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Kinetic method for enantiomeric determination of thyroid hormone (D,L-thyroxine) using electrospray ionization tandem mass spectrometry (ESI-MS/MS)

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Abstract

A rapid, sensitive, simple and accurate mass spectrometric analysis for the recognition and quantitation of D- and L-thyroxine (D- and L-T_4) was achieved by using kinetic method. The method uses the kinetics of competitive unimolecular fragmentations of trimeric transition metal ion-bound clusters formed under electrospray ionization (ESI). Singly charged cluster ions containing the divalent central metal ion Ca(II)/Mn(II), an amino acid/modified amino acid chiral reference, and the analyte D- and L-T_4 were generated by ESI. The cluster ion of interest was mass-selected, and subjected to collision-induced dissociation for undergoing dissociation by competitive loss of either a neutral reference or a neutral analyte. The chiral selectivity (R_{chiral}), the ratio of the two competitive dissociation rates (abundances of fragment ion) containing the analyte in one enantiomeric form expressed relative to that for the fragments of the other enantiomer, ranges from 0.46 to 3.03. Method by using fixed ligand such as peptide has also successfully improved chiral recognition and quantitative accuracy, which simplifies the dissociation kinetics, in which only the reference ligand or the analyte can be lost. The linear relationship between the logarithm of the fragment ion abundance ratio ($\ln R$) and enantiomeric compositions (ee%) of the T_4 allows the chiral purity of enantiomeric mixtures to be determined. The average relative errors were less than 2% between the actual and experimental enantiomeric compositions.

Keywords: Thyroid hormone; Enantiomeric determination; Kinetic method; ESI-MS/MS

1. Introduction

The thyroid hormones, thyroxine (T_4) and tri-iodothyronine (T_3) were tyrosine-based hormones produced by the thyroid gland. They act on the body to increase the basal metabolic rate [1], affect protein synthesis [2], and increase the body's sensitivity to catecholamines [3]. The major form of thyroid hormone in the blood was thyroxine (T_4). This was converted to active T_3 within cells by deiodinases [4]. Both T_3 and T_4 were used to treat thyroid hormone deficiency (hypothyroidism). Levothyroxine (L-T_4) sodium, the most commonly used form, has convenient pharmacokinetic properties, and when given in the proper dose, a high degree of effectiveness and small risk of adverse reactions [5]. The lack of production of these hormones causes hypothyroidism, leading to myxoedema with struma and debility. For the treatment thyroidism, thyroid hormone preparations were used.

T_4 exists as two enantiomeric forms, such as L-T_4 and D-T_4 (Scheme 1), which have different biological, pharmacological and therapeutic effects. L-T_4 was mainly used as replacement therapy in hypothyroidism while D-T_4 was used as anticholestraemic agent. In contrast to L-T_4, D-T_4 shows no basic metabolic rate enhancement but a significant decrease of cholesterol, phospholipids and apolipoprotein B in serum and of all lipids [6]. The therapeutic use of D-T_4 necessitates an optical purity to avoid side effects due to trace of L-T_4 [7]. Also, the clinical use of L-T_4 requires a check on the optical purity of the enantiomer during pharmaceutical formulations. In addition, a suppression of thyroid stimulating hormone (TSH) excretion caused by D-T_4 has been observed [8]. For these reasons, the sensitive analysis and quantitative determination of the individual enantiomers of thyroid hormones was essential for biological researches and pharmacokinetic studies.

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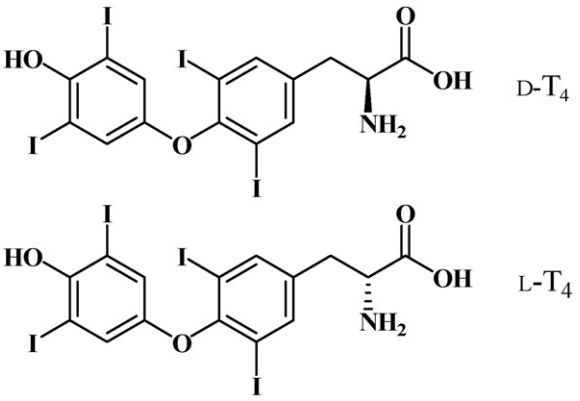

Scheme 1. Chemical structure of D and L-thyroxine (D, L-T_4).

