Isomeric Discrimination and Quantification of Thyroid Hormones, T₃ and rT₃, by the Single Ratio Kinetic Method Using Electrospray Ionization Mass Spectrometry

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The single ratio kinetic method is applied to the discrimination and quantification of the thyroid hormone isomers, 3,5,3'-triiodothyronine and 3,3',5'-triiodothyronine, in the gas phase, based on the kinetics of the competitive unimolecular dissociations of singly charged transition-metal ion-bound trimeric complexes $[M^{II}(A)(ref^*)_2-H]^+$ (M^{II} = divalent transition-metal ion; A = T₃ or rT₃; ref* = reference ligand). The trimeric complex ions are generated using electrospray ionization mass spectrometry and the ions undergo collisional activation to realize isomeric discrimination from the branching ratio of the two fragment pathways that form the dimeric complexes $[M^{II}(A)(ref^*)_2-H]^+$ and $[M^{II}(ref^*)_2-H]^+$. The ratio of the individual branching ratios for the two isomers R_{iso} is found strongly dependent on the references and the metal ions. Various sets are tried by choosing the reference from amino acids, substituted amino acids, and dipeptides in combination with the central metal ion chosen from five transition-metal ions (Co^{II}, Cu^{II}, Mn^{II}, Ni^{II}, and Zn^{II}) for the complexes in this experiment. The results are compared in terms of the isomeric discrimination for the T₃/rT₃ pair. Calibration curves are constructed by relating the ratio of the branching ratios against the isomeric composition of their mixture to allow rapid quantitative isomer analysis of the sample pair. Furthermore, the instrument-dependence of this method is investigated by comparing the two sets of results, one obtained from a quadrupole ion trap mass spectrometer and the other from a quadrupole time-of-flight mass spectrometer. (J Am Soc Mass Spectrom 2010, 21, 14–22) © 2010 American Society for Mass Spectrometry

The thyroid hormones, 3,5,3'-triiodothyronine (T₃) and 3,3',5'-triiodothyronine (rT₃; reverse T₃) (Scheme 1) are triiodo analogues of 3,5,3',5'-tetraiodothyronine (Thyroxine, T₄). T₄ is an important source of the T₃ and rT₃, as it is responsible for 80% of the whole amount. The two hormones secreted by the thyroid gland are produced by peripheral monodeiodination [1] of T₄, but alternative routes of enzymatic deiodination can also produce either T₃ or rT₃ [2]. The process involves the removal of an iodine atom from carbons 5' and 5 on the outer ring of T₄, respectively (Scheme 1). T₄ and T₃ are largely bound to protein in circulation and their concentrations in blood (typically 50–110 μg L⁻¹ for total T₄, and 0.5–2.0 μg L⁻¹ for total T₃) are measures of thyroid function.

T₃ is the most biologically potent thyroid hormone, produced from deiodination of T₄ by two deiodinases,

one exists within the liver and accounts for 80% of the deiodination of T₄ and the other is present within the pituitary. T₃ is metabolically active and important in regulating biological processes, including growth and development, body temperature, heart rate, oxygen consumption, protein synthesis, and fetal neurodevelopment [3]. Therefore, it is used to treat hypothyroidism or thyroid hormone deficiency in medical practices. Dratman and Gordon [4] have shown that T₃ is found in the junctions of synapses and regulates the amounts and activities of serotonin, norepinephrine, and γ-aminobutyric acid (GABA) in the brain.

On the other hand, rT₃ is currently conceived not to have hormonal activity itself (inactive), but rather to act as a major competitive inhibitor of T₃ at the cellular level [5]. Under normal conditions, 45% to 50% of the daily production of T₄ is transformed into rT₃ [6]. In some instances, there may be preferential formation of rT₃ to reduce energy output, that is, low T₃ syndrome. In addition, rT₃ may inhibit deiodinases, thereby directly interfering with the generation of T₃ from T₄, or

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Scheme 1. Generation of T_3 and rT_3 from T_4 .

with subsequent breakdown of T_3 [5, 7, 8]. Also, if the body fails to convert T_4 to T_3 , T_3 becomes less available in the bloodstream, while the level of the inactive rT_3 tends to build up [9]. A low T_3 to rT_3 ratio is associated with a lesser ability of the left ventricle to pump blood and is highly predictive of poorer short-term outcome in patients with severe chronic heart failure. Thus, the molar ratio of T_3 to rT_3 is an important diagnostic marker for the metabolism and function of thyroid hormones in clinical chemistry.

