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Direct separation and quantitative analysis of thyroxine and triiodothyronine enantiomers in pharmaceuticals by high-performance liquid chromatography

Helen Gika[a,1], Michael Lämmerhofer[a], Ioannis Papadoyannis[b], Wolfgang Lindner[a,*]

[a] *Institute of Analytical Chemistry, University of Vienna, Währinger Strasse 38, A-1090 Vienna, Austria*
[b] *Laboratory of Analytical Chemistry, Department of Chemistry, Aristotle University of Thessaloniki, Thessaloniki, Greece*

Abstract

A rapid reversed-phase type HPLC method for the simultaneous separation and analysis of D- and L-thyroxine (D- and L-T_4) and triiodothyronine (T_3) was developed using a quinine-derived chiral stationary phase and applied for a quantitative assay of the enantiomeric impurity of the drugs in pharmaceutical formulations of levothyroxine. The influence of operating parameters has been studied for the optimization of the separation and also in order to gain an insight into the retention mechanism. Validation of the method included linearity, precision and accuracy which revealed R.S.D. values of <3.3% for intra-assay precision and percent error ranging from −6 to +2.1% for various defined validation samples, proving satisfactory accuracy. Quantitation was performed over the range of 0.5–500 μg ml^{-1} with limits of detection and quantitation lower than 0.1 and 0.5 μg ml^{-1}, respectively, for both analytes. Further, the determination of 0.1% impurity, of D-T_4 as well as L- and D-T_3 in levothyroxine sodium tablets proved to be feasible.

Keywords: Enantiomer separation; Chiral stationary phases; LC; Thyroxine; Triiodothyronine

1. Introduction

The amino acid type hormones thyroxine (T_4) and triiodothyronine (T_3) (Fig. 1) secreted by the pituitary gland are compounds of major biological role as they are critically important for the normal development of the central nervous system (CNS) in infants, the skeletal growth and the maturation in children as well as for the normal function of multiple organ systems in adults.

The enantiomers of thyroid hormones are known to have quite different pharmacological effects. In contrast to L-thyroxine (L-T_4), D-thyroxine (D-T_4) shows no basic metabolic rate enhancement but was shown to alter levels of lipogenic enzymes in the liver and to reduce serum cholesterol significantly, acting by increasing the rate of degradation and oxidation of cholesterol [1–4]. In addition, a suppression of thyroid stimulating hormone (TSH) excretion caused by D-T_4 has been observed [5]. Besides, studies have shown that the biological effectiveness of D-T_3 is markedly less than that of L-T_3, corresponding to 5–30% of the activity of L-T_3 [6,7].

For these reasons, the sensitive analysis and quantitative determination of the individual enantiomers of thyroid hormones is essential for biological researches and pharmacokinetic studies, since the question of the in vivo conversion of D-T_4 to L-T_4 and the true biological activity of D-T_4 and its metabolites in humans is still not totally elucidated.

The enantiomeric purity of drug compounds in pharmaceutical formulations is consequently a critical issue. Some years ago increased numbers of sudden deaths occurred in hyperlipidemic patients treated with D-T_4 and led to a decrease of its use especially in the United States. It is not established with certainty if the observed side effects after therapeutic D-T_4 administration are a consequence of L-T_4 contaminations, but in any case it raises the need for enantiomerically highly pure D-T_4 if administered for hyperlipidemia treatment. Besides, L-T_4 is the most commonly prescribed form of hormone replacement therapy for

* Corresponding author. Tel.: +43-1-4277-52300; fax: +43-1-3151826.
E-mail address: wolfgang.lindner@univie.ac.at (W. Lindner).
[1] On temporary leave from the Laboratory of Analytical Chemistry, Department of Chemistry, Aristotle University of Thessaloniki, Greece.

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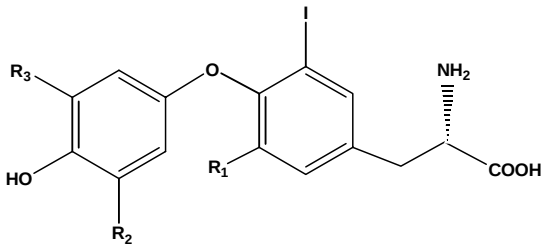

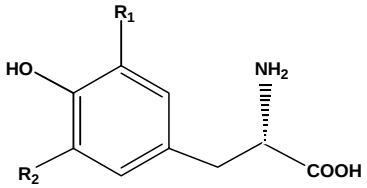

Thyroxine (T_4): R_1=I, R_2=I, R_3=I
Triiodothyronine (T_3): R_1=I, R_2=H, R_3=I
r-Triiodothyronine (rT_3): R_1=H, R_2=I, R_3=I
Diiodothyronine (T_2): R_1=I, R_2=H, R_3=H

Diiodotyrosine (DIT): R_1=I, R_2=I
Monoiodotyrosine (MIT): R_1=I, R_2=H
Tyrosine (Tyr): R_1=H, R_2=H

Fig. 1. Structures of investigated iodo-thyronines and -tyrosines.

hypothyroidism and in this case it seems similarly important to determine the content of D-T_4 in the commercially available drugs and in respective formulations.

In this context, the question of dosing is important as D-T_4 is administered in a dose about 40 times higher (0.05–0.1 mg kg^{-1} of body weight per day) than L-T_4, so the equivalence of 1% L-T_4 in D-T_4 is already of therapeutic significance, whereas in the reversed case the enantiomeric impurity of D-T_4 in L-T_4 seems of significance concerning side effects if it is present in a level of over 5%. With other words and for drug safety reasons the demand for enantiomerically highly enriched drug substances should be mandatory following recommendations for 99.9% enantiomeric purity, if meaningful.

