

**Characterization of thyroid hormones
and their precursors in terms of site-
specific parameters**

Theses of doctoral (PhD) dissertation

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1. Introduction

Bio- and drug molecules undergo highly specific reactions in the body. These reactions can only take place if the reacting species are in the appropriate microscopic protonation and conformation form. The concentrations of the microspecies and the related equilibrium constants can be determined in the process of microspeciation. Individual microspecies are highly elusive chemical entities. They occur only in continuous interconversion with nanosecond average individual lifetime which makes them practically coexisting. Therefore the microspecies cannot separate for each other by any currently known separation techniques. However, they act in their individual form and have their own physico-chemical and biochemical properties. Generally the non-charged form plays a role in the membrane transport, while ionic microspecies such as zwitterion are crucial in the protein binding. Microscopic equilibrium or kinetic parameters are important elements for the understanding of biochemical and analytical processes at the molecular

level, and also, for the therapeutic influencing of biological malfunctions.

The most important thyroid hormones are thyroxine (T4), liothyronine (T3) and reverse liothyronine (rT3). They produced in thyroid gland by iodination and coupling reactions of tyrosine (Tyr). T4 is formed upon coupling of two diiodotyrosine (DIT) molecules while T3 and rT3 by coupling DIT with monoiodotyrosine (MIT). The structure of the thyroid hormones and their precursors are in Figure 1.

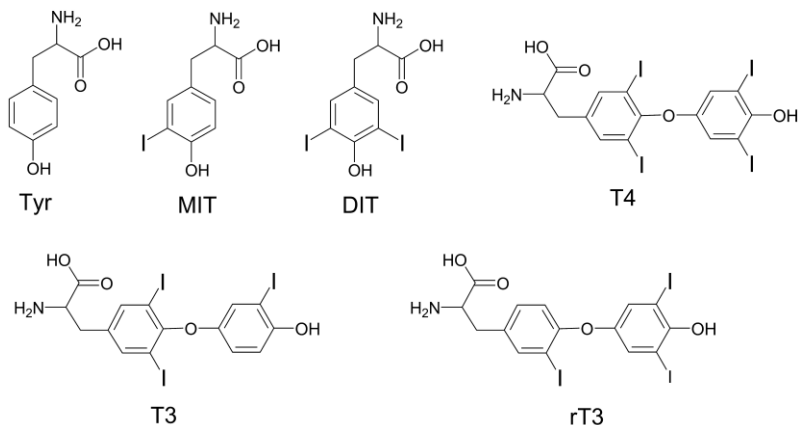


Figure 1. Constitutional formulas of the thyroid hormones and their precursors studied.

The physiological effects of thyroid hormones are well-known. They are crucial for the normal development of the central nervous system in infants, the skeletal growth in children and also for the normal function of multiple organ systems in adults. The site-specific physico-chemical properties of thyroid hormones have been underrepresented in the literature, in spite of the fact that ADME behavior, receptor binding and other biological processes depend on the acid–base properties.

In my PhD thesis the protonation macro- and microconstants as well as the site-specific partition coefficients of thyroid hormones and their biological precursors were determined.

The site-specific physico-chemical characterization of the thyroid hormones is of fundamental importance to understand their (patho)physiological behavior and also, to influence their therapeutic properties at the molecular level.

2. Objectives

The aim of this work was to investigate the possible relationship between the protonation states of thyroid hormones and their biosynthesis, receptor binding and membrane transport. Therefore our goal was the determination of site-specific physico-chemical parameters (basicity, lipophilicity and receptor affinity) of thyroid hormones and their precursors.

The protonation macroconstants were determined by ^1H NMR-pH titration using *in situ* indicator molecules. Microspeciation was carried out by ^1H NMR-pH and UV-pH titration techniques on the title compounds and their auxiliary derivatives of reduced complexity. In the knowledge of the protonation macro- and microconstants our purpose was to investigate how can the site-specific basicity influence the biosynthesis and protein binding of thyroid hormones.

For the investigation of receptor binding *in silico* modelling was planned. Thyroid hormone microspecies were docked to the thyroid hormone receptor isoforms

using Glide module in Schrödinger program. In this method the role of the ionization state of each basic site could be studied in the composite process of molecular recognition. Our results can quantitate at the molecular level how the ionization state and the charge distribution influence the protein binding.

