

Influence of thyroidectomy on thyroxine metabolism and turnover rate in rats

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Abstract

Little is known about the kinetics and metabolism of thyroid hormones in the hypothyroid state. To investigate these factors, we developed a reliable method for measurement of serum thyroxine (T₄), triiodothyronine (T₃), reverse-T₃ (rT₃) and stable isotope-labeled T₄ ([¹³C₉]T₄), using online solid-phase extraction liquid chromatography–mass spectrometry/mass spectrometry (online SPE LC–MS/MS). We measured supply and turnover rates of T₄ in thyroidectomized (Tx) rats using [¹³C₉]T₄ as a tracer. In rats, serum T₄, T₃ and rT₃ were decreased but not completely ablated after surgical Tx. Endogenous T₄ and T₃ levels in Tx rats were maintained at a constant low level throughout the experimental period. [¹³C₉]T₄ levels declined with a half-life of ~1.2 days after it was administered to Tx rats intravenously. These findings

strongly suggest that serum T₄ levels in Tx rats are maintained by T₄ supplied by extra-thyroidal tissues (e.g. secretion of extra-thyroidal storage, enhancement of enterohepatic recirculation, and production in extra-thyroidal tissues). Moreover, the turnover rate of T₄ in Tx rats was approximately twofold lower than in controls. This finding suggests that degradation of serum T₄ is repressed by Tx. In conclusion, serum T₄ is maintained at a constant low level by T₄ supply from extra-thyroidal tissues and repression of T₄ degradation in Tx rats. The powerful online SPE LC–MS/MS tool can be used to investigate thyroid hormones kinetics and metabolism, and thus has the potential to be used as a diagnostic tool and to investigate the pathogenesis of thyroid disease.

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Introduction

Thyroxine (T₄) is the main secretory product of thyroid follicular epithelial cells but has low biological activity. T₄ is converted to the more active triiodothyronine (T₃). Approximately 80% of the T₃ produced daily is formed by removal of one iodine atom from the outer ring of T₄ (outer-ring deiodination) in tissues outside the thyroid gland, such as the liver, kidney, muscle, and nervous system. The deiodination reaction is catalyzed by deiodinase enzymes. Two deiodinases, type 1 and type 2, catalyze the conversion of T₄ to T₃. Type 3 deiodinase catalyzes the conversion of T₄ to reverse T₃ (rT₃) and of T₃ to 3,3'-diiodothyronine (inner-ring deiodination) (Gereben *et al.* 2008); neither rT₃ nor 3,3'-diiodothyronine has biological activity (Bianco *et al.* 2002). In addition to deiodination, T₄ can be metabolized to its glucuronide forms by UDP-glucuronosyltransferases (UGTs). T₄ glucuronide is readily excreted in bile and is subsequently hydrolyzed by β-glucuronidases in the intestine, and may affect the enterohepatic circulation of T₄ (Visser 1994). It has been reported that UGT1A1 in the liver, UGT1A8 and

UGT1A10 in the intestine, and UGT1A7, UGT1A9, and UGT1A10 in the kidney mainly contribute to the T₄ glucuronidation activity in humans (Yamanaka *et al.* 2007). The change of these activities via inhibition and induction by administered drugs (Ohnhaus & Studer 1983, Isojarvi *et al.* 1992, Kiang *et al.* 2005) as well as genetic polymorphisms (Miners *et al.* 2002) may be a causal factor of interindividual differences in serum T₄ level.

Evans *et al.* (1960) reported that thyroidectomized (Tx) rats, whose growth had plateaued, could be made to grow again with a daily injection of large doses of inorganic iodide (3–5 mg/day). It has been subsequently shown that daily administration of 5 mg iodide to Tx rats partially restores their heart rate, metabolic rate, gonad and adrenal size and function, and pituitary acidophils (Evans *et al.* 1966). There are two potential mechanisms for this phenomenon: supply of thyroid hormones by extra-thyroidal tissues and repression of thyroid hormone degradation. Previously, T₃ generation from T₄ using radiolabeled T₄ and T₃ has been reported in detail (Silva & Matthews 1984a,b, Silva *et al.* 1984). However, little is known about the kinetics and metabolism of T₄ in the hypothyroid state.