The kinetic method was developed by Cooks and coworkers in 1977 [10] for the determination of relative thermochemical properties based on the rates of the competitive dissociation of mass selected ions [11–13], and is capable of differentiating processes with energy differences of <1 kJ/mol [9, 10, 14]. The thermochemical properties that the method can access include proton affinity [15–17], gas-phase basicity [17–20], ionization energy [21], and metal ion affinity [22–24] to name a few. It has also been found useful in the differentiation and determination of enantiomers [25–29] and isomers [29–34] under sterically-controlled conditions. The kinetic method has many interesting characteristics; it is fast, easy, and accurate, which are true even for polar and nonvolatile samples. Neither does it require isotopic labeling nor wet chemical processes. It is also tolerant to impurities and can be performed with commercial instruments. Furthermore, very low enantiomeric excess or isomeric molar ratio can be rapidly determined.

From the activities of thyroid hormones T_3 and rT_3 mentioned earlier, a sensitive analysis and quantitative determination of the isomers is essential in biologic researches and pharmacokinetic studies. From the literature survey, it is revealed that the analysis of thyroid hormones, T_3 and rT_3 , in a sample has been reported not so often unlike the separations of T_4 and T_3 . A novel on-line HPLC system incorporating the high-performance

frontal analysis (HPFA) system and electrochemical detection is developed for the simultaneous determination of unbound T_4 , T_3 , and rT_3 in human plasma [35]. Zhang et al. [36] developed an ESI tandem mass spectrometry (MS/MS) technique both in positive and negative ion modes to distinguish and quantify T_3 and rT_3 by utilizing the difference in percent ion contributions of the MS/MS fragment ions. In this work, the relative molar percentages of T_3 and rT_3 isomers in a mixture were determined using a system of two equations. Couldwell et al. [37] reported the fragmentation behavior of thyroid hormones T_4 , T_3 , rT_3 , [3, 5]- T_2 , and the non-iodinated thyronine (T_0) in negative ion mode using ESI-MS/MS. In their work, the isomers, T_3 and rT_3 , are differentiated by observing the characteristic fragments at m/z values 359.7 and 604.5 for rT_3 , which are absent in the spectrum of T_3 . However, this work concentrated mostly on interpretation of the MS/MS spectra and the isomeric compositional analysis for the mixture of the pair was not realized.

In our previous work, we have reported the enantiomeric analysis of thyroid hormones, D, L- T_4 [26] and chiral drug DOPA (D, L-DOPA) [27] by kinetic method using ESI-MS/MS. In the present report, the single ratio kinetic method is utilized as a simple but rapid and sensitive method for discriminating the two isomers and furthermore the molar percentages of T_3 and rT_3 isomers in the mixture without prior separation in mainly metal-mediated ionic complexes using an ion trap and a QTOF mass spectrometer.

Experimental

Reagents

Analytes, T_3 and rT_3 , reference compounds, including L-amino acids, substituted amino acids and dipeptides,

and central metal ions (transition-metal chlorides, CoCl_2 , CuCl_2 , MnCl_2 , NiCl_2 , and ZnCl_2) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). HPLC grade methanol (MeOH) was obtained from Merck (Darmstadt, Germany). All other chemicals and solvents were purchased from Aldrich Chemical Co. (St. Louis, MO, USA), and used without further purification.

Preparation of Solutions

Initially, the stock solutions of T_3 and rT_3 were prepared at a concentration of 1×10^{-3} M in a 1:1 (vol/vol) mixture of MeOH and 5 mM aqueous NaOH; these solutions were protected from light and stored at -20°C , and wrapped with aluminum foil. The stock solutions of reference compounds were prepared at a concentration of 4×10^{-4} M and 1×10^{-4} M concentrated stock solutions of the transition-metal ions, Co(II) , Cu(II) , Mn(II) , Ni(II) , and Zn(II) were prepared, all of which were stored at 4°C . The stock solutions were diluted with the same solvent to get the required concentration.

