than the others, bringing 97.8% and 97.6% to CSP1 and CSP2, respectively. In addition, at the time of measuring optical purity, the error investigated by RSD showed quite reliable 0.5% to 5.0%, as a result of measurements, where CSP2 had a higher level of reliability than CSP1. One interesting thing is that Table1 indicated the optical purity in CSP1 was higher, up about 0.1-0.3% than CSP2. In optical separation, the peak of the second-eluted enantiomer is far broader than the one of the first. In a test for measuring optical purity, the geometric reason why a tiny amount of the second-eluted enantiomer shows peak tailing occurring greater than the first-eluted enantiomer brings a small yet slightly different result in optical purity for the former and the latter. In this study, in fact, when the L-thyroxine sample aware of optical purity is optically separated, the optical purity measured in CSP1 was 99.0%, with the standard deviation of 1.4%, while in CSP2 the optical purity was 98.9%, with the standard deviation of 0.6%. This result meant that the optical purity in CSP1 was slightly higher and less precise than CSP2.

Therefore, as this experiment adopted L-T4 to analyze as a main ingredient and D-T4 is a tiny amount of ingredient, it is possible to say that the use of CSP2 provides more precise and accurate test results than the use of CSP1. Besides, as shown in this research findings, ensure that the optical purity has slightly more values in CSP1 than the results from CSP2 due to the peak tailing of the second-eluted enantiomer in the event of the optical separation with a tiny amount of the second-eluted enantiomer from CSP1. Hence,

make sure that the optical purity of analysis materials can come out higher, up about 0.1% to 0.3% when the elution order may not be reversed and only the elution order in CSP1 can bring in optical purity results.

Conclusively speaking, as presented in the outcomes taken from CSP2 in Table2, the optical purity (D-T4's enantiomer impurity : 0.3-0.7%) of levothyroxine sodium for sale at home and abroad represented high optical purity between 99.3% and 99.7%, except for the sample F, a product, demonstrated 97.6%. Of the samples with optical purity of over 99%, samples C and G had the highest optical purity of 99.7%, the sample H came out with the lowest level of optical purity of 99.3%. Measurement results for optical purity of levothyroxine sodium tablets measured in this research can be said to be satisfactory in general as compared to the result (92.7%-99.9%) reported by another research team (D-T4's enantiomer impurity : 0.1-7.3%) who applied a direct optical separation analysis. Fig.2 represents a leading chromatogram in which the identical samples to the levothyroxine sodium G are optically separated in CSP1 (D:L = 0.2:99.8) and CSP 2 (L:D = 99.7:0.3), respectively.

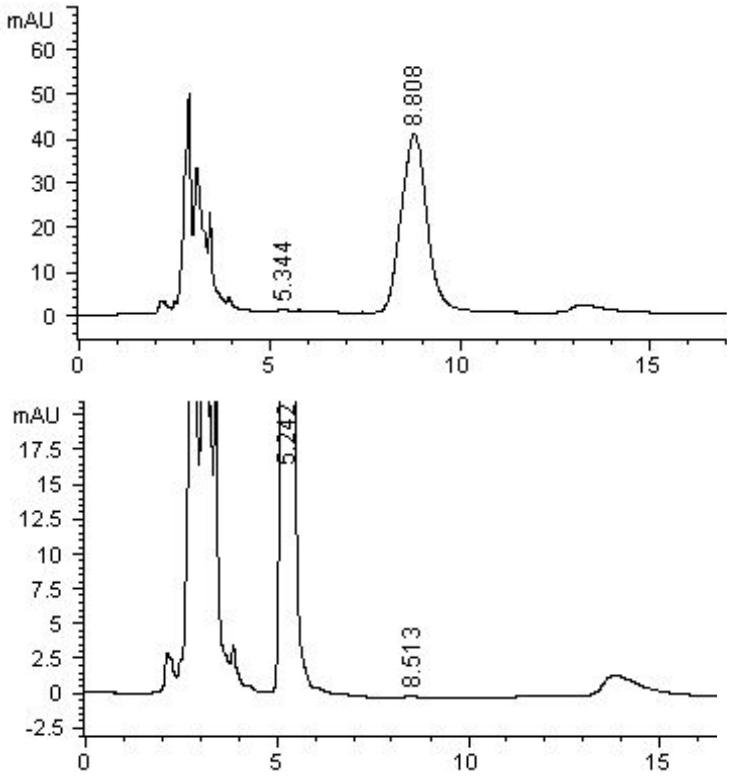

Fig. 4. Typical chromatograms of enantiomer separation of levothyroxine sodium tablet (G sample) on CSP 1 (the top) and CSP 2 (the bottom). Mobile phase: 100% methanol containing 10mM H_2SO_4. Flow rate: 1 mL/min. Detection UV 210 nm.