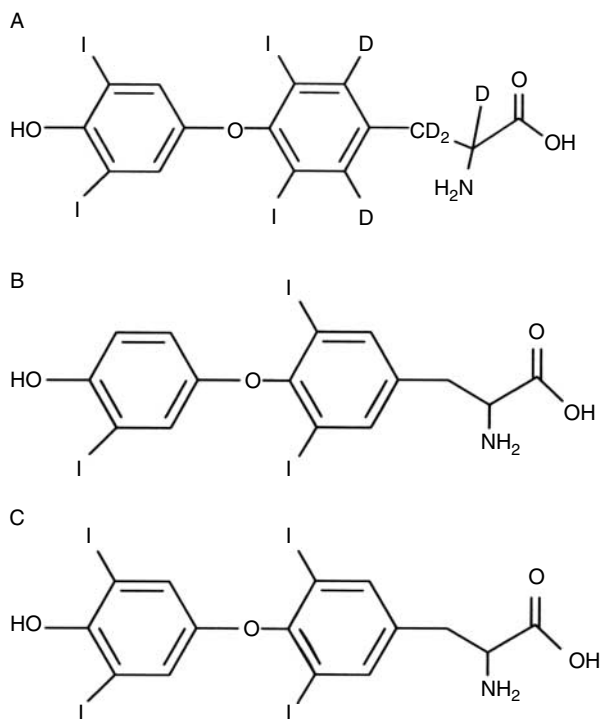


Figure 1 Chemical structures of [⁵H₂]T₄ (A), T₃ (B), and T₄ (C). T₄, thyroxine; T₃, triiodothyronine.

Although a sensitive and widely used immunoassay-based method exists for measuring serum thyroid hormones, there was a need for improved methods to investigate thyroid hormones kinetics and metabolism. Liquid chromatography–mass spectrometry/mass spectrometry (LC–MS/MS) is capable of simultaneously and accurately detecting specific chemicals in various specimens. One of the unique advantages of this method, which uses a stable isotope-labeled compound as a tracer, is that an endogenous compound and its exogenously administered labeled analog can be measured

separately using LC–MS/MS (Lin *et al.* 2010) or GC–MS/MS (Kasuya *et al.* 2005). Mass spectrometry of serum samples allows safe and precise *in vivo* measurement using stable isotopes, and such technology has contributed to a better understanding of metabolic diseases such as diabetes mellitus (Blaak *et al.* 2000, Hankard *et al.* 2000). Stable isotope-labeled tracers are commonly used to quantify the turnover rates of various metabolic intermediates and can provide information about physiological regulation. We have synthesized labeled T₄ by region-selective labeling with carbon 13. The biological tracer was T₄ with inner ring substitution with nine stable isotopes ([¹³C₉]T₄). Quantification methods for endogenous and exogenous (stable isotope-labeled) thyroid hormones in animal serum using online solid-phase extraction (SPE) LC–MS/MS were developed using [²H₅]T₄ as an internal standard. After *i.v.* administration of [¹³C₉]T₄ to Tx and control rats, serum samples containing endogenous and exogenous (labeled) thyroid hormones were analyzed using the double isotope dilution method. We investigated supply and turnover rates of T₄ in Tx and control rats by the stable isotope tracer method. The present study is the first to use stable isotope tracer injection in Tx rats.

The great potential of this technology can be extended to the use of T₄ labeled with ¹³C as a diagnostic tool for investigating pathogenesis of thyroid disease. The objectives of this study were to examine the kinetics and metabolism of T₄ in the hypothyroid state using Tx rats.

Materials and Methods

Chemicals

L-T₄-[L-tyrosine-²H₅]HCl (Fig. 1) was purchased from IsoSciences, LLC (King of Prussia, PA, USA). T₄, T₃ (Fig. 1), and rT₃ were purchased from Sigma–Aldrich Co. All other chemicals and reagents were of the highest analytical grade commercially available.