For the determination of site-specific lipophilicity the partition of some of the individual microspecies was mimicked by model compounds of the closest possible similarity (using C-methyl and O-methyl derivative of the parent molecules), then correction factors were determined and introduced. Based on our plan, the site-specific partition coefficients can interpret the membrane transport of thyroid hormone at molecular level.

3. Methods

All the measurement were carried out at 25°C. The ionic strength was adjusted to 0.15 M using KCl.

¹H NMR pH titration Titrations were carried out at 600 MHz on a Varian Inova NMR spectrometer, equipped with a broadband inverse detection pulse field gradient probehead. The solution contained 5 % (v/v) D₂O, the reference was DSS. Water resonance signal was suppressed by double spin echo pulse. For pH – measurement a Metrohm 6.0234.110 combined glass electrode was used in the pH range 3 to 12.5. In order to avoid the glass electrode distortion at pH < 3, *in situ* NMR – pH indicator molecules, dichloroacetic acid and chloroacetic acid, were used. Titration curves were evaluated by the OPIUM and Microcal OriginPro 8.0 softwares.

UV-pH titration Titrations were carried out on a Jasco V-550 diode-array spectrometer and 1-cm cuvettes at 25.0 °C. The pH of the solutions was adjusted and

measured in the same way as in the NMR – pH titrations. Absorption spectra were recorded between 250 and 350 nm, and the wavelength was chosen so that absorptions of the acidic and basic forms are as different as possible (~298 nm). UV – pH datasets were fitted with Statistica 6.0.

Partition coefficient measurements by the stir-flask method The distribution coefficients of the parent molecules and their derivatives were calculated from the absorbance of the molecules before and after partitioning at several octanol-water phase ratios. Before the measurement octanol and buffer phases were intensively stirred. After separation of the equilibrated phases (24h sedimentation) the concentration of the solute was determined by UV spectrophotometry, between 250 and 350 nm the complete spectra were recorded.

In silico receptor binding During the docking analysis each of the thyroid hormone microspecies were constructed and docked them flexibly to thyroid hormone receptor beta and alfa isoforms using Glide module in XP

(extra precise) mode (Schrödinger program). The ligand–protein cocystal structures for the thyroid hormones with both receptor isoforms are available in the Brookhaven Protein Data Bank (<http://www.pdb.org>). For protein geometry optimization, the OPLS2005 force field while for ligand geometry optimization MMFF94s force field was used. The best three poses for each protonation microspecies of the compounds as evaluated by the Glide scoring function were taken. Our method was also validated.

4. Results, new scientific statements

1. All the compounds studied are triprotic ones, with phenolate, amino and carboxylate protonation sites. Being a triprotic molecule, the total number of microspecies and microconstants are 8 and 12, respectively, as shown in Figure 2. All the protonation macro- and microconstants were determined by a multi-modal spectroscopic-deductive methodology.

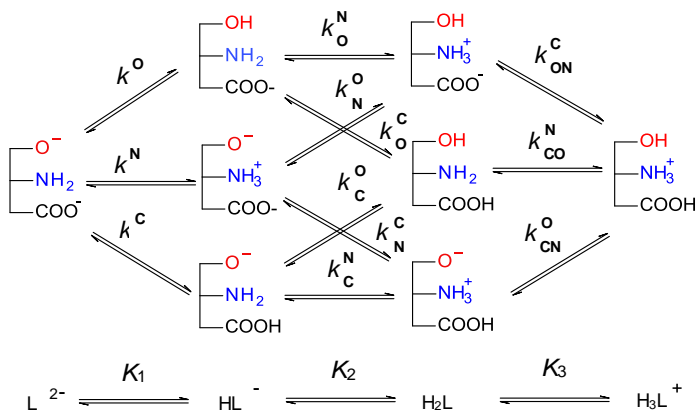


Figure 2. The micro- and macro-speciation schemes of the examined compounds, where microconstants with superscript O, N and C belong to the phenolate, amino and carboxylate site, respectively, and K_1 , K_2 and K_3 are stepwise macroconstants. The superscript on the microconstant indicate the protonating site, while the subscript (if any) stand for the site already protonated.