Ⅳ. Conclusions

The use of reverse-phase solvents as a mobile phase in the chiral stationary phase (CSP) induced from the crown ether has led to development of a new and direct optical separation analysis for measurements of optical purity of levothyroxine sodium, medical supplies of enantiomer for sale domestically and abroad. This has brought a good consequence (α=2.15, Rs=4.05). Only one single research finding has been reported until now, as to an experiment for measuring the optical purity of levothyroxine sodium available on the market, through methods for direct optical separation, by means of the chiral stationary phase (CSP). This study used a newly developed pre-treatment process and HPLC analysis conditions to measure the optical purity of medical tablets, commercially available at present and formulated primarily with levothyroxine sodium. The experiment for optical separation in measurement of optical purity of levothyroxine sodium was able to identify that all showed high optical purity of over 99% except for one product. It is expected that a new convenient and easy optical separation analysis will be very helpful for identifying qualities of levothyroxine sodium products available at home and abroad, developing chiral medicines and managing relevant products.

Ⅴ. References

1. Francotte, E. and W. Lindner, (Ed.) (2006) *Chirality in Drug Research*, Wiely-VCH, Weinheim.
2. Bantle, J., J. Oppenheimer, H. Schwartz, D. Hunninghake, J. Probstfield and R. Hanson (1981), TSH response to TRH in euthyroid, hypercholesterolemic patients treated with graded doses of dextrothyroxine, *Metabolism*, **30**, 63-66.
3. Abou-Basha, L. I. and H. Y. Aboul-Enein (1995), Enantiomeric separation and optical purity determination of thyroxine enantiomers in bulk and pharmaceutical formulations, *Pharm. Acta Helv.* **70**, 237-240.
4. Gika, H., M. Lämmerhofer, I. Papadoyannis and W. Lindner (2004), Direct separation and quantitative analysis of thyroxine and triiodothyronine enantiomers in pharmaceuticals by high-performance liquid chromatography, *J. Chromatogr.*, **800**, 193-201.
5. Aboul-Enein, H. Y., I. Ali, M. H. Hyun, Y. J. Cho and J. S. Jin (2002), Effect of acidity on the enantiomeric resolution of thyroxine and tocainide by HPLC on a (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid column, *J. Biochem. Bioph. Methods.* **54**, 407-413.
6. Jin, J. Y., C.-S. Baek, and W. Lee (2007), Development of a validated HPLC method for the simultaneous determination of D- and L-thyroxine in human plasma, *Bull. Kor. Chem.* Soc. **28**, 1070-1072.
7. Hyun, M.H., J. S. Jin and W. Lee (1998), Liquid chromatographic resolution of racemic amino acids and their derivatives on a new chiral stationary phase based on crown ether, *J. Chromatogr. A*, **822**, 155-161.
8. Lee, W., J. Y. Jin and C.-S. Baek (2005), Comparison of

enantiomer separation on two chiral stationary phases derived from (+)-18-crown-6-2,3,11,12-tetracarboxylic acid of the same chiral selector. *Microchemical Journal*, **80**, 213-217.

9. Jin, J. Y., W. Lee and M. H. Hyun (2006), Development of the antipode of the covalently-bonded crown ether type chiral stationary phase for the advantage of the reversal of elution order, *J. Liq. Chrom. & Rel. Tech.* **29**, 841-848.
10. Jin, J. Y. and W. Lee (2007), Liquid chromatographic enantiomer resolution of *N*-hydrazide derivatives of 2-aryloxypropionic acids on a crown ether derived chiral stationary phase, *Chirality*, **19**, 120-123.
11. Lee, T., W. Lee, M. H. Hyun and J. H. Park (2010), Enantioseparation of native α-amino acids on an 18-crown-6-tetracarboxylic acid-bonded silica by capillary electrochromatography, *J. Chromatogr. A*, **1217**, 1425-1428.
12. Perry, J. A., J. D. Rateike and T. J. Szczerba (1987), Eluting trace components before major constituents : I. Sensitivity enhancement in analytical determinations of optical purity, *J. Chromatogr.,* **389**, 57-64.
13. Jin, D., M. Zhang, S. Jin, M. Lee, G. Song, G. Back and Y. Lee (2007), Enantioselective resolution of thyroxine hormone by high-performance liquid chromatography utilizing a highly fluorescent chiral tagging reagent, *Chirality*, **19**, 625-631.