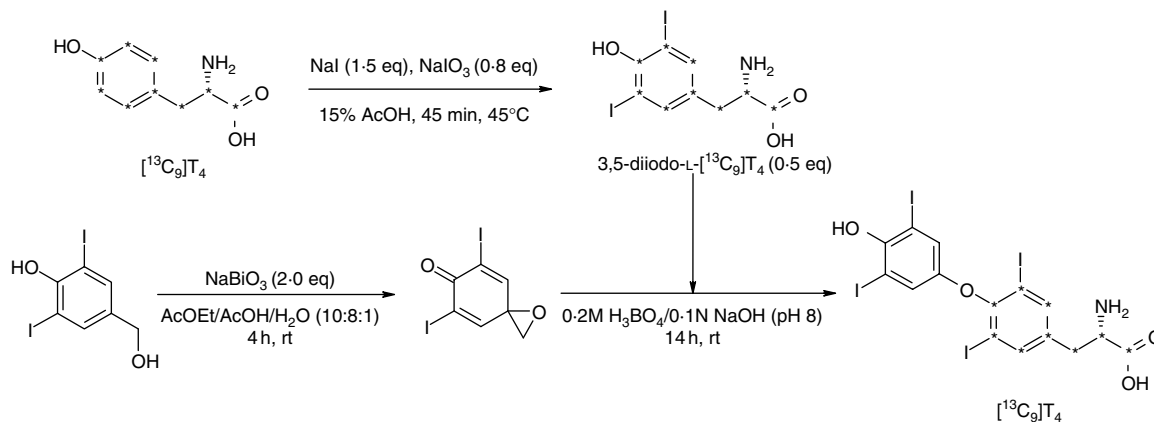


Figure 2 Chemical synthesis routes for [¹³C₉]T₄. rt, room temperature; asterisk denotes ¹³C.

Table 1 Liquid chromatography (LC) conditions used for the online solid-phase extraction LC–mass spectrometry (MS)/MS analysis

Time (min)	Flow rate (ml/min)	FCV-12AH valve position	Mobile phase		
			Pump A		Pump B
			A-1 (%)	A-2 (%)	B (%)
0:00	3:50	A	95	0	5
0:99	4:00	A	95	0	5
1:00	4:00	A	0	95	5
1:80	0:35	A	0	95	5
2:00	0:35	B	0	95	5
2:50	0:35	B	0	95	5
8:00	0:35	B	0	20	80
9:60	0:35	B	0	20	80
9:61	0:35	B	0	1	99
9:63	0:80	B	0	1	99
11:00	0:80	A	0	1	99
11:02	5:00	A	0	1	99
11:03	5:00	A	1	0	99
12:01	5:00	A	95	0	5
12:04	4:00	A	95	0	5
12:54	4:00	A	95	0	5

Valve position A, sample extraction or washing on the SPE column; valve position B, back-flush onto the analytical column and chromatographic separation; mobile phase A-1, 1.0 vol% formic acid; mobile phase A-2, 0.05 vol% acetic acid; mobile phase B, methanol; the pump C delivered 0.05 vol% acetic acid/methanol (95:5, v/v) at 0.35 ml/min to equilibrate the analytical column.

Chemical synthesis of [¹³C₉]T₄

[¹³C₉]T₄ was synthesized chemically from [¹³C₉] tyrosine using a modification of Salamonczyk's method (Salamonczyk *et al.* 1997; Fig. 2). The diiodo derivative of 1-oxaspiro [2,5]bicycloocta-4,7-dien-6-one reacted readily with [¹³C₉]3,5-diiodo-L-tyrosine. In turn, the diiodo derivative of 1-oxaspiro[2,5]bicycloocta-4,7-dien-6-one was prepared by sodium bismuthate oxidation of diiodinated *p*-hydroxybenzyl alcohol derivative.