Instrumentation

Ion Trap Mass Spectrometer

A commercial LCQ ion trap mass spectrometer (Thermo Finnigan Co., San Jose, CA, USA) equipped with an ESI source was operated in the positive ion mode in the following conditions: spray voltage, 4.5 kV; capillary voltage, 3 V; heated capillary temperature, 150°C ; and sheath gas (N_2), 20 arb; tube lens offset voltage, 10 V. Helium gas admitted directly into the ion trap was used both as the collision gas for collision-induced dissociation (CID) experiments and as the buffer gas to improve trapping efficiency. CID experiments were performed by setting the isolation width between 5 and 10 mass units depending on the species of interest and the relative collision energy at 14% to 23%, where 0% to 25% relative collision energy corresponds to the resonance excitation by the RF of 0 to 1.25 V (peak-to-peak). The ion lenses and octopole were also optimized for maximum trimer ion signal. All the mass spectra recorded were averaged for ~ 30 consecutive scans. Samples were infused using a syringe pump at a flow rate of $5 \mu\text{L}/\text{min}$.

Quadrupole Time-of-Flight Mass Spectrometer

The mass spectrometry was performed on a QTOF Premier (Waters, Manchester, UK) that has a quadrupole followed by a time-of-flight tandem mass spectrometer with orthogonal acceleration in V mode. The system was operated under MassLynx 4.1 software (Waters-Micromass, Manchester, UK) in positive ion electrospray mode. High purity nitrogen was used as the nebulizer and auxiliary gas; Argon was used as the

collision gas. The nebulization gas was set to 200 L/h at a temperature of 200°C , the cone gas set to 20 L/h and the source temperature set to 90°C . The capillary, extraction and sample cone voltages were set to 3.0 kV, 5.0 V, and 25 V, respectively. The instrument was operated in full-scan mode with the QTOF data being collected between m/z 100–1500 with collision energy of 4 eV. The data were collected in the centroid mode, with a scan time of 0.5 s, interscan delay of 0.1 s. The MS/MS experiments were performed using required collision energies (8–12 eV) which were optimized for every experiment and kept constant for both isomers to measure R_{iso} values. Samples were infused at a flow rate of $5 \mu\text{L}/\text{min}$. The QTOF detector (MCP) was operated at 2100 V.

Gas-phase transition-metal ion complexes of T_3/rT_3 were generated by electrospray ionization of 50:50 water-MeOH solutions containing a mixture of analyte, T_3/rT_3 , and chiral references at a concentration of 100 μM each, with the metal salt (M(II)Cl_2) present at 25 μM . The analyte, chiral reference, and metal ion solutions were mixed before infusion into the mass spectrometer.

Results and Discussion

Isomeric Discrimination of T_3/rT_3

Isomeric discrimination of T_3/rT_3 by kinetic method is based on the formation of trimeric complex ions followed by fragmentation with CID measuring the abundance ratio of the two fragmented dimeric complex ions in the mass spectrometer. Scheme 2 presents the schematic representation for the formation of the metal-bound trimeric complex ions of T_3/rT_3 and their competitive unimolecular dissociations to dimeric complex ions using CID (MS/MS).

Singly charged trimeric complex ions, $[\text{M}^{\text{II}}(\text{ref}^*)_2(\text{T}_3/\text{rT}_3)-\text{H}]^+$, are generated by electrospray ionization of an 50:50 aqueous methanolic solution containing the analyte, T_3/rT_3 , the divalent transition-metal ion (M^{II}), and the reference, ref^* . These ions are mass selected and collisionally excited in a quadrupole ion trap where they undergo competitive dissociation by CID to form the dimeric complexes, $[\text{M}^{\text{II}}(\text{ref}^*)(\text{T}_3/\text{rT}_3)-\text{H}]^+$ and $[\text{M}^{\text{II}}(\text{ref}^*)_2-\text{H}]^+$ by the loss of a neutral reference compound, ref^* , or analyte, T_3/rT_3 , respectively. The relative branching ratio R for the two dimeric complexes is presented in eq 1.