Several chromatographic enantiomer separation methods of thyroid hormones have been reported in the past [5,8–16]. Among them the use of copper(II)acetate and L-proline as chiral mobile phase additive adapting a chiral ligand exchange chromatography (CLEC) principle [10,11] and the employment of a covalently bound L-proline copper(II) chelate type chiral stationary phase (CSP) have become popular [12]. As alternative to the direct enantiomer separation by CLEC also an indirect approach by derivatising the iodinated thyronines with L-leucine as chiral reagent resulting in diastereomeric dipeptides separable on a reversed-phase column has proven useful [13,14]. Enantioselective analysis of D- and L-T_4 in pharmaceutical formulations was achieved also on an ovomucoid containing protein type chiral stationary phase [15], whereas in another attempt D- and L-thyroxine were determined in pharmaceuticals quasi indirectly after de-iodination by catalytic hydrogenation followed by the separation of the resulting thyronines on a CSP with serum albumin covalently bound to silica or aminopropyl silica [16]. Recently, an amperometric biosensor based on an L-amino acid oxidase [17] and a chemiluminescence flow-through immunosensor based on a competitive assay using enantioselective antibodies [18] were proposed for the enantioselective assay of L-T_3 and -T_4.

The aim of the present study was to develop a novel, robust, rapid and reliable approach for the simultaneous separation and the quantitative analysis of thyroxine and triiodothyronine enantiomers in drug substances and for-

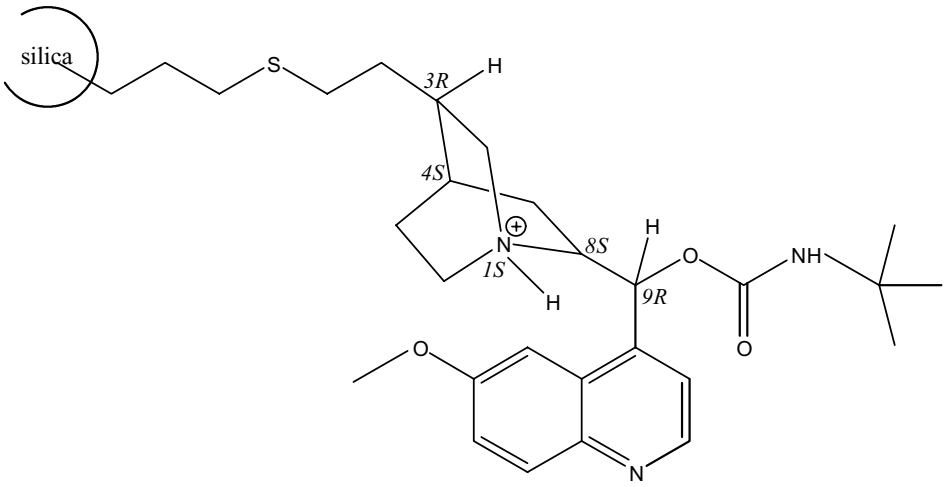

Fig. 2. Structure of chiral stationary phase with *tert*-butyl carbamoylated quinine as selector.

mulations. The method involves direct separation of the enantiomers of T_4 and T_3 on a chiral anion exchange-type stationary phase with *tert*-butyl carbamoylated quinine (*t*-BuCQN) as chiral selector. Due to the chromophoric substituents of the compounds, the HPLC method coupled to UV detection should be sensitive enough. Moreover, no further derivatization of the amino function should be necessary to support the overall molecular recognition mechanism leading to enantiomer separation.

Chiral stationary phases based on carbamoylated cinchonan derivatives (Fig. 2) as so-called chiral selectors represent a category exhibiting high stereoselectivity in the reversed-phase or polar organic mode using buffered or organic acid/base containing mobile phases. This has been successfully demonstrated for the separation of N-protected amino acids, peptides and diverse other types of chiral acidic analytes [19–23].

For these anion-exchange type CSPs and chiral selectors (SOs), ionic interactions with complementary charged chiral analytes, hereafter called selectands (SAs), are a major driving force for intermolecular SO–SA interactions together with several other interactions such as hydrogen bonding, π–π and van der Waals interactions becoming active in the binding regions of the chiral selector.

This study deals with the optimization and validation of the HPLC method utilizing a cinchonan based CSP for the enantiomer separation of the thyroid hormones T_4 and T_3 as well as related compounds (Fig. 1). In addition the application of the validated stereoselective HPLC method for the quantitative analysis of levothyroxine and liothyronine (L-T_3) sodium containing pharmaceuticals will be given.

2. Experimental

2.1. Chemicals

L-Thyroxine (L-T_4), D-thyroxine (D-T_4), 3,3′,5-triiodo-L-thyronine (L-T_3), 3,5-diiodo-L-thyronine (L-T_2), 3,5-diiodo-L-tyrosine (L-DIT) and 3-iodo-L-tyrosine (L-MIT) were obtained from Fluka (Sigma–Aldrich, Vienna, Austria) and 3,3′,5′-triiodo-L-thyronine (L-rT_3) was from Calbiochem (Darmstadt, Germany). Solvents for chromatography were of HPLC grade and from Merck (Darmstadt, Germany). All other reagents used were of analytical grade. Racemic mixtures of 3,3′,5-triiodo-thyronine, 3,3′,5′-triiodo-thyronine, 3,5-diiodo-thyronine, 3,5-diiodo-tyrosine and 3-iodo-tyrosine were prepared from the enantiomerically enriched parent compounds by racemization according to a procedure described earlier in the literature [24,25].