1. Based on the determined microconstant the effect of the different iodination patterns was investigated. The iodine atom reduces the basicity of every protonating group, mainly the phenolate. At compounds where one iodine atom occurs only near the phenolic hydroxyl

group (T3 and MIT) the diprotonated (OH , NH_3^+ , COO^-) zwitterionic form predominates at a wider pH interval ($2 < \text{pH} < 8$) relative to the analogous pH interval of T4, DIT and rT3 ($2 < \text{pH} < 6.5$). This also means that T4, DIT and rT3 exist mainly in monoprotated (O^- , NH_3^+ , COO^-) anionic form in the blood ($\text{pH} = 7.4$) while the phenolate site can mainly be found in its protonated form in T3 and MIT under identical circumstances.

2. The relationship between thyroid hormone biosynthesis and the ionization states of precursors was studied. As a result of our phenolate microconstants, the aromatic hydroxyl group of DIT exists in the deprotonated form (phenolate) in 93% at the pH of the blood plasma ($\text{pH} 7.40$). The analogous value of MIT is 14% only. The biosynthesis of the thyroid hormones is a complex, enzyme-catalyzed process, but it has long been generally accepted that anionic iodotyrosine residues are required for their biosynthesis. On the grounds of our phenolate microconstants 90% of the biochemically produced thyroid hormones needs to be T4 (formed by linking of two molecules of DIT), which is in good

agreement with 95% in physiological data. The microconstant of DIT shows that this molecule is largely ionized at the pH of the thyroid gland; providing plausible explanation why T4 is the main thyroid hormone, which is formed under physiological conditions. Therefore biosynthetic data can be interpreted in terms of site-specific basicities.

3. The receptor binding of thyroid hormone microspecies was investigated by molecular modeling study. Our results quantitate at the molecular level how the ionization state and the charge distribution influence the protein binding. The anionic form of the carboxyl group (i.e., carboxylate site) is essential for protein binding, whereas the protonated form of amino group worsens the binding. The protonation state of the phenolate plays a less important role in the receptor affinity. The combined results of docking and microspeciation studies show that microspecies with the highest concentration at the pH of blood are not the strongest binding ones.

4. The site-specific lipophilicity of iodinated amino acids was determined using distribution coefficients of model compounds of the closest possible similarity. The octanol-water partition coefficient is a good predictor of the membrane transport. Our data show that the iodinated aromatic ring system is the definitive structural element that fundamentally determines the lipophilicity of thyroid hormones, whereas the protonation state of the aliphatic part plays a role of secondary importance. On the other hand, the lipophilicity of the precursors is highly influenced by the protonation state due to the relative lack of overwhelmingly lipophilic moieties. Our determined $\log p$ values also show that contributions of the zwitterionic forms to the overall lipophilicity are much higher than those of the non-charged ones. The site-specific partition coefficient values of thyroid hormones show that the thyroid hormones are amphipathic molecules due to their duality – hydrophilic side chain and lipophilic aromatic ring. This property and the low distribution of the non-charged forms can explain why thyroid hormones cannot cross membranes by

passive diffusion. In fact, they even “get stuck” and become constituents of biological membranes in vertebrates. Relationships were also found between the species-specific lipophilicity and the membrane transport capabilities of thyroid hormones at the submolecular level.

5. Summary

In my PhD thesis surveys the site-specific physico-chemical parameters – basicity, lipophilicity and receptor affinity - and related biological functions of thyroid hormones (thyroxine, liothyronine and reverse liothyronine) and their biological precursors (tyrosine, monoiodotyrosine and diiodotyrosine). The biosynthesis, membrane transport and receptor binding of thyroid hormones can be interpreted in terms of site-specific parameters.

The protonation macroconstants of thyroid hormones and their precursors were determined by ^1H NMR-pH titration while the microconstants were determined by a

multi-modal spectroscopic-deductive methodology using auxiliary derivatives of reduced complexity. Our determined protonation microconstants show that the ionization state of phenolic hydroxyl group in precursors is crucial in the thyroid biosynthesis.