$$R = \frac{[\text{M}^{\text{II}}(\text{ref}^*)(\text{T}_3/\text{rT}_3)-\text{H}]^+}{[\text{M}^{\text{II}}(\text{ref}^*)_2-\text{H}]^+} \quad (1)$$

Due to the two isomeric configurations of T_3 and rT_3 , different energies are required to generate the dimeric complexes that result in a difference in their abundances. Therefore, the relative abundance ratios (R_{T_3} or R_{rT_3}) for the T_3 and rT_3 isomers are calculated, respectively. The ratio of the individual ratios, R_{T_3} to R_{rT_3} , is

Scheme 2. Schematic representation for the formation of metal-bound trimeric complex ions of T_3/rT_3 by electrospray ionization of 50:50 aqueous methanol solution containing of analyte, T_3/rT_3 ; reference molecule, Ref; and divalent transition metal ion, M^{II} , and followed by their competitive unimolecular dissociation to dimeric complex ions using collision induced dissociation (MS/MS).

defined as R_{iso} (eq 2) and indicates the degree of isomeric discrimination achievable in a particular experiment, and is strongly dependent on the reference and metal used in the system.

$$R_{iso} = \frac{R_{T_3}}{R_{rT_3}} \quad (2)$$

$R_{iso} = 1$ means no isomeric discrimination and the larger the deviation of the R_{iso} value from 1, the higher the degree of the isomeric recognition.

Formation and Dissociation of Trimeric Complex Ions of T_3/rT_3

Abundant complexing, including the desired transition-metal bound trimeric cluster ions, was observed in the ESI mass spectra taken for the sample mixtures containing T_3/rT_3 , either on of the reference ligand compounds and one of the five divalent transition-metal ions (Co^{II} , Cu^{II} , Mn^{II} , Ni^{II} , and Zn^{II}), and the resultant ions were probed by the kinetic method to discriminate the hormone isomers by two types of ESI mass spectrometers, an ion trap mass spectrometer and a quadrupole time-of-flight mass spectrometer (QTOF) in this study. A typical electrospray mass spectrum of a mixture including manganese (II) chloride, T_3 , and Phe-Gly using the ion trap mass spectrometer is illustrated in Figure 1.

The ESI-MS spectrum shows several ions, including the most interesting singly-charged transition-metal-bound trimeric complex ion, $[Mn^{II}(Phe-Gly)_2(T_3)-H]^+$ and dimeric complex ions, $[Mn^{II}(Phe-Gly)(T_3)-H]^+$, and $[Mn^{II}(Phe-Gly)_2-H]^+$, formed by the deprotonation of one of the constituent ligands. In addition to the trimeric complex ion species of interest, several other ions such as the protonated T_3 , $[(T_3) + H]^+$; its proton-bound dimer with the reference ligand, $[(Phe-Gly)(T_3) + H]^+$ were detected alongside. The ions that are without the analyte are also observed, which include the proton-bound reference ligand, $[(Phe-Gly) + H]^+$, its dimer, $[(Phe-Gly)_2 + H]^+$, and the sodiated dimer of the reference $[Na(Phe-Gly)_2]^+$, and its trimeric metal complex, $[Mn^{II}(Phe-Gly)_3-H]^+$.

Figure 2 shows the product ion spectra of the singly charged trimeric complex ions, $[Cu^{II}(Phe-Gly)_2(T_3)-H]^+$ (Figure 2a) and $[Cu^{II}(Phe-Gly)_2(rT_3)-H]^+$ (Figure 2b) formed from the solution containing pure T_3 and rT_3 , respectively. The trimeric ions of T_3/rT_3 were mass selected and fragmented by CID to generate two dimeric complex ions, $[Cu^{II}(Phe-Gly)(T_3/rT_3)-H]^+$ and $[Cu^{II}(Phe-Gly)_2-H]^+$ by the loss of Phe-Gly and T_3/rT_3 , respectively. The difference in the branching ratios for these two dimeric complex ions is obvious, i.e., the R values, $[Cu^{II}(Phe-Gly)(T_3/rT_3)-H]^+ / [Cu^{II}(Phe-Gly)_2-H]^+$, are different for T_3 and rT_3 and that reflects the difference in the stabilities of the trimeric complex ions. The