Animals

Seven-week-old male Sprague–Dawley rats were obtained from Charles River Laboratories Japan (Kanagawa, Japan). Animals were fed a commercial diet (AIN-93G, Oriental Yeast Co., Tokyo, Japan) and distilled water *ad libitum*. The cages were located in a light (0800–2000 h lights on),

temperature (23 ± 5 °C) and humidity (60 ± 20%) controlled room.

The rats were allowed to acclimatize for 1 week before starting experiments.

All experimental procedures were approved by the Animal Research Committee of ASKA Pharmaceutical Co., in accordance with the Basic Guidelines for Proper Conduct of Animal Testing and Related Activities in the Research Institutions under the Jurisdiction of the Ministry of Health, Labour and Welfare of Japan.

Thyroidectomy

Rats were anesthetized with Isoflurane (Escain; Mylan, Pittsburgh, PA, USA). Thyroid glands were resected from the tracheal tube. After surgery, serum TSH rapidly increased and then achieved a steady state (~13.4 ± 3.6 ng/ml) after 7 days. Complete resection of the thyroid in the Tx rats

Table 2 Precision and accuracy of the intra-day assay. Data are expressed as the mean values ± s.d. (n=5). Figures in parentheses are expressed as coefficient of variance (%)

Concentration (ng/ml)	% Accuracy (mean ± s.d.)			
	T ₄	T ₃	rT ₃	[¹³ C ₉]T ₄
0.1	103.0 ± 4.0 (3.9)	95.0 ± 4.0 (4.2)	96.0 ± 2.0 (2.1)	101.0 ± 6.0 (5.9)
0.2	105.5 ± 5.5 (5.2)	94.0 ± 5.5 (5.9)	93.0 ± 4.5 (4.8)	104.0 ± 4.5 (4.3)
4	104.4 ± 2.0 (1.9)	99.4 ± 2.8 (2.8)	98.0 ± 2.6 (2.7)	104.2 ± 1.9 (1.8)
80	97.6 ± 2.0 (2.1)	95.1 ± 2.1 (2.2)	97.6 ± 1.4 (1.4)	98.2 ± 1.3 (1.4)

T₄, thyroxine; T₃, triiodothyronine; rT₃, reverse-triiodothyronine.

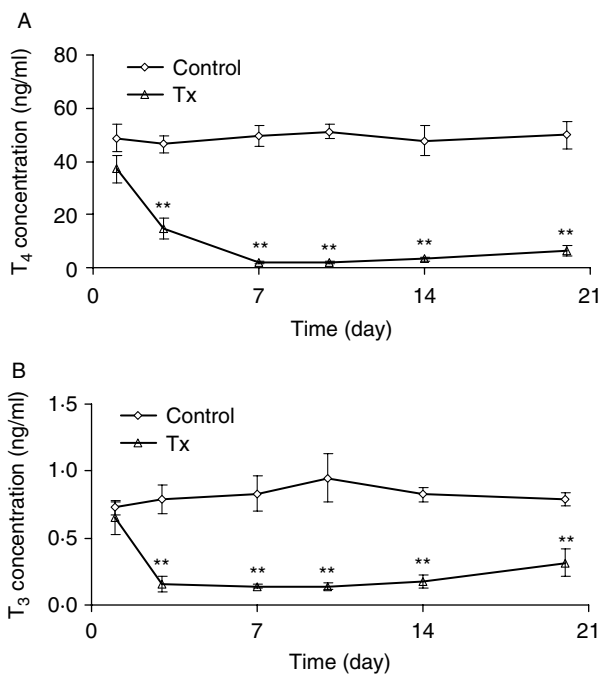


Figure 3 Changes of serum thyroid hormones concentration in control and Tx rats (A) T₄ and (B) T₃. Tx, thyroidectomy; T₄, thyroxine; T₃, triiodothyronine. All data represent the mean \pm s.d. of five animals. ***P* < 0.01.

was confirmed at the end of the experiment by macroscopic observation at necropsy. In addition, serial sections from tracheal tubes (area of thyroid glands in Tx rats) were reviewed by a pathologist and hypertrophy or hyperplasia of follicular epithelial cells were not observed.

