Journal of Chromatography B, 878 (2010) 1725–1730

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

A method for the analysis of six thyroid hormones in thyroid gland by liquid chromatography–tandem mass spectrometry

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ARTICLE INFO

Article history:
Received 10 February 2010
Accepted 20 April 2010
Available online 28 April 2010

Keywords:
Thyroid hormones
Thyroid gland
Perchlorate
Iodide deficiency
LC–MS/MS

ABSTRACT

Perchlorate can competitively inhibit iodide uptake by the thyroid gland (TG) via the sodium/iodide symporter, consequently reducing the production of thyroid hormones (THs). Until recently, the effects of perchlorate on TH homeostasis are being examined through measurement of serum levels of TH, by immunoassay (IA)-based methods. IA methods are fast, but for TH analysis, they are compromised by the lack of adequate specificity. Therefore, selective and sensitive methods for the analysis of THs in TG are needed, for assessment of the effects of perchlorate on TH homeostasis. In this study, we developed a method for the analysis of six THs: L-thyroxine (T_4), 3,3′,5-triiodo-L-thyronine (T_3), 3,3′,5′-triiodo-L-thyronine (rT_3), 3,5-diiodo-L-thyronine (3,5-T_2), 3,3′-diiodo-L-thyronine (3,3′-T_2), and 3-iodo-L-thyronine (3-T_1) in TG, using liquid chromatography (LC)–tandem mass spectrometry (MS/MS). TGs used in this study were from rats that had been placed on either iodide-deficient diet or iodide-sufficient diet, and that had either been provided with perchlorate in drinking water (10 mg/kg/day) or control water. TGs were extracted by pronase digestion and then analyzed by LC–MS/MS. The instrumental calibration range for each TH ranged from 1 to 200 ng/ml and showed a high linearity ($r > 0.99$). The method quantification limits (LOQs) were determined to be 0.25 ng/mg TG for 3-T_1; 0.33 ng/mg TG for 3,3′- and 3,5-T_2; and 0.52 ng/mg TG for rT_3, T_3, and T_4. Rats were placed on an iodide-deficient or -sufficient diet for 2.5 months, and for the last 2 weeks of that period were provided either perchlorate (10 mg/kg/day) in drinking water or control water. Iodide deficiency and perchlorate administration both reduced TG stores of rT_3, T_3, and T_4. In iodide-deficient rats, perchlorate exacerbated the reduction in levels of THs in TG. With the advances in analytical methodology, the use of LC–MS/MS for measurement of hormone levels in TG will allow more comprehensive evaluations of the hypothalamic-pituitary–thyroid axis.
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1. Introduction

It is well known that thyroid hormones (THs) are essential for regulation of biological processes such as growth, metabolism, neurodevelopment, and protein synthesis. L-Thyroxine (T_4), synthesized in the thyroid gland (TG), is carried by the bloodstream to the target tissues and is biotransformed by deiodinase type I or II (D1, D2) into the active hormone, 3,3′,5-triiodo-L-thyronine (T_3) [1]. It is estimated that about 20% of T_3 produced daily is formed in the TG and is circulated by the bloodstream. The remainder is produced from metabolism of T_4. Thus, the cytoplasmic pool of T_3 in the target tissue includes both T_3 from the plasma and T_3 generated by the actions of D1 and D2 [1]. T_4 and T_3 measurements in the blood can supply clinically important information on TH homeostasis. Thus far, immunoassay (IA) methods have been commonly used for T_4 and T_3 determinations, but the specificity of the antibodies used in these assays limits selectivity. The assay results for T_4 and T_3 reported by the College of America Pathologists varied widely, depending on the antibodies used [2,3]. Furthermore, it has been suggested that the measurement of T_4 and T_3 using IA methods in samples from pregnant women can lead to false diagnosis of maternal thyroid levels, because of complex changes in circulating steroid hormones and in thyroid-binding globulin (TBG) that occur during pregnancy [3]. In recent years, analytical methods using gas chromatography–mass spectrometry (GC–MS) [4], liquid chromatography–mass spectrometry (LC–MS) [5], and tandem mass spectrometry (LC–MS/MS) [2,3,6] have been developed to measure T_4 and T_3 in human and animal serum or plasma. The LC–MS/MS methods are, in general, shown to be more accurate and reliable than the IA methods [3,7].