Up to date, many methods have been mainly developed for chiral recognition of T_4, including high-performance liquid chromatography using a protein column [9], a chiral ligand ion-exchange system with chiral mobile phase [10], a quinine-derived chiral stationary phase [11], and a capillary electrophoresis [12]. Chiral recognition by mass spectrometry was currently an active area of research because of its sensitivity, speed, and simplicity. Moreover, mass spectrometry eliminates the interference from solvent or a stationary phase such as chromatographic method, therefore allowing an intrinsic evaluation of the interactions in the diastereomeric complex and providing structural confirmation in the gas phase. The introduction of soft ionization approaches to mass spectrometry, including fast atom bombardment and electrospray ionization, allows formation of the gas phase ions of non-covalent or weakly bound complexes. These ionization methods were also helpful to the chiral recognition studies of some important nonvolatile chiral compounds such as amino acids and amines [13,14].

There were many methods for chiral recognition by mass spectrometry, however, such as host–guest diastereomeric adduct formation with a chiral reference compounds [13], guest–host-type ion/molecule reactions [15], tandem mass spectrometric experiments using collision-induced dissociation [16], and kinetic methods [17–22]. Electrospray ionization mass spectrometry [14,23] (ESI-MS), especially electrospray ionization tandem mass spectrometry [16,18,19,21,22] (ESI-MS/MS), has recently emerged as a powerful technique for chiral recognition. Especially, the kinetic method was very simple, rapid, and accurate for chiral recognition and quantitation. For the chiral analysis of chiral hormone, T_4, chromatographic methods were usually employed [8,24]. In this study, first effort for chiral analysis of hormone by electrospray ionization tandem mass spectrometry, which uses kinetic method and a single ratio method, was compared with the fixed ligand method.

2. Experimental

2.1. Materials

D, L-T_4, chiral references (L-phenylalanine, L-tyrosine, 3,4-dihyroxy-L-phe, L-tryptophan, L-abrine), fixed ligands (phe-gly, gly-phe) and central metal ions ($CaCl_2$, $MnCl_2$) were purchased from Sigma–Aldrich Chemical Co. (Milwaukee, WI, USA) and used without purification. Methanol (HPLC grade) was obtained from Aldrich.

2.2. Electrospray ionization tandem mass spectrometry

All experiments were performed using a LCQ-Advantage ion trap mass spectrometer (Thermo Finnigan Co., San Jose, CA, USA) equipped with an electrospray interface. Operation conditions were as follows: spray voltage, 4.5 kV; capillary voltage, 3 V; heated capillary temperature, 150 °C; sheath gas (N_2), 20 arb; and tube lens offset voltage, 10 V. Helium gas admitted directly into the ion trap was used as buffer gas to improve trapping efficiency and as the collision gas for CID experiments. CID experiments were performed by setting the isolation width between 5 and 10 mass units depending on the interest species and the relative collision energy at 15–23%, where 0–25% relative collision energy corresponds to 0–1.25 V peak-to-peak of resonance excitation RF voltage. The ion lenses and octapole were also optimized to provide the maximum trimer ion signal. All mass spectra recorded were the average of ~30 consecutive scans. Samples were infused using a syringe pump at a flow rate of 5 μL min^{-1}. Gas-phase central metal ion and adduct ions of T_4 were generated simply by electrospraying 50:50 water–methanol solutions containing a mixture of T_4, at a concentration of 1×10^{-4} mol L^{-1} (methanol:H_2O with 4 mM NaOH = 50:50), chiral references and fixed ligands, at a concentration of 1×10^{-4} mol L^{-1} respectively, and 2.5×10^{-5} mol L^{-1} central metal ions.

3. Results and discussion

The kinetic method can be applied in two distinct ways for enantiomeric measurement. These were termed the single ratio method and the quotient ratio method [18]. In this study, we only use the simpler single ratio method since this allows mixtures to be analyzed by simply recording a ratio of fragment ion abundances in a single (tandem) mass spectrum. The application of Cook's kinetic method can conveniently fulfilled the chiral analysis, based on the generation of the trimeric cluster ions $[M^{II}(ref)_2(T_4)-H]^+$ of the following equation:

$$T_4 \xrightarrow{M^{II}, ref} [M^{II}(ref)_2(T_4)\text{-}H]^+ \begin{cases} \xrightarrow{k_1} [M^{II}(ref)(T_4)\text{-}H]^+ + ref \\ \xrightarrow{k} [M^{II}(ref)_2\text{-}H]^+ + T_4 \end{cases} \quad (1)$$

where ref was a suitable reference compound and M^{II} was a divalent central metal ion produced by electrospray ionization (ESI) of a tandem mass spectrometer. The mass-selected $[M^{II}(ref)_2(T_4)-H]^+$ ion dissociate to form two dimeric clusters $[M^{II}(ref)(T_4)-H]^+$ and $[M^{II}(ref)_2-H]^+$ exclusively by collision-induced dissociation without other competitive or consecutive fragmentations.