Figure 1. ESI mass spectrum of a sample mixture containing T_3 (100 μ M), Phe-Gly (100 μ M) and manganese (II) chloride (25 μ M) in a 1:1 methanol/water solution. Major complex ions are designated.

corresponding R_{T_3} and R_{rT_3} values for the competitive dissociations of T_3 and rT_3 are 1.71 (Figure 2a) and 0.78 (Figure 2b), respectively. The ratio of these branching ratios represents the isomeric selectivity ($R_{iso} = R_{T_3}/R_{rT_3}$) and is 2.19 for this isomeric pair using Phe-Gly as a reference ligand and Cu^{II} as a central metal ion.

Isomeric Recognition of T_3/rT_3 Using Different Reference Ligands

The best combination of the reference ligand and metal ion should be chosen to obtain highly abundant signal for the complex ions comprising each isomer along with significant stereospecificity (R_{iso}). Various reference molecules, including amino acids, L-Phe, L-Trp, L-Tyr, L-Lys, L-Arg, L-Val, L-Ser, L-Asn, L-Leu, and L-Thr, substituted amino acids, N-Ace-L-Phe, N-Methy-L-

Trp, 3-Phe-L-Lactic acid, 3,4-Dihydroxy-L-Phe, *N,N*-Dimethyl-L-Phe, and 3,5-Diiodo-Tyr, and dipeptides, Phe-Phe, Phe-Ala, Phe-Arg, and Phe-Gly, were tried in terms of the complexing capability in presence of the divalent transition-metal ions, Co^{II} , Cu^{II} , Mn^{II} , Ni^{II} , and Zn^{II} to optimize the conditions for the isomeric discrimination. Table 1 shows the R_{iso} values for the isomers, T_3/rT_3 with the above reference ligands and metal ions.

The results in Table 1 indicate the discrimination was successful for thyroid hormone pairs, T_3/rT_3 , with the R_{iso} values that also demonstrate the strong dependence of the branching ratios on the structure of the ligands. The effect of the reference ligand and central ion on ease of the isomeric recognition was reflected in the R_{iso} values. Reference ligands were chosen such that they have potentiality to produce large steric interactions and for structural similarity to the analytes. Such simi-

Figure 2. MS/MS product ion spectra of (a) $[Cu^{II}(Phe-Gly)_2(T_3)-H]^+$ (m/z 1157.6); (b) $[Cu^{II}(Phe-Gly)_2(rT_3)-H]^+$ (m/z 1157.6). The CID activation level is chosen as 16%.

Table 1. R_{iso} (isomeric recognition of T_3/rT_3) values using different reference compounds and five transition metal ions