Experiment 1

Ten rats were divided into two groups (*n* = 5), the Tx and control groups. Serum samples were collected for 3 weeks. In control rats, serum TSH was maintained at \sim 2.2 ng/ml over the experimental period. All samples were obtained between 0900 and 1100 h to minimize fluctuations in thyroid hormones (Jeremiah *et al.* 1990).

Experiment 2

Five Tx rats were allowed 4 weeks to achieve steady-state plasma thyroid hormone concentrations after Tx. [¹³C₉]T₄ was administered intravenously to Tx and control rats at a dose of 1.5 μ g/500 μ l/kg (*n* = 5; Nguyen *et al.* 1993). Serum samples were obtained 5 min after [¹³C₉]T₄ administration, then at every 24 h for 2 weeks, and every 48 h for an additional week. All samples were kept frozen at -20° C until analysis.

Online SPE LC-MS/MS

The HPLC system (Shimadzu, Kyoto, Japan) consisted of a SCL-10Avp system controller, three LC-20AD pumps

connected to an FCV-11AL reservoir selection valve, an SIL-HTc autosampler, and a CTO-20A column oven equipped with an FCV-12AH six-port switching valve for online extraction. The SPE column was a Shim pack MAYI-ODS, 2.0 mm I.D. \times 10 mm, 50 μ m (Shimadzu) maintained at 45 $^{\circ}$ C. The analytical column was a Synergi Polar-RP 80A, 2.0 mm I.D. \times 50 mm, 4 μ m (Phenomenex, Utrecht, The Netherlands) maintained at 45 $^{\circ}$ C. The mobile phases were 1.0 vol% formic acid (A-1), 0.05 vol% acetic acid (A-2), methanol (B), and 0.05 vol% acetic acid/methanol (95:5, v/v), respectively. The LC conditions are listed in Table 1. An API5000 triple-quadrupole mass spectrometer (AB SCIEX, Foster City, CA, USA) equipped with a TurboIonSpray source was operated in the positive ion multiple reaction monitoring (MRM) mode to perform the analysis. The transitions to monitor were selected for each compound: *m/z* 777.70 > 731.50 for [¹³C₉]T₄, *m/z* 651.77 > 605.90 for [¹³C₉]T₃, *m/z* 651.77 > 507.73 for [¹³C₉]rT₃, *m/z* 786.71 > 739.90 for [¹³C₉]T₄, and *m/z* 782.75 > 735.90 for [²H₅]T₄.

Sample preparation

A 20 μ l aliquot of rat serum was mixed with 60 μ l of internal standard solution (2 ng/ml of [²H₅]T₄). After vortex mixing, the mixture was centrifuged (16 000 *g* for 5 min at 10 $^{\circ}$ C). Then, 40 μ l of 0.1 vol% formic acid was added to the mixture and vortex mixed. After centrifugation (16 000 *g* for 5 min at 10 $^{\circ}$ C), 80 μ l of the supernatant was injected into the LC-MS/MS system. Ten calibration standards ranging from