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doi:10.1016/j.jchromb.2010.04.031

Disruption of normal TH function following exposure to xenobiotic chemicals is an issue of public health concern. The presence of perchlorate anion (ClO_4^-) in US drinking water supplies has raised concern about potential adverse thyroid-related health effects [8–10]. In addition to drinking water, diet is a source of human exposure: milk [11], vegetables [12,13], fruits [14], seaweeds [15], fish [16], and dietary supplements and flavor enhancers [17] have all been shown to contain perchlorate. Indeed, perchlorate has been detected in human milk [11], blood [18], urine [19,20], and saliva [21]. The US National Academy of Sciences (NAS) recommended an oral reference dose (RfD) of 0.0007 mg/kg/day; this RfD was adopted by the US Environmental Protection Agency (EPA) in February 2005 [10,22], based on the no-observed-effect level (NOEL) estimated in a dose-response study for perchlorate inhibition of thyroidal iodide uptake in humans [23].

Perchlorate can competitively inhibit iodide uptake by the TG via the sodium/iodide (Na^+/I^-) symporter (NIS), reducing the downstream synthesis and secretion of T_4 and T_3 [24]. An *in vitro* study, in which human NIS was transfected into a Chinese hamster ovary cell line, found that the relative potency of perchlorate, in inhibition of $^{125}I^-$ uptake, was 30 times higher than the potency of iodide [25]. Because perchlorate is actively transported across cell membranes by the NIS, this anion can cross lactating breast tissue, thereby exposing breastfed neonates [26]. A recent study reported that perchlorate exposure in women with low iodide intake caused changes in TH levels [19]. In experimental studies using rats, perchlorate exposure decreased serum levels of T_4 and T_3 and increased thyroid stimulating hormone (TSH) levels [27,28]. High doses of perchlorate in rodents during fetal development resulted in permanent detriment to the brain function of the pup [29]. Oral administration of perchlorate to rats via drinking water increased TG weight and induced accompanying histopathological changes in the TG at a dose level of 10 mg/kg/day, but changes were not seen in other tissues and organs; this finding confirmed that the TG is the principal target organ for perchlorate toxicity, in the rat [27]. Nevertheless, documentation of TH perturbations in the TG following perchlorate exposure is not available. Given that D1 and D2 regulate the activity of THs via removal of specific iodine moieties from T_4 and T_3 [1], measurement of not only T_4 and T_3 but also inactive 3,3′,5′-triiodo-L-thyronine (rT_3) and the deiodinated forms, diiodo-L-thyronine (T_2) and monoiodo-L-thyronine (T_1), in the TG is important, for assessment of the overall effects of perchlorate on TH homeostasis.

In this study, we have developed a method for the determination of six THs, namely, T_4, T_3, rT_3, 3,3′-T_2, 3,5-T_2, and 3-T_1, in the TG, using methods of pronase digestion and LC–MS/MS detection. TGs from rats exposed to perchlorate and from unexposed control rats were used in the development of the analytical method. Using this method, we also investigated any differences in TH levels in the TG from rats exposed to perchlorate in drinking water, and fed either an iodide-deficient diet or an iodide-sufficient diet. The toxicological findings of these studies will be reported elsewhere.

2. Materials and methods

2.1. Chemicals and reagents

TH standards were purchased from several vendors: T_4 (purity: 98%) and T_3 (purity: 95%) from Sigma–Aldrich (St. Louis, MO), rT_3 (purity: 98%) 3,3′-T_2 (purity: 98%) and 3,5-T_2 (purity: 98%) from the United States Biological Inc. (Swampscott, MA), and 3-T_1 (purity: 95%) from Toronto Research Chemicals Inc. (North York, ON, Canada). Ammonium perchlorate (purity: 99.8%) was obtained from Sigma–Aldrich. Methanol (MeOH) was purchased from Mallinckrodt Baker Inc. (Phillipsburg, NJ) and ammonium hydroxide was from Mallinckrodt Inc. (Hazelwood, MO). All other chemicals were of analytical grade and were purchased from Sigma–Aldrich. Deionized (DI) water was generated with a NANOpure Diamond ultrapure water system (Barnstead International, Dubuque, IA), and had a resistance of 18.2 MΩ cm.