2.2. Pharmaceuticals

Thyro-4 tablets (Faran, Greece), T4-200 tablets (Uni-Pharma, Greece), Thyrex tablets (Biochemie, Austria), labeled to contain 0.1, 0.2 and 0.1 mg of levothyroxine sodium salt (L-T_4 sodium), respectively, per tablet and Dithyron tablets (Uni-Pharma, Greece) labeled to contain 0.05 mg of L-T_4 sodium salt and 0.125 mg of liothyronine sodium salt (L-T_3 sodium) per tablet were analyzed. Seven tablets of each drug formulation were pulverized and an aliquot amount of powder corresponding to 0.1 and 0.2 mg of levothyroxine sodium was weighed. The powder was suspended in MeOH–NaOH 10 mM (1:1 (v/v)), ultrasonicated at room temperature for 5 min and finally undissolved particles were removed by filtration. The filtrate that contained L-T_4 at a nominal concentration of 194.5 or 97.2 μg ml^{-1} L-T_4 was directly used for injection.

2.3. Instrumentation and chromatography

Chromatography was performed in an isocratic elution mode employing a Hewlett-Packard 1050 HPLC system (Agilent Technologies, Waldbronn, Germany) equipped with a UV detector. Operation, data acquisition and data analysis were carried out by the HP ChemStation software. The detection wavelength used was 240 nm, while temperature was maintained at 25 °C with the aid of a column thermostat.

Separation was obtained on a Prontosil Chiral AX QN-1, 150 mm × 4 mm column containing *tert*-butyl carbamoylated quinine (*t*-BuCQN) modified silica (Prontosil 120–5 μm) as chiral stationary phase that is now commercially available from Bischoff Chromatography (Leonberg, Germany) or as Chiris^{TM}-QN Chiral AX from IRIS Technologies (Lawrence, KS, USA).

A 60:40 (v/v) mixture of acetonitrile and 0.05 M ammonium acetate was used as standard mobile phase with a flow-rate of 0.7 ml min^{-1}. The apparent pH (pH_a) of the mixture was adjusted to 4.5 by addition of glacial acetic acid.

2.4. Standard solutions/procedure

Stock solutions of L-T_4, D-T_4 and L-T_3 were prepared at a concentration of 1 mg ml^{-1} in a mixture of MeOH and 10 mM NaOH (1:1 (v/v)). Working standard solutions of the individual enantiomers or their mixtures were prepared by dilution of the stock solutions with the same solvent to the concentration range between 0.5 and 500 μg ml^{-1}. All solutions were protected from light and stored at 4 °C. At these basic storage conditions no racemization could be detected within a period of 8 weeks.

Racemic mixtures of T_4, T_3, rT_3, T_2, DIT and MIT were obtained according to the following procedure [24,25]: racemization of L-iodo amino acids was carried out by heating them at 50 °C for 1 h in glacial acetic acid in the presence of 0.2 mol equivalent of salicylaldehyde. The reaction mixture was then evaporated to dryness and the residue redissolved in MeOH–10 mM NaOH (1:1 (v/v)). Under these conditions a complete racemization occurred and only a minor and negligible chemical degradation could be monitored.

3. Results and discussion

3.1. Chiral recognition mechanism: influence of mobile phase composition on chemo- and enantio-selectivity

Several studies have shown that the retention mechanisms for selectands with acidic functional groups on cinchona alkaloid type CSPs is based on electrostatic interactions, hydrogen bonding, π–π and hydrophobic interactions between the enantiomers of the analytes and the chiral selector [26–28]. For many separations, variations in mobile phase pH or organic modifier concentration could affect analyte retention and resolution. For this reason, all the parameters that might have a significant influence on the effectiveness of the chromatographic separation on these CSPs including pH, ionic strength and organic modifier content were studied in order to evaluate the underlying chiral recognition mechanism in the case of the iodo-thyronines and -tyrosines separations.

3.1.1. CSP selection

Out of the portfolio of diverse cinchonan-type anion exchanger stationary phases developed in our laboratory and tested for the separation of the L- and D-enantiomers of T_4, T_3, rT_3, T_2, MIT and DIT, the *t*-BuCQN derived CSP exhibited the best results in a pre-screening with regards to complete enantio-discrimination. In this context it must be noticed that the so-called pseudoenantiomeric behavior of the corresponding quinidine-derived chiral selector and CSP surprisingly failed. These data are not shown but pinpoint once more the stringent and exquisite stereochemical requirements of a chiral selector to actually generate enantioselectivity.

Besides enantioselectivity also sufficient chemoselectivity was required to separate the pairs of D,L-T_4 and -T_3 and possibly also of D,L-rT_3 and -T_2 simultaneously. The latter analytes, however, will only be of relevance for biological samples, in which these solutes are present, as they are formed by metabolic de-iodination of T_4 and T_3, but not for pharmaceutical formulations.

3.1.2. Effect of mobile phase pH

As previously examined for N-protected amino acids, it was observed that retention and selectivity were directly depending on the pH of the mobile phase. Fig. 3(a) depicts the effect of mobile phase pH on retention, enantioselectivity and resolution for T_4 serving as model compound for the whole series of thyronine derivatives.

Retention was found to increase as the pH was raised from 4 to 7 while above pH 7 the retention started to decrease and the peaks became broad and asymmetrical. In addition, selectivity and resolution deteriorated above pH 5 (Fig. 3(a)).

This behavior can readily be explained by the degree of ionization of analyte and selector and an ion-exchange mechanism.