The branching ratio (R) can be represented by the fragment ion abundance ratio in the following equation:

$$R = \frac{[M^{II}(ref)(T_4) - H]^+}{[M^{II}(ref)_2 - H]^+} \quad (2)$$

The difference in energy between the diasteromeric ions $[M^{II}(ref)(D\text{-}T_4)-H]^+$ and $[M(ref)(L\text{-}T_4)-H]^+$, results in differences in their rates of formation and hence, in the relative abundance ratios (R_D or R_L) for the D- and L-isomers, defined in Eqs. (3) and (4), respectively.

$$R_L = \frac{[M^{II}(ref)(L\text{-}T_4) - H]^+}{[M^{II}(ref)_2 - H]^+} \quad (3)$$

$$R_D = \frac{[M^{II}(ref)(D\text{-}T_4) - H]^+}{[M^{II}(ref)_2 - H]^+} \quad (4)$$

The ratio of the individual ratios, R_D to R_L, was defined as R_{chiral} (Eq. (5)) and indicates the level of chiral discrimination achievable in a particular experiment. $R_{chiral} = 1$ means a lack of chiral discrimination and the more different the R_{chiral} value from 1, the higher the degree of chiral recognition. Chiral references were chosen for their capability to produce large steric interactions and for structural similarity to particular analytes. Such similarity allows the complexes to form easily and it also allows accurate relative abundance ratios to be measured; otherwise, dissociation proceeds overwhelmingly to form the more stable product.

$$R_{chiral} = \frac{R_D}{R_L} \quad (5)$$

The chiral selectivity, the ratio of the two fragment ion abundances for the complex containing one enantiomer of analyte expressed relative to that for the fragments of the corresponding complex containing the other enantiomer. The sensitive nature of the methodology and the linear relationship between the logarithm of the fragment ion abundance ratio and the optical purity, which was intrinsic to the kinetic method, allows mixtures to be analyzed for small enantiomeric excess (ee) by simply recording ratios of fragment ion abundances in a mass spectrum [18].

And the method by using fixed ligand kinetic method such as peptide has also been successfully improved chiral recognition, and simplifies the dissociation kinetics, in that only the reference ligand or the analyte can be lost [25,26].

$$T_4 \xrightarrow[L_{fixed}]{M^{II},\ ref} [(M^{II}+L_{fixed})(ref)_2(T_4)\text{-}H]^+ \begin{array}{l} \xrightarrow{k_1} [(M^{II}+L_{fixed})(ref)(T_4)\text{-}H]^+ + ref \\ \xrightarrow{k_2} [(M^{II}+L_{fixed})(ref)_2\text{-}H]^+ + T_4 \end{array} \quad (6)$$

$$R^{fixed} = \frac{[(M^{II} + L_{fixed})(T_4) - H]^+}{[(M^{II} + L_{fixed})(ref)_2 - H]^+} \quad (7)$$

$$R^{fixed}_{chiral} = \frac{R^{fixed}_D}{R^{fixed}_L} = \frac{[(M^{II} + L_{fixed})(ref)(D\text{-}T_4) - H]^+ / [(M^{II} + L_{fixed})(ref)_2 - H]^+}{[(M^{II} + L_{fixed})(ref)(L\text{-}T_4) - H]^+ / [(M^{II} + L_{fixed})(ref)_2 - H]^+} \quad (8)$$

Under these conditions, Eq. (1) takes the form of Eq. (6); and accordingly, Eq. (2) and Eq. (5) become Eq. (7) and Eq. (8), respectively. Here, R^{fixed}_D and R^{fixed}_L were the branching ratios for the analytes of D-T_4 and L-T_4 when the fixed ligand was used.