Analyte	Reference	R_{iso}				
		Co ^{II}	Cu ^{II}	Mn ^{II}	Ni ^{II}	Zn ^{II}
T_3/rT_3	L-Phe	0.48 ± 0.19	0.45 ± 0.26	0.58 ± 0.33	0.49 ± 0.25	0.66 ± 0.44
	L-Trp	0.56 ± 0.22	0.38 ± 0.12	0.56 ± 0.16	0.60 ± 0.62	1.55 ± 0.52
	L-Tyr	1.44 ± 0.32	2.36 ± 0.11	2.18 ± 0.19	0.56 ± 0.34	0.61 ± 0.48
	L-Lys	0.04 ± 0.21	0.07 ± 0.15	0.26 ± 0.23	0.14 ± 0.24	0.36 ± 0.25
	L-Arg	0.54 ± 0.39	0.65 ± 0.28	0.05 ± 0.17	1.97 ± 0.21	1.68 ± 0.33
	L-Val	0.59 ± 0.44	0.20 ± 0.19	1.84 ± 0.27	1.78 ± 0.38	0.72 ± 0.29
	L-Ser	0.35 ± 0.32	0.48 ± 0.36	0.25 ± 0.15	0.56 ± 0.29	0.44 ± 0.22
	L-Asn	0.64 ± 0.25	0.56 ± 0.28	0.38 ± 0.35	0.40 ± 0.37	0.78 ± 0.36
	L-Leu	1.75 ± 0.37	0.36 ± 0.15	0.47 ± 0.18	2.02 ± 0.34	0.83 ± 0.20
	L-Thr	0.70 ± 0.48	0.24 ± 0.18	1.23 ± 0.22	0.53 ± 0.25	0.87 ± 0.18
	N-Ace-L-Phe	2.42 ± 0.32	0.54 ± 0.27	0.43 ± 0.34	0.38 ± 0.22	0.90 ± 0.11
	N-methy-L-Trp	2.16 ± 0.16	1.33 ± 0.45	1.25 ± 0.39	2.03 ± 0.34	0.92 ± 0.22
	3-Phe-L-lactic acid	1.75 ± 0.28	2.16 ± 0.33	1.78 ± 0.28	2.05 ± 0.28	0.65 ± 0.44
	3,4-Dihydroxy-L-Phe	0.27 ± 0.11	2.10 ± 0.38	0.15 ± 0.25	0.22 ± 0.17	0.23 ± 0.36
	N,N-dimethy-L-Phe	0.22 ± 0.26	0.17 ± 0.25	0.44 ± 0.49	0.24 ± 0.25	0.63 ± 0.25
	3,5-Diiodo-L-Tyr	0.34 ± 0.34	0.69 ± 0.44	0.61 ± 0.32	1.64 ± 0.38	1.12 ± 0.34
	Phe-Phe	2.43 ± 0.21	2.61 ± 0.28	2.49 ± 0.12	0.18 ± 0.29	1.88 ± 0.36
	Phe-Ala	2.62 ± 0.07	2.14 ± 0.06	1.92 ± 0.52	1.98 ± 0.15	0.37 ± 0.19
	Phe-Gly	2.11 ± 0.19	2.19 ± 0.25	1.64 ± 0.65	1.74 ± 0.22	1.72 ± 0.28
	Phe-Arg	2.40 ± 0.23	0.10 ± 0.14	0.09 ± 0.25	2.56 ± 0.14	0.28 ± 0.21

larity allows the complexes to form easily, and it also allows accurate relative abundance ratios to be measured. The intrinsic interactions between the ligands and the metal ion i.e., metal–ligand and ligand–ligand, form the basis for the isomeric differentiation [38]. From Table 1, clearly substituted amino acids and dipeptides show a significant enhancement in the isomeric recognition than simple amino acids, which is due to the increased electron density in the π -system. In the case of substituted amino acids, it was proposed that the interactions between the π -electron of the aromatic ring and the metal cation promote large isomeric interactions by maximizing steric factors. The peptides were reported to have higher metal ion affinities than amino acids as their special coordination properties allow them to bind strongly at multiple sites to form metallopeptides [39, 40]. As a result, they further facilitate the π -cation interaction to increase isomeric discrimination.

In the present work, the choice of the metal ion also had a significant influence on the isomeric recognition of T_3/rT_3 . The dissociation behavior of the trimeric ions of interest changed with the selection of metal ion, and hence the isomeric recognition was affected. The different orbital configurations including d-orbital occupancy and splitting in transition-metal ions have great influence on the stability of the resulting complexes and prescribe the interactions between the ligands and the metal ions. To investigate the best transition-metal ions on the isomeric recognition, five first-row transition-metal ions, Co(II), Cu(II), Mn(II), Ni(II), and Zn(II), were examined. As shown in Table 1, Co(II) and Cu(II) are the best choices for the isomeric recognition of T_3/rT_3 . The higher isomeric differentiation using Co(II) and Cu(II) indicates that there are much stronger interactions between the two metal ions and the ligands than within the other combination.