The pK_a values of T_4 are reported to be 2.1 for the carboxylic function, 6.7 for the phenolic hydroxyl function and 8.9 for the amino function. Accordingly, the ionization status of the T_4 analyte will vary with pH, possessing a negatively charged carboxylic group capable for ionic interactions at pH >4. Similarly, above pH 7 the tertiary nitrogen of the quinuclidine ring of the chiral selector starts to deprotonate, so that the ionic interaction between the selector and selectand will be reduced. At the optimal working pH between 4 and 6 the phenolic group of T_4 is largely non-dissociated, so that it does not competitively disturb the preferred binding mechanism with ionic interaction of the carboxylic group.

At lower pH (pH $<$ 4) the carboxylic group will become increasingly protonated reducing again the primary Coulomb interaction with the protonated and charged selector moiety (quinuclidine residue) resulting in a decrease of retention time and in sharper peaks. As optimum operating conditions a mobile phase of pH 4.5 was selected due to the higher resolution.

3.1.3. Effect of buffer concentration

In accordance with the anion-exchange mechanism, retention was found to decrease with an increase of ammonium acetate buffer concentration as expected, while selectivity remained unaffected over a range of 25–100 mM (Fig. 3(b)). This strongly suggests that there are long-range electrostatic interactions in force that are balanced by high counter-ion (acetate) concentrations. Apparently, the other stereoselective binding increments (hydrogen bonds and π–π interactions) do not get strongly affected as materialized in a constant enantioselectivity within the studied ionic strength range. A minor effect on resolution is revealed as depicted in Fig. 3(b).

3.1.4. Effect of organic modifier

Methanol and acetonitrile were used as organic modifiers representing polar protic and aprotic solvents. Methanol provided results similar to acetonitrile, but the peak shapes became significantly broader. For this reason, acetonitrile was used for further studies. The concentration of organic modifier of the hydro-organic mobile phase was found to influence enantiomer retention and resolution significantly. As the concentration of acetonitrile in the mobile phase was increased a corresponding decrease in retention was observed, accompanied also by a significant loss of enantioselectivity. This leads us to the assumption that besides the primary ion-exchange retention mechanism also a hydrophobic interaction contribution is superimposed, typical for a reversed phase system. As can be extracted from Fig. 3(c), α is negatively affected by a weakening of these hydrophobic interaction contributions, which indicates that this interaction occurs stereoselectively. Most likely π–π interactions of one of the aromatic groups of the analyte and the quinoline ring of the quinine selector and purely hydrophobic interactions between the bulky carbamate residue and the amino acid side chain are to be made responsible for such a behavior.