2.2. Standard solution

A stock solution of each TH standard was prepared at 1 mg/ml using 40% ammonium hydroxide (v/v) in MeOH; solutions were stored at −20 °C. The calibration standards, ranging from 1 to 200 ng/ml, were prepared from the stock solution through dilution with MeOH, every time a batch of samples ($n = 12$) was injected into the LC–MS/MS instrument.

2.3. Experimental design and sample collection

Male Sprague–Dawley rats (body weights between 100 and 120 g) were obtained from Charles River Laboratories Inc. (Wilmington, MA). The animals used in this study were handled in accordance with the guidelines of the University of Georgia Institutional Animal Care and Use Committee (IACUC), AUP number A2006-10079. Rats ($n = 48$) were separated into two groups and were fed either with regular diet (AIN76A-D10003) or iodide-deficient diet (AIN76A-D18503R), both from Research Diet Inc. (New Brunswick, NJ). The regular and iodide-deficient chows respectively contained 0.270 μg and 0.025 μg iodide/g chow, or about 5.4 μg and 0.5 μg iodide intake per day [30], and were fed for 2.5 months. For the last 2 weeks of that period, the half of the rats in each group was provided either drinking water without or with perchlorate at 10 mg/kg/day. The rats were thus categorized into four exposure groups: group 1 iodide-deficient diet + perchlorate-treated water (IDP); group 2 iodide-deficient diet + water (IDW); group 3 regular diet + perchlorate-treated water (RP); group 4 regular diet + water (RW). After 24 h or 14 days of perchlorate treatment, 6 rats from each group were sacrificed by CO_2 gas and whole TGs ($n = 48$) were harvested.

In this experimental design, other TGs were harvested for genomic studies. Serum samples were also collected, for the analysis of THs and TSH by an IA method. Some rats were housed in metabolism cages, to enable collection of urine for the analysis of iodide and perchlorate. These aspects of the study and toxicological findings will be reported elsewhere.

Twelve additional TG samples were collected from rats provided with regular research diet (900 μg iodide/g chow, Research Diet, Inc.) and plain water for refinement of the extraction procedure for THs from the TG, by the pronase-digestion method described below. All of the samples were stored at −20 °C until analysis.

2.4. Sample preparation

TGs were thawed at 4 °C overnight. Because it was necessary to hydrolyze the TGs, to dissociate THs from thyroglobulin, we used a pronase (protease from *Streptomyces griseus*; Sigma–Aldrich). The digestion method for the extraction of THs has been reported earlier [31]. Similar procedures have been used to hydrolyze rat TGs [32] and whole-body homogenates from fish and tadpoles [33]. First, we tested the digestion efficiency of pronase, by assaying various levels of the enzyme. TG samples of approximately 5 mg ($n = 5$) or 7 mg ($n = 5$) were placed in 1.5-ml snap-cap microcentrifuge tubes. Pronase (≥4 units/mg solid) was dissolved in the digestion buffer, which consisted of 153.65 mg L-glutathione, 42.55 mg N-phenylthiourea, 1.21 g tris(hydroxymethyl)aminomethane, and 1 ml Triton X-100 in 100 ml DI water. Pronase buffer (0.35 ml) was added to each tube. The amount of pronase per tube was set to be 1.0, 2.5, 5.0, 7.5, or 10-times the weight of the TG sample (either

Table 1
MS/MS parameters optimized for analysis of thyroid hormones from thyroid gland tissue.