Quantitative discrimination can be achieved by relating the relative branching ratio to the difference in free energy change of the system by the kinetic method expression, given in the following equation:

$$\ln R = \frac{\Delta(\Delta G)}{RT_{eff}} \quad (9)$$

here, R was the branching ratio of competitive dissociation from trimeric cluster ion, R was the gas constant, T_{eff} was the effective temperature of the activated trimeric cluster, and ΔG was the difference in free energy change for each pathway of dissociation for the diastereomeric trimeric clusters to the two dimeric clusters. When a binary mixture was used, enantiomeric excess was given by ee, a combined expression for $\Delta(\Delta G)$ can be given as follows:

$$\begin{aligned} \Delta(\Delta G) &= [\Delta(\Delta G)_D]ee + (1 - ee)[\Delta(\Delta G)_L] \\ &= \Delta(\Delta G)_L + [\Delta(\Delta G)_D - \Delta(\Delta G)_L]ee \end{aligned} \quad (10)$$

where, $\Delta(\Delta G)_D$ and $\Delta(\Delta G)_L$ refer to the pure enantiomers, D-T_4 and L-T_4. By then combining Eqs. (9) and (10), one obtains convenient linear relationship relating the enantiomeric excess to the natural logarithm of the branching ratio, as shown in the following equation:

$$\begin{aligned} \ln R &= \frac{\Delta(\Delta G)_L}{RT_{eff}} + \left[\frac{\{\Delta(\Delta G)_D - \Delta(\Delta G)_L\}}{RT_{eff}}\right] ee \\ &= \ln R_L + [\ln R_{chiral}]ee \end{aligned} \quad (11)$$

This equation can be used to construct a calibration curve ($\ln R$ versus ee) with mixtures of known enantiomeric excess. For the relationship given in Eq. (11), $\ln R_{chiral}$ becomes the slope of the curve and $\ln R_L$, or the branching ratio determined for the pure enantiomer L-T_4 becomes the y-intercept. A similar derivation can be made for the fixed ligand method.

$$\ln R^{fixed} = \frac{\Delta(\Delta G^{fixed})}{RT_{eff}} \quad (12)$$

$$\begin{aligned} \ln R^{fixed} &= \frac{\Delta(\Delta G^{fixed})_L}{RT_{eff}} + \left[\frac{\{\Delta(\Delta G^{fixed})_D - \Delta(\Delta G^{fixed})_L\}}{RT_{eff}}\right] ee \\ &= \ln R^{fixed}_L + [\ln R^{fixed}_{chiral}]ee \end{aligned} \quad (13)$$

The reference ligands (ref*) were chosen to allow deprotonation as well as to give measurable ion abundance and dissociation ratios for the fragmentation channels. Fig. 1 shows the ESI mass spectra of a mixture of T_4 as an analyte, tryptophan as a reference, and Mn^{2+} as a central metal ion. The

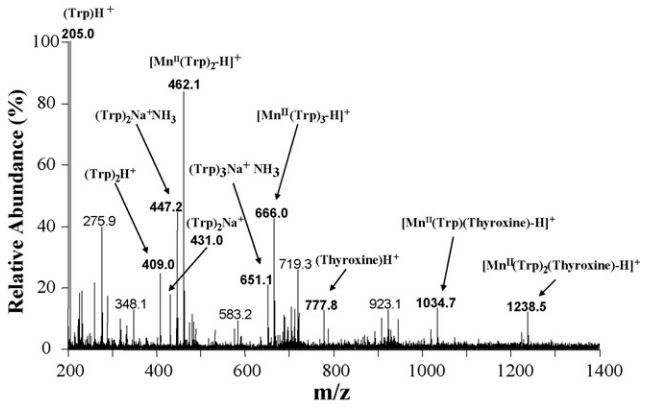

Fig. 1. Electrospray mass spectrum of a sample containing manganese(II) chloride, typtophan and thyroxine in a 50:50 methanol/water solution.

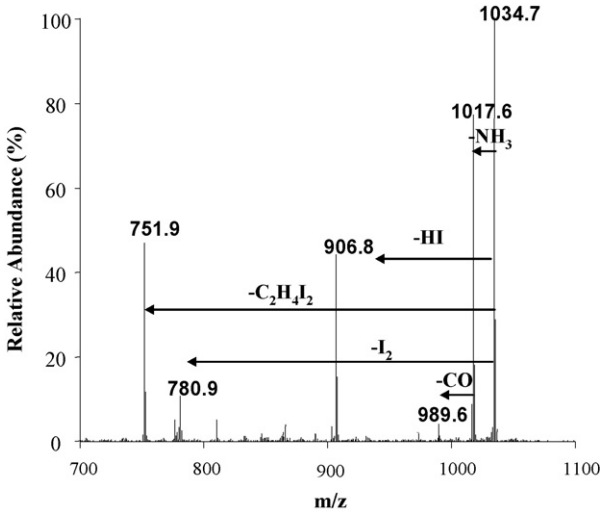

Fig. 2. MS/MS spectrum of dimeric cluster ion $[Mn^{II}(trp)(T_4)-H]^+$ (*m/z* 1034.7). The collision-induced dissociation energy was chosen as 23%.