Effect of Concentration on Isomeric Distinction of T_3/rT_3

The concentrations of the analyte and reference must not have any impact on the isomeric differentiation if this method should be generalized as one of the analytical methodologies. Therefore, a systematic study on the influence of the change in the concentration ratios of T_3 and a reference ligand Phe-Phe was carried out. The isomeric selectivity was investigated and the results are summarized in Table 2. This study shows that the isomeric selectivity is virtually independent of the relative concentration ratio of the analyte, T_3 , and the reference, Phe-Phe, in the solution. Therefore, clearly the addition of different concentrations of reference compounds to the unknown sample has little effect on the accuracy of measurements, a necessity for a practically useful method of isomeric analysis.

Effect of the Collision Energy on Isomeric Distinction of T_3/rT_3

The effect of the collision energy on the isomeric distinction of T_3/rT_3 was studied; it was observed that the

Table 2. Concentration effects of the analyte, T_3/rT_3 , reference ligand, Phe-Phe using Cu^{II} as a central metal ion

Analyte (T_3/rT_3):reference (Phe-Phe)	R_{iso} *
4:1	2.584
3:1	2.575
2:1	2.602
1:1	2.588
1:2	2.612
1:3	2.606
1:4	2.596

*Average of five measurements.

Table 3. Comparison of R_{iso} values using the ion trap and the QTOF mass spectrometers

Analyte	Metal	Reference	R_{iso}			
			Ion trap	RSD (%) [*]	QTOF	RSD (%) [*]
T_3/rT_3	Cu	L-Trp	0.38	2.85	0.31	0.62
	Mn	L-Trp	0.56	2.94	0.50	0.72
	Co	Phe-Ala	2.62	2.66	2.74	0.44
	Cu	Phe-Ala	2.14	2.78	2.27	0.58

^{*}RSD calculated for five determinations.

total fragment ion abundance increased with increasing collision energy but the effectiveness of the isomeric recognition decreased gradually (data is not shown here). So the increased total fragment ion abundance at increased collision energies is identified as an offsetting factor. This behavior is in good agreement with the expectation that the higher the collision energy, the higher the effective temperature for the activated complex ion [41, 42] and is a common phenomenon in cases where thermochemical determinations are made using the kinetic method.

Comparison of Isomeric Distinction of T_3/rT_3 Using an Ion Trap and QTOF Mass Spectrometers

The methodology described thus far was also carried out using a QTOF mass spectrometer, and the results were compared with the data taken using the ion-trap mass spectrometer. It was observed that there is no significant difference in the MS/MS spectra between the ion trap and QTOF. Table 3 presents the comparison results of the R_{iso} values of the ion trap with the QTOF, there is no appreciable difference in the R_{iso} values between the ion trap and QTOF mass spectrometers, either. Similar results were obtained in the reported method by Wu and Cooks [29]. However, there is a little improvement in isomeric discrimination between T_3 and rT_3 using QTOF mass spectrometer.

Reproducibility of R_{iso} values has been evaluated in terms of precision by calculating relative standard deviation (RSD) both in the ion trap and QTOF mass spectrometers. The results presented in Table 3 indicate that the reproducibility of R_{iso} values from QTOF mass spectrometer (RSD was less than 1%) was better than that of ion-trap mass spectrometer (RSD was up to 3%). In addition to this, QTOF mass spectrometer provided high mass accuracy, enhanced sensitivity, and fast acquisition in both the MS and MS/MS experiments. Therefore, QTOF mass spectrometer is a slightly better alternative to ion trap mass spectrometer for the isomeric or enantiomeric compositional analyses using the kinetic method.

Quantification of T_3/rT_3 in Mixtures

The relationship between the relative branching ratio (R) and the molar fraction of one isomer is given in eq 3.

$$\ln R = \frac{\Delta(\Delta G)}{RT_{\text{eff}}} \quad (3)$$

Here, R is the gas constant, T_{eff} is the effective temperature of the two activated complexes for the two competitive reactions of the activated trimers [43], and $\Delta(\Delta G)$ is the difference in the free energies for the reactions, which involves loss of either the analyte isomer or the reference molecule from the complex and is associated with a characteristic fragment ion branching ratio.