The role of the protonation state in the receptor binding was investigated by *in silico* docking method. Our results quantitate at the molecular level how the ionization stage and the charge distribution influence the protein binding. The combined results of docking and microspeciation studies show that microspecies of the highest concentration at the pH of blood are not the strongest binding ones.

The site-specific lipophilicity of our investigated molecules was determined with the measurement of distribution coefficients at different pH using carboxymethyl- and *O*-methyl-derivatives to mimic the partition of some of the individual microspecies. Correction factors were determined and introduced. The membrane transport of thyroid hormones can be well

interpreted with the site-specific lipophilicity. At physiological pH these biomolecules are strongly amphipathic due to the lipophilic aromatic rings and hydrophilic amino acid side chains which can well be the reason why thyroid hormones cannot cross membranes by passive diffusion.

The site-specific physico-chemical characterization of the thyroid hormones is of fundamental importance to understand their (patho)physiological behavior and also, to influence their therapeutic properties at the molecular level.

5. Publications

Papers of the thesis work

Tóth G, Hosztafi S, Kovács Zs, Noszál B (2012) The site-specific basicity of thyroid hormones and their precursors as regulators of their biological functions. *J Pharm Biomed Anal* 61:156-164.

Mazák K, **Tóth G**, Kökösi J, Noszál B (2012) Thyroxine lipophilicity is dominated by its zwitterionic microspecies. *Eur J Pharm Sci* 47:921-925.

Tóth G, Mazák K, Hosztafi S, Kökösi J, Noszál B (2013) Species-specific lipophilicity of thyroid hormones and their precursors in view of their membrane transport properties. *J Pharm Biomed Anal* 76: 112-118.

Tóth G, Baska F, Schretner A, Rácz A, Noszál B (2013) The site-specific basicity of thyroid hormones as regulators of their receptor binding: in silico investigation. *Eur Biophys J* 42: 721-730

Tóth G, Noszál B (2013) Thyroid hormones and their precursors I. Biochemical properties. *Acta Pharm Hung* 83: 35-45

Related papers

Rusu A, **Tóth G**, Szócs L, Kökösi J, Kraszni M, Gyéresi Á, Noszál B (2012) Triprotic site-specific acid-base equilibria and related properties of fluoroquinolone antibacterials. *J Pharm Biomed Anal* 66: 50-57

Neumayer G, Sohajda T, Darcsi A, **Tóth G**, Szente L, Noszál B, Béni Sz (2012) Chiral recognition of dapoxetine enantiomers with methylated- γ -cyclodextrin: A validated capillary electrophoresis method, *J Pharm Biomed Anal* 62: 42-47

Béni Sz, **Tóth G**, Noszál B, Hosztafi S (2012) Preparation of benzoate esters of morphine and its derivatives. *Monatsh Chem* 143: 1431-1440

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Váradí A, Lévai D, **Tóth G**, Horváth P, Noszál B, Hosztafi S (2013) Glucosides of morphine derivatives: synthesis and characterization. *Monatsh Chem* 144: 255-262

Váradí A, Hosztafi S, Rouzic LV, **Tóth G**, Urai Á, Noszál B, Pasternak WG, Grinnell GS, Majumdar S (2013) Novel 6 β -acylamino-morphinans with analgesic activity *Eur J Med Chem* 69C: 786-789

Rusu A, Hancu G, Völgyi G, **Tóth G**, Noszál B, Gyéresi Á (2013) Separation and determination of quinolone antibacterials by capillary electrophoresis *J Chrom Sci* in press DOI: 10.1093/chromsci/bmt10

Riethmüller E, Alberti Á, **Tóth G**, Béni Sz, Ortolano F, Kéry Á (2013) Characterisation of diarylheptanoid-and

flavonoid-type phenolics in *Corylus avellana* L. leaves and bark by HPLC/DAD–ESI/MS *Phytochem Anal* 24: 493-503

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Szabó B, Kállai N, **Tóth G**, Hetényi G, Zelkó R (2014) Correlation between the changes of the free volume and the drug release characteristics of cast and freeze dried buccal films, *J Pharm Biomed Anal* 89C: 83-87.