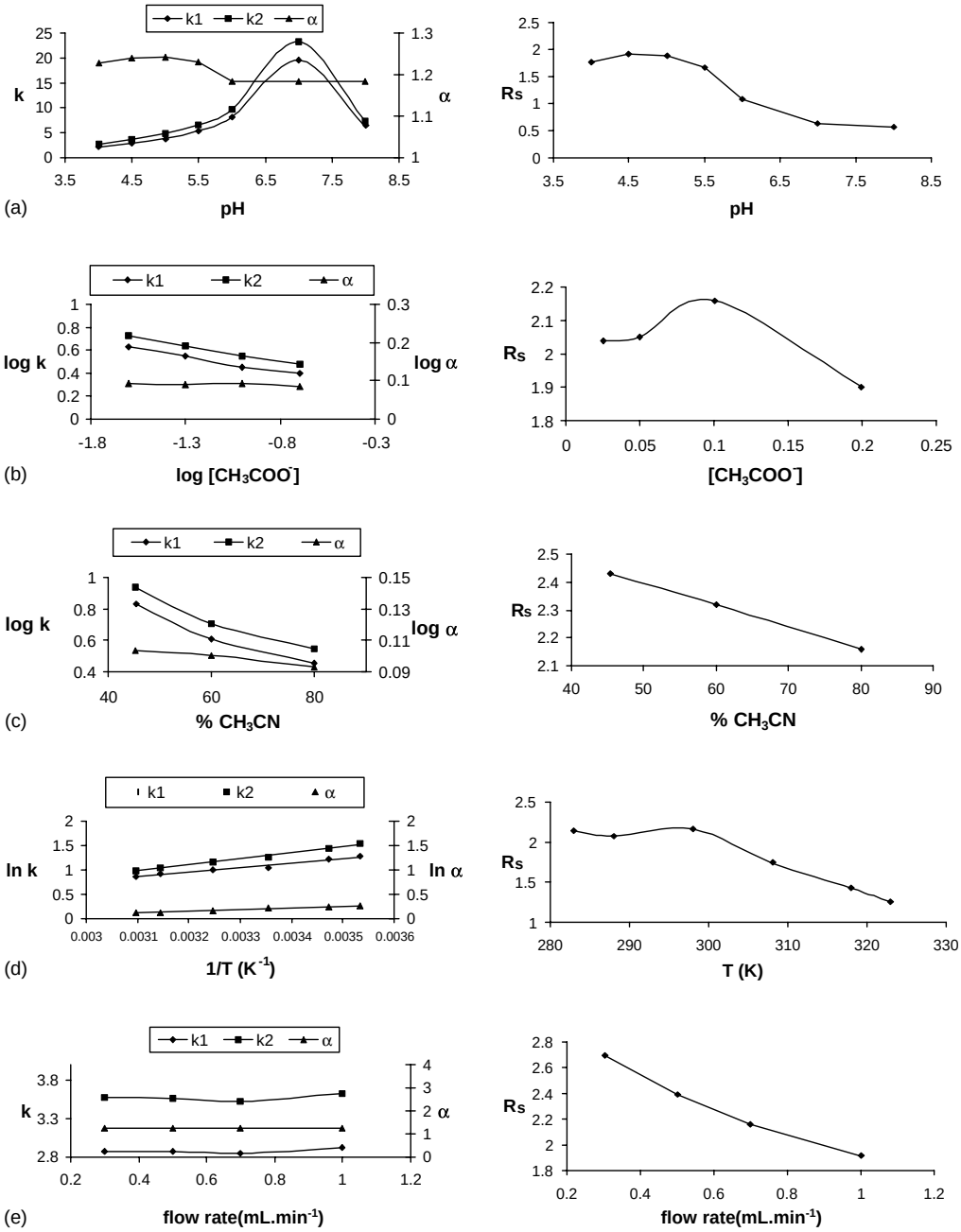

Fig. 3. Influence of liquid phase variables on retention, selectivity and resolution of thyroxine (T_4) enantiomers on quinine-derived chiral stationary phase. Chromatographic conditions: (a) mobile phase, CH_3CN/0.05 M CH_3COONH_4 80:20 (v/v); pH adjusted to the respective pH value by addition of glacial acetic acid or ammonia; flow rate, 1 ml min^{-1}; temperature, 25 °C; (b) mobile phase, CH_3CN/0.025–0.2 M CH_3COONH_4 80:20 (v/v); pH 4.5; flow rate, 0.7 ml min^{-1}; temperature, 25 °C; (c) mobile phase, CH_3CN/0.1–0.04 M CH_3COONH_4 80:20–50:50 (v/v); pH 4.5; flow rate, 0.7 ml min^{-1}; temperature, 25 °C; (d) mobile phase, CH_3CN/0.05 M CH_3COONH_4 60:40 (v/v); pH 4.5; flow rate, 0.7 ml min^{-1}; temperature, 10–50 °C; (e) mobile phase, CH_3CN/0.05 M CH_3COONH_4 60:40 (v/v); pH 4.5; flow rate, 0.3–1 ml min^{-1}; temperature, 25 °C. Detection was performed at 240 nm.

3.1.5. Effect of column temperature and flow rate of the mobile phase

The effect of column temperature on retention and resolution was studied over the range of 10–50 °C. The results as shown in Fig. 3(d) prove a common behavior with a decrease of enantioselectivity as temperature increases, which indicates an enthalpic control of the separation. A working temperature of 25 °C was selected where the system gave sufficient resolution values.

The improvement of the separation efficiency with reduced flow rate on this type of CSP has been studied previously [29]. As expected, the flow rate did not alter the overall enantio-separation characteristics (retention factors and enantioselectivity), but the resolution was significantly