spectrum shows several ions, including relatively abundant protonated, sodiated clusters and metal bound clusters, especially those involving the reference tryptophan, which has high abundance in the spectrum. The most interesting clusters of ions were the singly charged metal-bound trimeric clusters and dimeric clusters formed by deprotonation of one of the constituent ligands. Singly charged dimeric cluster ions, such as $[Mn^{II}(trp)(T_4)-H]^+$ (*m/z* 1034.7) and $[Mn^{II}(trp)_2-H]^+$ (*m/z* 462.0), were shown in the mass spectra. Especially, singly charged trimeric cluster ion, $[Mn^{II}(trp)_2(T_4)-H]^+$ in *m/z* 1238.5. The trimeric ion was mass-selected and fragmented by collision-induced dissociation in a quadruple ion trap to form the pair of dimeric ions.

Fig. 2 shows the CID spectrum of a typical Mn(II)-bound dimeric cluster ion, $[Mn^{II}(trp)(T_4)-H]^+$. Instead of the loss of an intact neutral molecule, the fragment ions in this case were NH_3, HI, C_2H_4, I_2, etc. The loss of small molecules indicates that the ligands were strongly bound in the Mn(II) dimeric cluster. Such type of CID fragmentation behavior of singly charged transition metal-bound dimeric cluster ions has been observed for all the systems so far examined [19,27,28]. By contrast with the behavior of the dimeric cluster ions, the Mn-bound trimeric cluster ions, $[Mn^{II}(trp)_2(T_4)-H]^+$, fragment simply by the loss of a neutral ligand to form deprotonated dimeric cluster ions. Because they appear loosely bound, as evidenced by the occurrence of low energy CID compared with its dimeric cluster high energy CID, these ions were of great interest from the standpoint of the kinetic method [18].

Fig. 3 shows the CID spectra of trimeric cluster ion $[Mn^{II}(trp)_2(T_4)-H]^+$ (Fig. 3a) and $[Mn^{II}$(3,4-dihydroxy-L-phe$)_2(T_4)-H]^+$ (Fig. 3b). The results indicate that the branching ratio for these fragmentations depends strongly on the stereo-

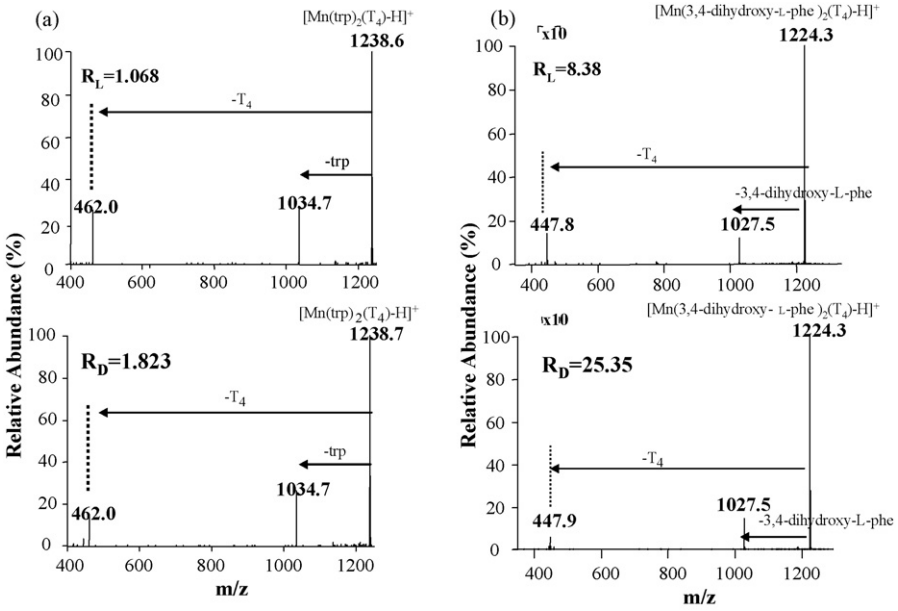

Fig. 3. MS/MS spectrum of trimeric cluster ion $[Mn^{II}(trp)_2(T_4)-H]^+$ (a) and $[Mn^{II}$(3,4-dihydroxy-L-phe$)_2(T_4)-H]^+$ (b). The collision-induced dissociation energy was chosen as 17% (a) and 16% (b).