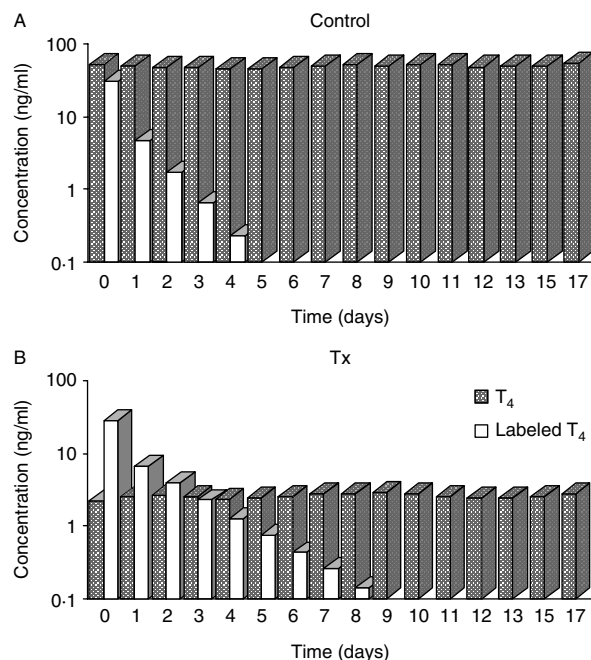


Figure 4 Serum concentrations of T₄ and [¹³C₉]T₄ after an i.v. administration of [¹³C₉]T₄ to control (A) and Tx (B) rats. Tx, thyroidectomy; T₄, thyroxine.

Table 3 Supply rates and turnover of serum thyroxine (T₄) in control and Tx rats. Data are expressed as the mean values ±s.d. (n=5)

Group	kel (day ⁻¹)	t _{1/2} (day)	Vd (ml)	SR (ng/day)
Control	1.19 ± 0.10	0.59 ± 0.05	29.8 ± 4.8	1723.4 ± 87.7
Tx	0.60 ± 0.02	1.16 ± 0.05	26.4 ± 3.8	39.9 ± 5.9

kel, turnover rate; t_{1/2}, half-life; Vd, distribution volume; SR, supply rate; Tx, thyroidectomy.

0.1 to 100 ng/ml were prepared from charcoal-stripped rat serum. Similarly, four quality control samples were prepared with concentrations of 0.1, 0.2, 4 and 80 ng/ml. These levels were chosen to demonstrate the precision and accuracy of the method at the lower limit of quantitation (LLOQ) as well as at low, medium and high concentrations on the calibration curve.

Data analysis

The turnover rate (kel) was determined by plotting serum concentration of [¹³C₉]T₄ against time in a semilog plot,

$$C = C_0 \times e^{-kel \times t}$$

where C, serum concentration of [¹³C₉]T₄ at time (t); C₀, serum concentration of [¹³C₉]T₄ at time zero; t, time (day) and kel, turnover rate. The half-life (t_{1/2}) of [¹³C₉]T₄ was calculated as t_{1/2} = 0.693/kel (Kasuya *et al.* 2005).

The supply rate (SR) of T₄ was calculated as follows:

$$SR = C_{ss} \times Vd \times kel,$$

where C_{ss}, steady state serum T₄ concentration; Vd, volume of distribution and kel, turnover rate. Vd was calculated by the equation

$$Vd = \frac{X_0}{C_0},$$

where X₀, dose of [¹³C₉]T₄ administered and C₀, serum concentration of [¹³C₉]T₄ at time zero.

Statistical analysis

The data are expressed as the mean ±s.d. Statistical significance was calculated by unpaired Student's *t*-test. A *P* value <0.01 was considered significant.

Results

Online SPE LC-MS/MS

For T₄, T₃, rT₃ and [¹³C₉]T₄, intra-day precision and accuracy were evaluated by analysis of the 0.1, 0.2, 4 and 80 ng/ml quality control samples. The results are summarized in Table 2. Overall, the intra-day precision was <5.9% for each analyte at each quality control level, and the accuracy

was between 93.0 and 105.5%. Therefore, the LLOQ of each analyte was established at 0.1 ng/ml.

These methods represent a specific and reliable technique for measurement of endogenous and stable isotopically labeled thyroid hormones in serum with a high degree of precision and accuracy.

Serum concentrations of endogenous thyroid hormones

After Tx, T₄ and T₃ levels decreased rapidly and achieved a steady state 7 days after treatment (Fig. 3). The steady-state T₄ and T₃ concentrations were ~2 and 0.1 ng/ml, respectively. The rT₃ concentration also rapidly decreased in Tx rats, dropping a level lower than the quantification limit (0.1 ng/ml). In control rats, T₄, T₃, and rT₃ concentrations were maintained at ~50, 0.82, and 0.76 ng/ml, respectively, throughout the experimental period.