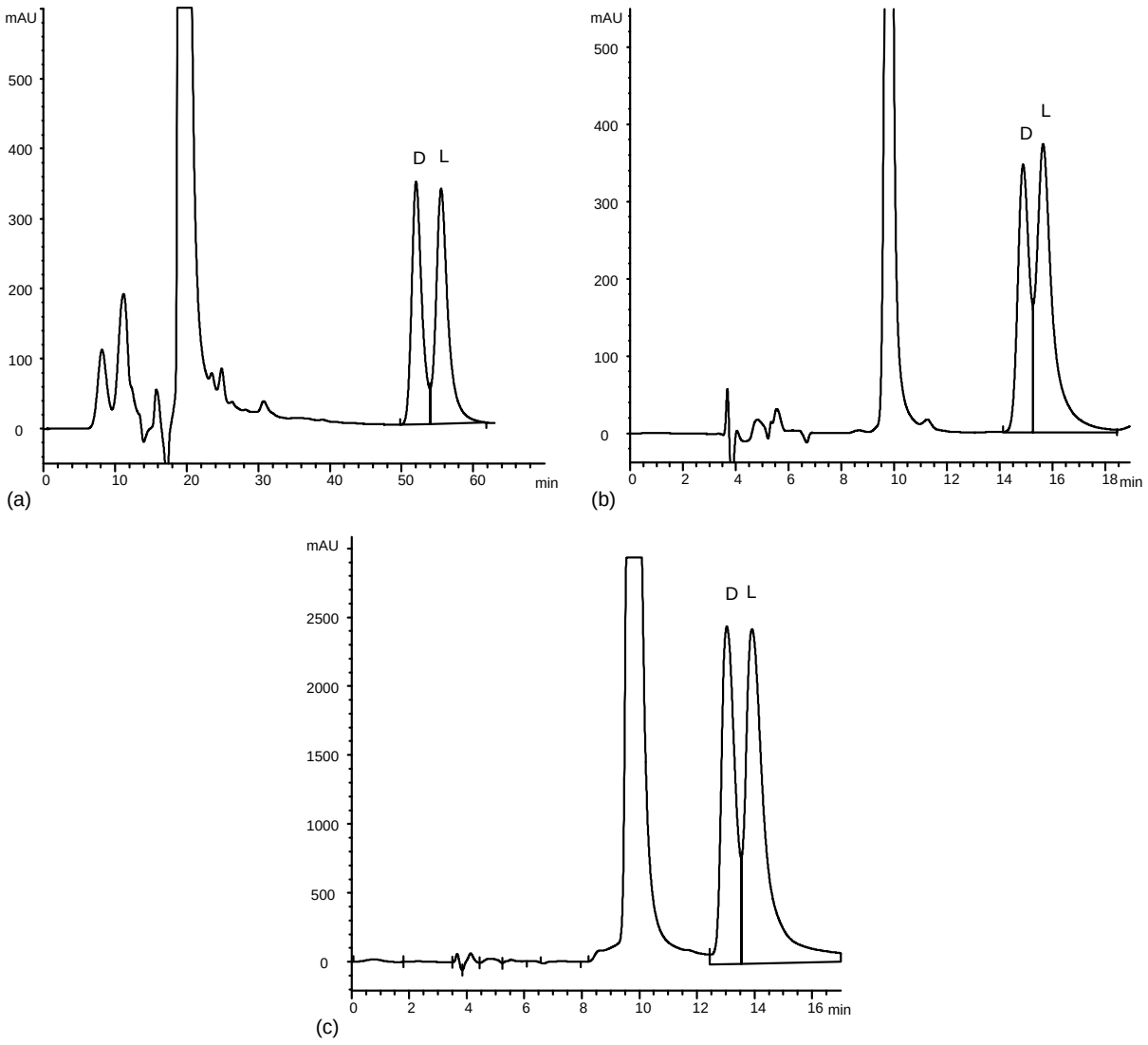

Fig. 4. Chromatographic separation of the pair of the enantiomers of: (a) rT_3 with mobile phase, CH_3CN/0.05 M CH_3COONH_4 60:40 (v/v); pH 4.5; flow rate, 0.1 ml min^{-1} ($\alpha = 1.14$, $R_s = 1.32$); (b) T_2 with mobile phase, CH_3CN/0.03 M CH_3COONH_4 40:60 (v/v); pH 4.5; flow rate 0.3 ml min^{-1} ($\alpha = 1.07$, $R_s = 0.69$); (c) DIT at the same conditions as for Fig. 4(b) ($\alpha = 1.10$, $R_s = 0.92$); $T = 25\,^{\circ}C$, detection at 240 nm.

better at lower flow rates as shown in Fig. 3(e) due to the smaller plate heights.

3.1.6. Structure of the analyte

In Fig. 1, the structure variations are visualized. From the chromatographic data at optimized conditions it becomes evident that the so-called chemical selectivity of the CSP for separating the pairs of enantiomers of T_4, T_3, rT_3, T_2 and DIT simultaneously is not sufficient. However, it seems that the numbers of iodine atoms as well as their positions on the aromatic rings of the analyte molecule are playing a decisive role in the overall stereoselectivity of the system. The number of iodines of the analyte, the size and the π acidity of the aromatic moiety obviously affect the π–π interaction and thus the chiral recognition substantially. The enantioselectivity for T_3 is approximately the same as for T_4, while it starts to decrease for T_2. Moreover, the enantiomers of reversed T_3 (rT_3) are not baseline resolved in contrast to T_3 enantiomers, which supports the importance of the ally-controlled steric interactions being somewhat close to the actual multiple binding sites.

MIT and Tyr enantiomers could not be resolved apparently due to the relatively low π acidity, whereas DIT and T_2 were found to be separated to the same degree which reveals that the “inner” aromatic ring is most probably more important to explain the separation.

In Fig. 4 representative enantio-separations for the pairs of rT_3, T_2 and DIT are illustrated. The D-enantiomers are always eluted first.

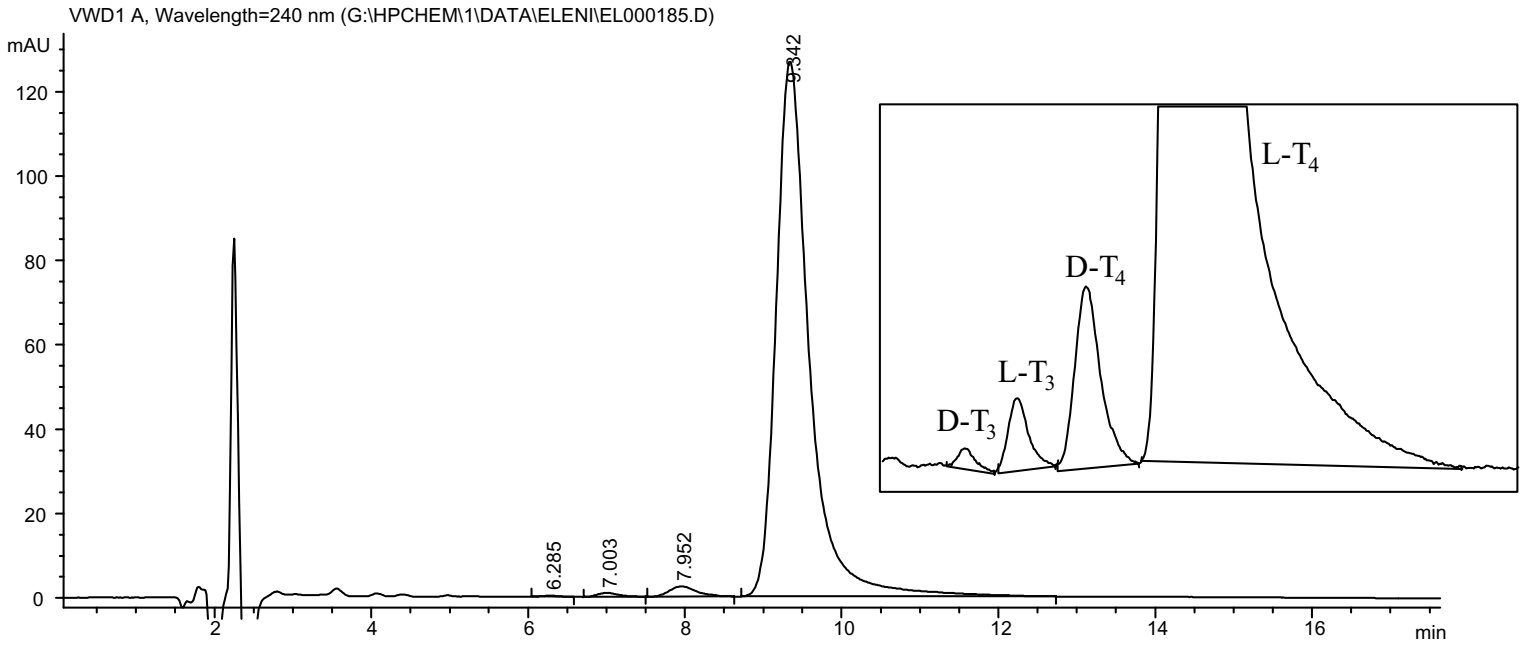

Fig. 5. Identification of impurities in pharmaceutical formulation of levothyroxine sodium. Quantitative data can be taken from Table 2.