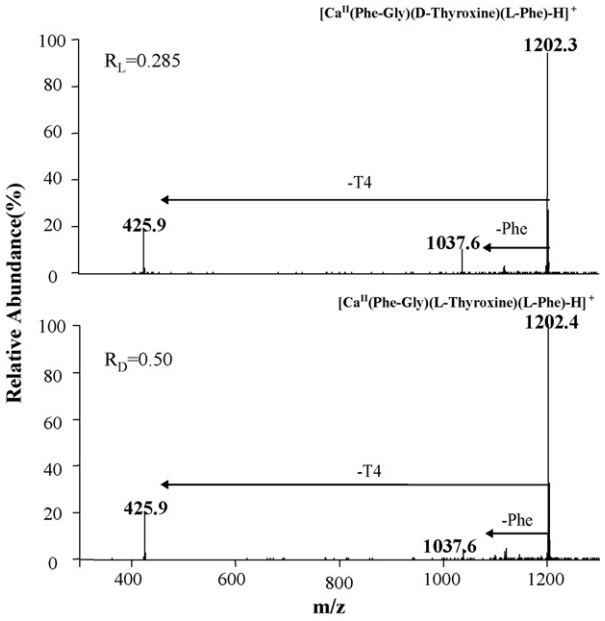

Fig. 4. MS/MS spectrum of trimeric cluster ion with fixed ligand, Phe-gly, $[Ca^{II}(phe\text{-}gly)(phe)(T_4)\text{-}H]^+$ (*m/z* 1202). The CID energy was chosen as 15%.

chemistry of the ligands, when the same reference ligand was used, that depends specifically on the chirality of the T_4. In this case, chiral recognition factor (R_{chiral}) was 1.71 (Fig. 3a) using tryptophan as a reference ligand and 3.03 (Fig. 3b) using 3,4-dihydroxy-L-phe as a reference ligands, Mn(II) as a central metal ion.

Fig. 4 shows the CID spectra of the trimeric cluster ion with fixed ligand $[Ca^{II}(phe\text{-}gly)(phe)(T_4)\text{-}H]^+$. Peptides often have higher metal ion affinities than amino acids, especially when bonding to the metal ions, where their special coordination properties allow them to bind strongly at multiple bonding sites to form metallopeptides [29,30]. Therefore, by selecting peptides as fixed ligands, only the reference ligand and analyte T_4 will be lost upon collision activation. In the fixed-ligand experiments, the branching ratio for these fragmentations of the trimeric cluster ion depends strongly on the stereochemistry of T_4 when the same reference and suitable fixed ligands were employed [26]. At a relative CID activation energy of 15%, when analyte was the pure D-T_4, the branching ratio R_D was 0.50, whereas R_L was 0.285 for the pure L-T_4. The chiral selectivity, R_{chiral}^{fixed}, was therefore 1.82 in this case.

The absence of solvent in these mass spectrometry experiments means that direct interactions between the ligands and the metal ion must form the basis for chiral differentiation. Because of this, the electronic and steric effects of the chiral reference ligand dictate chiral recognition. Note that if the difference between the metal ion affinities of the analyte and the reference are too large, dissociation of the trimeric cluster ions overwhelmingly favors only one fragmentation channel and thus prevents accurate measurement of the abundance of the other fragment ion [31]. It is, therefore, essential to choose a reference ligand of appropriate metal ion affinity. Chiral recognition was achieved for T_4, indicated by the chiral selectivity values R_{chiral}, summarized in Table 1. Previous results from chiral analysis showed that

Table 1
Influence of the chiral reference and central metal ion on chiral recognition

Reference	R_{chiral}				
	Ca^{2+}	Mn^{2+}	Cu^{2+}	Ni^{2+}	Zn^{2+}
L-Phe	1.30	0.78	0.64	0.58	0.71
L-Tyr	0.75	0.46	0.58	0.45	0.42
L-Trp	1.19	1.71	0.93	0.57	0.88
L-Abrine	1.05	1.17	0.84	0.62	0.69
3,4-Dihydroxy-L-phe	0.52	3.03	2.67	1.82	0.94

CID activation energy level is optimized in each experiment and then held constant for measurement of each pair of enantiomers.

reference ligands with aromatic functional groups often result in greater chiral discrimination than non-aromatic chiral references [32]. For this reason aromatic side chain amino acid was chosen as the references.