	T_4	T_3	rT_3	3,5-T_2	3,3′-T_2	3-T_1
Curtain gas (CUR)	20	25	25	25	25	20
Collision gas (CAD)	8	8	8	8	8	8
Ionspray voltage (IS)	−4500	−4500	−4500	−4500	−4500	−4500
Temp. (TEM)	400	400	400	400	400	400
Ion source gas 1 (GS1)	50	50	50	50	50	50
Ion source gas 2 (GS2)	55	55	55	55	55	55
Declustering potential (DP)	−40	−30	−30	−30	−30	−45
Focusing potential (FP)	−350	−350	−350	−350	−350	−350
Entrance potential (EP)	−11	−10	−10	−10	−10	−10
Collision energy (CE)	−70	−80	−80	−70	−70	−50
Collision cell exit potential (CXP)	−2.5	−3	−3	−3	−3	−3

5 mg or 7 mg, above). After vortexing, the TG with pronase buffer was incubated at 37 °C for 24 h. Then, 1 ml of cold MeOH was added, vortexed, and kept at −20 °C overnight. The mixture was centrifuged at 13,000 × g (at 4 °C) for 10 min. The supernatant was decanted into a new 1.5-ml snap-cap microcentrifuge tube and was stored at −20 °C. A 100-μl aliquot of the supernatant was diluted with 100 μl of MeOH and then injected into a LC–MS/MS.

To determine whether the distribution of THs within a lobe of the TG were similar, if the sample was divided for analysis, two TG lobes weighing 8.4 and 8.8 mg from two normal rats were each subdivided into two near-equal portions, weighing 3.8 and 4.6 mg, and 5.0 and 3.8 mg, respectively. These four portions were extracted by the pronase-digestion procedure described above. The amount of pronase was set to be 5.0-times the weight of each TG portion.

2.5. LC–MS/MS conditions

An API 2000 electrospray triple quadrupole mass spectrometer (ESI-MS/MS, Applied Biosystems, Foster City, CA) equipped with an Agilent 1100 Series HPLC system (Agilent Technologies Inc., Santa Clara, CA) was used for measurement of six THs. The negative ion multiple reaction monitoring (MRM) mode was used and the MRM transitions monitored were 776 > 127 for T_4, 650 > 127 for T_3 and rT_3, 524 > 127 for 3,3′- and 3,5-T_2, and 398 > 127 for 3-T_1. Nitrogen was used as both curtain and collision gas. MS/MS parameters were optimized for every TH standard, by infusion of 1 μg/ml-standard solution. The optimized MS/MS parameters are summarized in Table 1.

2.6. LC–MS/MS procedure

Twenty microliters of TG extract were injected onto an Agilent ZORBAX Extend-C18 (150 mm length × 2.1 mm internal diameter, 5 μm particle diameter) chromatographic column serially connected with a guard column (20 mm × 2.1 mm, 5 μm, Thermo Electron Co., Bellefonte, PA), at a flow rate of 500 μl/min. The mobile phase was 0.01% (v/v) ammonium hydroxide in MeOH (solvent A) and deionized water (solvent B); the gradient parameters are shown in Table 2.

Table 2
HPLC gradient parameters optimized for analysis of thyroid hormones.

	Time (min)				
	2	5	6	8	10
Mobile phase A (%)	10	50	70	75	80
Mobile phase B (%)	90	50	30	25	20

Mobile phase A: 0.01% ammonium hydroxide in MeOH.
Mobile phase B: deionized water.
Injection volume: 20 μl.
Flow rate: 500 μl/min.

2.7. Data analysis

The analytes were quantified from an external calibration curve prepared at TH concentrations ranging from 1 to 200 ng/ml, when the retention time of TH peaks detected in TG matches that of TH standard within ±0.05 min. Data processing was performed with the Analyst 1.4.1 software package. Statistical analyses were conducted with Statistica V. 06J (StatSoft Inc., Tulsa, OK). The statistical significance of differences in concentrations of THs, between perchlorate-treated and control rats (IDP vs. IDW, and RP vs. RW), was evaluated by *t*-test. Because the *F*-test detected significant differences, for both the IDP versus IDW and RP versus RW groups exposed to perchlorate for 14-days, we re-examined the significances using the Mann–Whitney *U*-test. For the evaluation of the effect of perchlorate on TH concentrations, one-way analysis of variance (ANOVA) and the Scheffe test were used, for the 1-day exposure groups, after the homoscedasticity among the four groups was verified using the Brown–Forsythe and Levene's tests. A p value <0.05 was considered to denote significance.