Supply and turnover rates

After a single i.v. administration of 1.5 µg/kg [¹³C₉]T₄ to Tx and control rats, [¹³C₉]T₄ level was maintained at ~2.6 and 50 ng/ml, respectively, throughout the experimental period (Fig. 4). In contrast, [¹³C₉]T₄ levels in both Tx and control rats rapidly decreased after administration of [¹³C₉]T₄, and were below the threshold for quantification by 9 days after treatment.

The turnover rate of serum [¹³C₉]T₄ in Tx rats was 0.60 ± 0.02 day⁻¹, approximately twofold slower than in controls (1.19 ± 0.10 day⁻¹), and the t_{1/2} was ~1.2 days (Table 3 and Fig. 5).

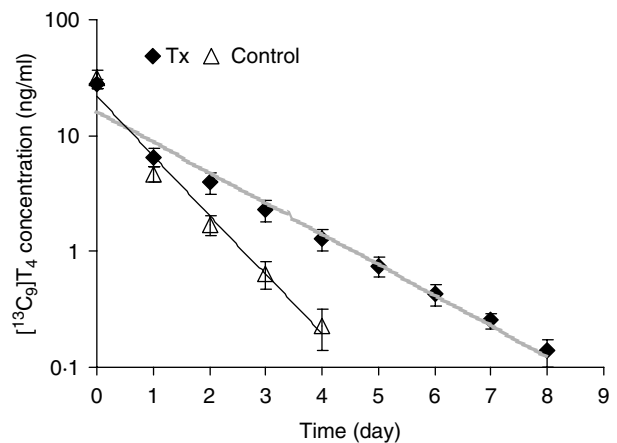


Figure 5 Turnover of serum T₄ in control and Tx rats. Tx, thyroidectomy; T₄, thyroxine.

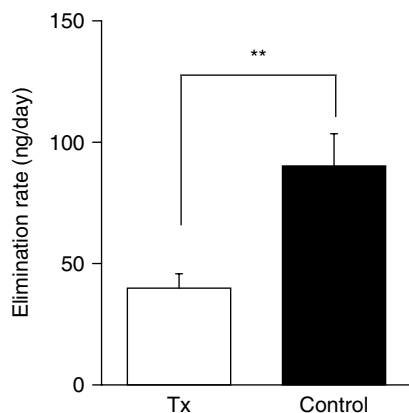


Figure 6 Suppression degree of elimination of serum T_4 in Tx rats. Tx, thyroidectomy; T_4 , thyroxine. All data represent the mean \pm s.d. of five animals. $**P < 0.01$.

The T_4 SR was 39.9 ± 5.9 ng/day (2.3% of that in control rats). The T_4 elimination rate in controls (ER_{cnt}), which was calculated using the kinetics parameters of control rats, was ~ 2.3 -fold faster than ER_{tx} (Fig. 6 and Table 4).

Discussion

Concentrations of thyroid hormones in the plasma are strictly controlled by TSH, which is subject to negative feedback regulation by thyroid hormones. The major form of thyroid hormone secreted from the thyroid gland is T_4 . In hypothyroidism, T_4 is orally administered to maintain plasma thyroid hormones at a normal level. Although thyroid hormone levels are typically utilized to diagnose thyroid disease, thyroid hormone kinetics may also provide valuable information about the causes of thyroid hormone disorders.

To investigate the turnover rate of T_4 and changes in T_4 metabolism resulting from hypothyroidism, we synthesized a stable isotope-labeled compound and established an online SPE LC-MS/MS method. Conventional HPLC sample preparation is still labor-intensive and time-consuming, and requires many steps. Protein precipitation (PPT) is considered to be the fastest and simplest approach for extraction of hydrophilic and hydrophobic compounds. However, the extraction ratio, reproducibility, and selectivity of PPT are

Table 4 Suppression degree of elimination of serum thyroxine (T_4) in Tx rats. Data are expressed as the mean values \pm s.d. ($n=5$)