Metal ions play a key role in achieving chiral distinction by dissociation of trimeric complex ions. The different metal ions have different electronic configurations and hence, different stereochemical effects, as was clearly shown in solution phase [33]. It was expected that the choice of core metal ions would have a significant effect on chiral recognition in this system. To probe the intriguing effects of metal ions on chiral recognition five metal ions, Ca(II), Mn(II), Ni(II), Cu(II), and Zn(II) were examined. From the results in Table 1, it can be seen that Mn(II) have more chiral selectivity, because their metal complexes show the required competitive fragmentations for the ligands under consideration and it often results in large chiral selectivity. In previous study, Mn(II) also showed good chiral selectivity to the some antibiotics with fixed ligands [25]. Among these metal ions, Cu(II) might have the largest geometry distortion [34] and two of the axial binding sites in its six coordinate complexes were unusually far from the central ion, resulting in inefficient overlap between the π-orbital on the carbonyl group, and d-orbitals of Cu(II) ion and hence in less chiral recognition than Mn(II).

The Mn(II), central metal ion and 3,4-dihydroxy-L-phe, chiral reference combination shows largest chiral recognition, when using Mn(II) as a central metal ion, higher R_{chiral} value was obtained according to Ca(II). The phenomenon can be explained from the effect of π–d interaction between d-orbital of Mn(II) and electrons of ligand [32]. The method depends strongly on the choice of chiral reference compound and the interactions of metal–ligand, ligand–ligand for chiral recognition and quantitative analysis, because there was no solvent in the mass spectrometer. The effect of the choice of chiral reference and central ion on ease of chiral recognition was reflected in the R_{chiral} values. Structural similarity to the analyte was also a desirable quality because it usually facilitates ready complex formation and gives similar rates of competitive dissociation that allow accurate measurements of relative abundance ratios. In the case of tyrosine and 3,4-dihydroxy-phenyl alanine, the –OH group on the ring tends to increase the electron density, increased chiral recognition was observed. Tryptophan, whose indole residue should provide good overlap with the π-orbital of the carboxylic group, shows a similar effect [18].

Table 2
Chiral recognition of T_4 using a fixed ligand

Central metal ion	Fixed-ligand	Reference	R^{fixed}_{chiral}
Ca	Phe-gly	L-Phe	1.82
	Gly-phe	L-Phe	0.66
	Phe-gly	L-Phe	0.64
Mn	Gly-phe	L-Trp	1.39
	Phe-gly	L-Tyr	1.60

CID activation energy level is optimized in each experiment and then held constant for measurement of each pair of enantiomers.

Chiral discrimination of T_4 using fixed ligands, phe-gly and gly-phe, was investigated and R^{fixed}_{chiral} values shown in Table 2. To provide metal–ligand interaction and ligand–ligand interaction, aromatic terminus residue dipeptides were selected as a fixed ligand in this experiment. Using fixed ligands, the chiral recognition of the thyroxine has more improved. In each case, Ca(II) as a metal ion, aromatic N-terminus residue have more chiral selective than aromatic C-terminus residue. It might be proved that aromatic N-terminus residue in fixed ligand serve more interaction with other ligand such as ligand–ligand interaction than C-terminus residue.

The first step in quantitative analysis of T_4 mixtures was the construction of the calibration curve between R and the enantiomeric excess (ee%) of one enantiomer. If a relatively simple relationship between ln R and ee% can be found, then rapid determination of mixture components will be possible by simply measuring the branching ratio R value in a single tandem mass spectrum. Fig. 5 shows the calibration curves for the analysis of T_4 enantiomer by 3,4-dihydroxy-L-phe as a reference, Mn(II) as a metal mediator (Fig. 5a) and L-phe as a reference, phe-gly as a fixed ligand and Ca(II) as a metal mediator (Fig. 5b) for kinetic method. The results indicate good and comparable linearity (correlation coefficient $r^2 = 0.9970$ for (Fig. 5a) and $r^2 = 0.9967$ for (Fig. 5b)). This increased correlation will enable more accurate measurement of ee, and hence a lower detection limit. Table 3 lists the actual ee values of the mixtures and the values obtained through use of calibration curves. Good results were observed, with consistently good agreement between the actual and the measurement values (average relative error for (Fig. 5a) was 1.8% and 1.6% for (Fig. 5b)). This linear relationship of ln R versus ee% was intrinsic to the kinetic method and it can be derived from consideration of the kinetics and energetics of dissociation. Each technique, in the situation