3.2. Quantitative analysis of T_4-formulations and determination of enantiomeric impurities

With the optimized chromatographic system the α-values of the pairs of T_4 and T_3 enantiomers reached 1.28 and 1.16, respectively, with peak resolution values of 2.32 and 1.28, revealing that the system is suitable for the determination of trace enantiomeric impurities where reasonable resolution values are essential. In addition, the chemoselectivity was sufficient for the simultaneous determination of T_3 and T_4 in drug substances and formulations thereof. The separation of T_4 and T_3 enantiomers in a pharmaceutical formulation of L-T_4 is shown in Fig. 5 corroborating that significant chemical and enantiomeric impurities are present.

3.2.1. Linearity, limit of detection and limit of quantitation

Quantifications of the minor peaks and the main peak were performed by applying two different calibration methods, based on peak areas or peak heights of the analytes and the so-called self-internal standard method [30] and the resulting data were thus compared. Calibration curves were first established for L-T_4 and D-T_4 based on the peak areas, peak heights and on the peak area ratios using thus the one enantiomer as internal standard for the other one. For L-T_3, the calibration curves were also based on the peak areas, the peak heights and the peak area ratios but using L-T_4 as internal standard. Linearity was evaluated using eight standard solutions over a concentration range of 0.5–500 $\mu g\,ml^{-1}$. Injection volume was always 10 μl and each calibration sample was injected in triplicate. All calibration curves were corrected in the cases where the enantiomeric impurity of the calibration standard was significant (>0.1%).

It was found that the data from the different calibration methods were in good agreement (Table 1). In all cases, over the concentration range of 0.5–500 $\mu g\,ml^{-1}$ of L,D-T_4 or L-T_3, correlation coefficients estimated by linear regression analysis ranged from 0.9999 to 0.9936. However, better linearity was noticed when data were obtained from peak area based calibration (R^2 is 0.9994 for L-T_4, 0.9998 for D-T_4 and 0.9999 for L-T_3). The linearity of the method spans a range of three orders of magnitude, thus making the quantification of 0.1% enantiomeric impurity easily possible.

In the case where L-T_4 is used as internal standard for the D-T_4 calibration curve, linearity was found to deviate in higher concentrations and the determination seems less sensitive as a result of an inaccurate integration of peak areas, since the second peak is eluted on the tailing edge of the first main peak.

Limits of detection (LOD) and quantitation (LOQ) were determined as signal-to-noise ratios equal to 3 and 10, respectively. LOQs lower than 0.5 $\mu g\,ml^{-1}$ and LODs around 0.1 $\mu g\,ml^{-1}$ were achieved.

3.2.2. Repeatability and accuracy

Method validation regarding intra-assay precision was achieved by replicate injections of standard solutions of the analytes at three concentration levels (0.5, 100 and 500 $\mu g\,ml^{-1}$) within the calibration curve range. R.S.D. was calculated for peak areas, peak heights and retention times and found to be in all cases <1.5%.

Repeatability and intra-assay precision was also evaluated in a pharmaceutical preparation sample (0.2 mg sodium

Table 1
Precision and accuracy data of the method obtained by intra-day repeatability assay ($n = 6$) and estimated with two calibration methods

Analyte	Injected ($\mu g\,ml^{-1}$)	Calibration based on peak areas			Calibration based on peak heights			Calibration based on self internal standard method		
		Measured ± S.D. ($\mu g\,ml^{-1}$)[a]	R.S.D. (%)	Error (%)	Measured ± S.D. ($\mu g\,ml^{-1}$)[a]	R.S.D. (%)	Error (%)	Measured ± S.D. ($\mu g\,ml^{-1}$)[a]	R.S.D. (%)	Error (%)
L-T_4	0.5	0.47 ± 0.02	4.2	−6	0.52 ± 0.01	1.9	4	0.47 ± 0.03	6.4	−6
D-T_4	100	101.6 ± 0.24	0.3	1.6	98.8 ± 0.08	0.1	−1.2	102.1 ± 1.8	1.8	2.1
L-T_3	500	497.0 ± 4.5	0.9	−0.6	501.0 ± 2.5	0.5	0.2	503.4 ± 4.6	0.9	0.7

[a] Mean value of six measurements.

Table 2
Results of L-T_4 and -T_3 analysis and enantiomeric impurity determination in pharmaceutical formulations of levothyroxine sodium (sodium salt of L-T_4)

Commercial sample of L-T_4	Nominal concentration of L-T_4 ($\mu g\,ml^{-1}$)	Measured concentration of L-T_4 ± S.D. ($\mu g\,ml^{-1}$)[a]	R.S.D. (%)	Error (%)	Enantiomeric impurity of D-T_4 (%)	Nominal concentration of L-T_3 ($\mu g\,ml^{-1}$)	Measured concentration of L-T_3 ± S.D. ($\mu g\,ml^{-1}$)[a]	R.S.D. (%)	Error (%)
1	194.5	193.5 ± 1.4	0.7	0.5	1.6	–	1.6 ± 0.1	6.2	–
2	194.5	197.6 ± 1.7	0.7	−1.6	0.1	–	2.9 ± 0.1	3.4	–
3	97.2	98.7 ± 0.6	0.6	−1.5	0.4	–	0.9 ± 0.1	11.1	–
4	97.2	95.8 ± 0.8	0.8	1.4	7.3	24.2	22.8 ± 1.9	0.9	–5.7

Results obtained with calibration method based on peak areas.
[a] Mean value of three measurements.

levothyroxine per tablet) with replicate injections ($n = 8$) giving R.S.D. of 2.1% and standard error of only ±0.012 indicating satisfactory accuracy. Table 1 summarizes the results of the method validation regarding accuracy (percent error) and intra-day precision assays.