The relationship between R and α (isomeric molar fraction) is given in eq 4

$$\ln R = \frac{\Delta(\Delta G)_{rT_3}}{RT_{\text{eff}}} + \frac{\Delta(\Delta G)_{T_3} - \Delta(\Delta G)_{rT_3}}{RT_{\text{eff}}} \cdot \alpha \quad (4)$$

Equation 4 predicts a linear relationship between the natural logarithm of the branching ratio R and isomeric composition α on the applicability of the single ratio kinetic method relationship.

The branching ratio for the two competitive fragmentations of the trimeric complex ions depends strongly on the isomeric configurations of T_3/rT_3 and can be used to characterize the composition of an isomeric mixture. Quantification of the thyroid hormone isomers, T_3/rT_3 , was also achieved using Cu^{II} and Co^{II} as the central metal ions, and Phe and Phe-Ala as the reference ligands, respectively, in ion trap and QTOF mass spectrometers, respectively. The ratio R of the two fragmented dimeric complex ions was measured in a single tandem (MS/MS) spectrum as a function of the isomeric purity of the T_3/rT_3 and calibration curves for the quantification of T_3 in the mixture of T_3/rT_3 were constructed. Figure 3 shows the quantitative results for isomeric discrimination of T_3/rT_3 using Cu^{II} as the central metal ion and Phe as the reference ligands in the ion trap mass spectrometer. As shown in Figure 3, a linear relationship of $\ln R$ versus isomeric

Figure 3. Calibration curve for quantitative analysis of T_3/rT_3 isomers using phenylalanine as the reference and Cu^{II} as the central metal ion in ion trap mass spectrometer.

Figure 4. Calibration curve for quantitative analysis of T_3/rT_3 isomers using Phe-Ala as the reference and Co^{II} as the central metal ion using the QTOF mass spectrometer. The region where the molar fraction of T_3 is above 90% is shown in the inset.

molar fraction of T_3 was obtained (as indicated in eq 4) with a correlation coefficient (r^2) of 0.9912 in the mixture of T_3/rT_3 (0/100, 5/95, 10/90, 25/75, 50/50, 75/25, 90/10, 95/5, and 100/0). The linear relationship of ln R versus isomeric molar fraction coincides with the theoretical consideration as depicted in eq 4. Furthermore, the linearity was also evaluated in lower amounts using QTOF mass spectrometer by choosing Phe-Ala as the reference ligand and Co^{II} as the transition-metal ion. As illustrated in Figure 4, a linear relationship of ln R versus α values was confirmed with a correlation coefficient (r^2) of 0.9924, a calibration curve (Figure 4, inset) was constructed when the percentage of T_3 was above 90% (90%, 92%, 95%, 97%, 99%, 99.2%, 99.4%, 99.6%, 99.8%, and 100% of T_3), it shows an improvement in the linearity as judged by from the correlation coefficient obtained, 0.9977. By using these calibration curves recorded under optimized experimental conditions, the thyroid hormone isomers, T_3/rT_3 , can be readily quantified.

Conclusions

The kinetic method is successfully applied for the isomeric discrimination and quantification of the thyroid hormone isomers, T_3/rT_3 , using an ion trap and a QTOF mass spectrometer. The application of the kinetic method using the QTOF mass spectrometer is an alternative approach to the ion trap mass spectrometer. This method is based on the generation, selection, and fragmentation of specific metal-bounded trimeric complex ions using electrospray ionization-tandem mass spectrometry. Results indicate that there is a significant difference in the isomeric differentiation of thyroid

isomers using different reference ligands, and also transition-metal ions playing an important role in the present study. Further, it is also expected that the use of analyte/reference systems with similar metal ion affinity will allow the extension of the kinetic method for discriminating chemical and biologically isomeric compounds as well. The present study, based on the kinetic method, provides a useful addendum to the previously reported methods [37, 38] in the analysis of thyroid hormone isomers in that the analysis of mixtures was facilitated with the help of metal-bound complex formation. Finally, the current experiments used for discrimination of isomers are not restricted to any particular metal ion for complex formation, generalizing the methodology, and thus confirm the readiness of the kinetic method for differentiation and quantification of other chemical compounds of pharmaceutical and biologic importance.

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