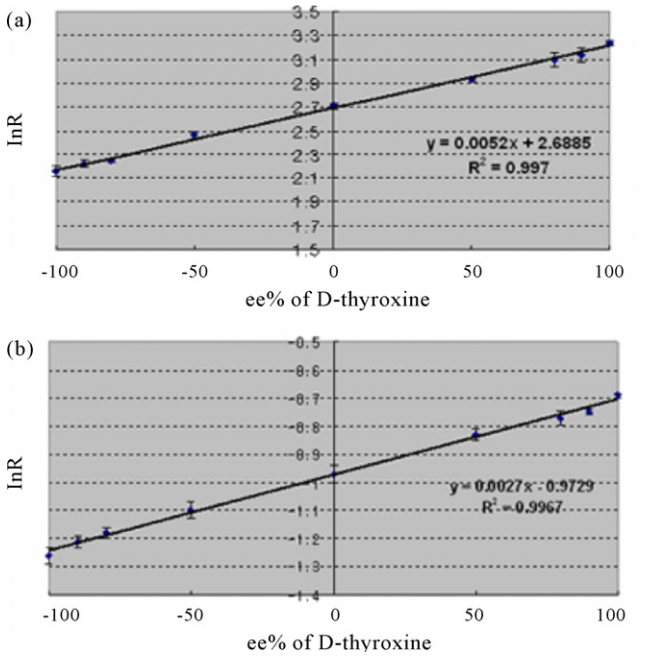

Fig. 5. Calibration curves for quantitative analysis of D, L-T_4. (a) Using 3,4-dihydroxy-L-phe as a reference, Mn(II) as a central metal. (b) Using phe-gly as a fixed ligand, phe as a reference, Ca(II) as a central metal.

Table 3
Enantiomeric excess (ee) measurements on T_4 using (a) Mn(II)/3,4-dihydroxy-L-phe

Actual	Experimental			Average (% ee)	Relative error (%)
	1	2	3		
(a) Mn(II)/3,4-dihydroxy-L-phe					
−95	−94.99	−95.80	−97.65	−96.15	1.2
−90	−86.72	−86.17	−91.41	−88.10	2.1
−80	−82.68	−80.96	−83.96	−82.53	3.2
80	82.49	83.38	78.49	81.45	1.8
90	90.77	88.51	93.02	90.77	0.9
95	95.62	93.96	91.62	93.73	1.3
				Average (%)	1.8
(b) Ca(II)/phe-gly/phe					
−95	−91.46	−97.86	−96.58	−95.30	0.3
−90	−88.39	−92.15	−95.69	−92.08	2.3
−80	−76.37	−84.05	−80.50	−80.31	0.4
80	89.24	79.70	74.28	81.07	1.3
90	94.21	89.16	92.46	91.94	2.2
95	93.68	95.81	87.23	92.24	2.9
				Average (%)	1.6

of this particular reference/analyte/metal system and fixed ligand/reference/analyte/metal system, provides acceptable results for quantitative analysis of T_4 enantiomers.

4. Conclusions

Tandem mass spectrometry of metal-bound cluster ions using kinetic method provides a rapid, sensitive, and accurate method for recognition and quantitation for the D- and L-T_4. The measurements were performed by a commercial instrument, they use tandem mass spectrometry equipped with electrospray ionization source, and they require only very small amounts of sample for analysis. The subject reported in this paper was of wide relevance, especially to the pharmaceutical industry and clinical application, for the general demonstration of a simple method for the chiral recognition and enantiomeric quantitation. In future, another important thyroid hormone, T_3 and its isomer reverse T_3 will be applied to isomeric discrimination and determination. Furthermore, simultaneous chiral analysis and isomeric determination of a mixture containing thyroid hormones, D, L-T_4, T_3 and r-T_3, will be attempted without chromatographic separations.

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