3.2.3. Application to pharmaceutical formulations

The method was applied for the determination of the enantiomeric impurities in commercial preparations of L-T_4 that are used as replacement therapy in hypothyroidism. In all cases, 0.1% enantiomeric impurity or even more was found together with impurities referred to as D- and L-T_3. Respective results are summarized in Table 2.

4. Conclusions

The reported chromatographic method, comprising the principles of enantioselective cinchona-type anion exchanger CSPs, enables a simultaneous baseline separation of the enantiomers of thyroid hormones T_4 and T_3. In this chromatographic system, the ionic as well as the π–π interactions play a key role in the chiral recognition mechanism. The method has been validated and shows good performance with respect to linearity, repeatability and accuracy. It is a method suitable for the quality control of the enantiomeric purity of thyroxine containing formulations but also particularly promising for stereoselective pharmacokinetic studies.

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References

[1] J. Rabinowitz, T. Rodman, R. Myerson, J. Am. Med. Assoc. 183 (1963) 758.
[2] T. Koschinsky, K. Vogelberg, F. Gries, Deut. Med. Wochenschr. 99 (1974) 494.
[3] C. Gorman, N. Jiang, R. Ellefson, L. Elveback, J. Clin. Endocrinol. Metab. 49 (1979) 1.
[4] J. Bantle, J. Oppenheimer, H. Schwartz, D. Hunninghake, J. Probstfield, R. Hanson, Metabolism 30 (1981) 63.
[5] G. Leb, E. Lankmayr, R. Goebel, H. Pristautz, F. Nachtmann, G. Knapp, Klin. Wochenschr. 59 (1981) 861.
[6] G. Boyd, M. Oliver, J. Endocrinol. 21 (1960) 25.
[7] C. Greenberg, C. Bocker, J. Kerwin, S. Greenberg, T. Lin, Am. J. Physiol. 201 (1961) 732.
[8] E. Oelrich, H. Preusch, E. Wilhelm, J. High Resolut. Chromatogr., Chromatogr. Commun. 3 (1980) 269.
[9] H. Aboul-Enein, V. Serignese, Biomed. Chromatogr. 8 (1994) 317.
[10] W. Rong, J. Zheng-Ping, H. Xiao-Li, X. Li-Ting, L. Yong-Min, C. Li-Ren, J. Chromatogr. B 785 (2003) 353.
[11] I. Hay, T. Annesley, N. Jiang, C. Gorman, J. Chromatogr. 226 (1981) 383.
[12] G. Gübitz, F. Juffmann, J. Chromatogr. 404 (1987) 391.
[13] E. Lankmayr, K. Budna, F. Nachtmann, J. Chromatogr. 198 (1981) 471.
[14] E. Lankmayr, B. Maichin, G. Knapp, F. Nachtmann, J. Chomatogr. 224 (1981) 239.
[15] L. Abou-Basha, H. Aboul-Enein, Pharm. Acta Helv. 70 (1995) 237.
[16] L. Dalgaard, J. Hansen, J. Pedersen, J. Pharm. Biomed. Anal. 7 (1989) 361.
[17] H. Aboul-Enein, R. Stefan, S. Litescu, G. Radu, J. Immunoassay Immunochem. 23 (2002) 181.
[18] H. Silvaieh, R. Wintersteiger, M. Schmid, O. Hofstetter, V. Schurig, G. Gübitz, Anal. Chim. Acta 463 (2002) 5.
[19] M. Lämmerhofer, W. Lindner, J. Chromatogr. A 741 (1996) 33.
[20] V. Piette, M. Lämmerhofer, K. Bischoff, W. Lindner, Chirality 9 (1997) 157.
[21] N. Maier, L. Nicoletti, M. Lämmerhofer, W. Lindner, Chirality 11 (1999) 522.
[22] A. Peter, G. Torok, G. Toth, W. Lindner, J. High Resolut. Chromatogr. 23 (2000) 628.
[23] C. Czerwenka, M. Lämmerhofer, W. Lindner, J. Pharm. Biomed. Anal. 30 (2003) 1789.
[24] S. Yamada, C. Hongo, R. Yoshioka, I. Chibata, J. Org. Chem. 48 (1983) 843.
[25] G. Mitulovic, M. Lämmerhofer, N. Maier, W. Lindner, J. Labelled Compd. Radiopharm. 43 (2000) 449.
[26] A. Mandl, L. Nicoletti, M. Lämmerhofer, W. Lindner, J. Chromatogr. A 858 (1999) 1.
[27] E. Zarbl, M. Lämmerhofer, F. Hammerschmidt, F. Wuggenig, M. Hanbauer, N. Maier, L. Sajovic, W. Lindner, Anal. Chim. Acta 404 (2000) 169.
[28] C. Czerwenka, M. Lämmerhofer, N. Maier, K. Rissanen, W. Lindner, Anal. Chem. 74 (2002) 5658.
[29] M. Lämmerhofer, E. Tobler, W. Lindner, J. Chromatogr. A 887 (2000) 421.
[30] J. Pazourek, C. Perrin, D. Massart, Chromatographia 56 (2002) 597.