ER (ng/day)		S (ng/day)
ER _{tx}	ER _{cnt}	
39.9 ± 5.9	90.2 ± 13.3	50.3

Tx, thyroidectomy; ER, elimination rate; ER, supply rate (SR) (in steady state); ER_{tx}, SR for Tx rats. $ER_{cnt} = C_{ss} \times V_d \times kel$; where C_{ss} is 2.6 ng/ml for Tx rats, kel and V_d are each value for control rats. S, suppression degree for elimination of T_4 , $S = ER_{cnt} - ER_{tx}$.

not sufficient to determine the concentrations of each analyte. Thus, we developed an online SPE assay using automated sample preparation instead of additional manual sample cleanup. The Shim Pak MAYI-ODS column was chosen for this assay because it can accommodate a large injection volume of PPT sample. Chromatographic focusing in the gradient elution produced very narrow peaks despite the large injection volume of 80 μ l. Completely resolved peaks were obtained for T_4 , T_3 , rT_3 and [$^{13}C_9$] T_4 . Thorough cleanup by online SPE with high-efficiency LC separation and specific MRM detection conferred high selectivity. We used a novel combination of stable isotope-labeled tracer and online SPE LC-MS/MS to study thyroid hormone supply and turnover rates in Tx rats. Serum thyroid hormones (T_4 , T_3 and rT_3) decreased as a result of surgical Tx, but were not completely abolished. At the steady state, hormone SRs are assumed to be in equilibrium with the elimination rates. Thus, we are able to estimate the elimination rate of labeled T_4 . In Tx rats, although endogenous T_4 , T_3 and rT_3 maintained at a constant level throughout the experimental period, the [$^{13}C_9$] T_4 level declined with a half-life of about 1.2 days. These findings indicate that serum T_4 in Tx rats is maintained at a constant low level due to supply of T_4 in extra-thyroidal tissues (e.g. secretion of extra-thyroidal storage, enhancement of enterohepatic recirculation and production in extra-thyroidal tissues) (SR: 39.9 ± 5.9 ng/day). A few previous reports in the literature have also suggested an unknown extrathyroidal source of thyroid hormone (Evans *et al.* 1966, Taurog & Evans 1967, Obregon *et al.* 1981). In recent years, Meischl *et al.* (2008) reported that cardiomyocytes can produce thyroid hormone under specific experimental conditions. More recently, expression of transporters that act through thyroid hormone secretion (Visser *et al.* 2008, Cosmo *et al.* 2010) and I^- intake (de Carvalho & Quick 2011) have been reported in extrathyroidal organs. However, to the best of our knowledge, these studies have never been followed up to quantitatively elucidate the extrathyroidal sources of T_4 . Moreover, in Tx rats, the turnover rate of T_4 was approximately twofold slower than in controls. This suggests that disappearance of serum T_4 is suppressed by Tx, and the magnitude of the suppression was estimated to be 50.3 ng/day.

Tx may upregulate deiodination from T_4 to T_3 to mitigate hypothyroidism. Conversely, T_4 glucuronidation and sulfation may be inhibited by Tx. Changes in urinary excretion of T_4 and deiodination of T_4 to rT_3 following Tx warrant further investigation. In addition to these changes in T_4 metabolism, Vranckx *et al.* (1994) reported that Tx induces expression of T_4 -binding globulin (TBG), and suggested that regulation of TBG may thus be important for thyroid hormone homeostasis. Further study is required to completely determine the mechanisms of thyroid hormone homeostasis after Tx.

In conclusion, we have synthesized stable isotope-labeled T_4 and have established a reliable and simple online SPE LC-MS/MS method. We have also characterized thyroid hormone kinetics in Tx rats using a stable isotope tracer. In Tx rats, serum T_4 was maintained at a constant low level due to thyroid

hormone supply from extra-thyroidal tissues and decreased degradation of T₄. The powerful online SPE LC–MS/MS tool can be used to investigate thyroid hormones kinetics and metabolism, and thus has the potential to be used as a diagnostic tool and to investigate the pathogenesis of thyroid disease. Our methods can be applied to future kinetic and metabolic studies of T₄ in the hyperthyroid and hypothyroid states in humans.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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