3. Results and discussion

3.1. Instrumental calibration and limit of detection

Calibration standards injected at seven different concentrations, ranging from 1 to 200 ng/ml, for each of the THs showed high linearity ($r > 0.99$). When 20 μl of 1 ng/ml standard (*i.e.*, 20 pg of each TH) was injected, the signal to noise (S/N) ratio was 9 for 3-T_1; 7 for both 3,3′-T_2 and 3,5-T_2; and 5 for each of rT_3, T_3, and T_4. Thus, the instrumental detection limits in the range of 10–20 pg could be established for these THs. The limits of detection (LOD) and quantification (LOQ) for the analytical method were determined based on the standard deviations from five replicate analyses, using the lowest calibration standard, 1 ng/ml. The LOD and LOQ were calculated as 3*S* and 10*S*, respectively, where *S* is the standard deviation. The calculated LOD and LOQ were respectively 0.16 and 0.53 ng/ml for 3-T_1; 0.20 and 0.70 ng/ml for both 3,3′-T_2 and 3,5-T_2; and 0.33 and 1.1 ng/ml for each of rT_3, T_3, and T_4. Because of the 2.35-fold dilution of the sample and the use of 5 mg-TG samples (*i.e.*, small sample size, see the next section) for extraction, the actual LOD and LOQ in the samples analyzed in this study were determined to be 0.08 and 0.25 ng/mg TG for 3-T_1; 0.10 and 0.33 ng/mg TG for both 3,3′-T_2 and 3,5-T_2; and 0.16 and 0.52 ng/mg TG for each of rT_3, T_3, and T_4.

3.2. Pronase-digestive efficiencies and distribution of THs in the TG

The digestion efficiencies for the pronase enzyme used at various levels for the extraction of THs from the TG are shown in Fig. 1. 3-T_1, 3,3′-T_2, and 3,5-T_2 were not detected in rat TG, by our

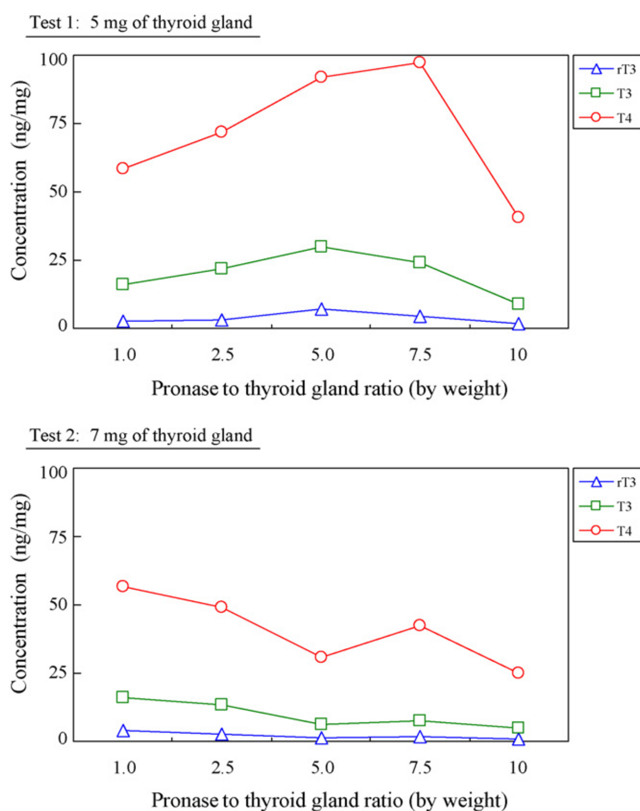

Fig. 1. Thyroid hormone concentrations in thyroid gland of rats, after digestion by pronase, at various pronase to thyroid gland ratios (by weight). 3-T_1, 3,3′-T_2, and 3,5-T_2 were not detected in the thyroid gland tested.

methodology. When 5-mg TG was used for extraction, the highest concentrations of rT_3 and T_3 were found in the sample that had been hydrolyzed with pronase added at 5-times the TG mass (*i.e.*, 25 mg, 71.4 mg/ml digestive solution). T_4 level was also higher at this level of pronase than in the samples with pronase added at 1.0-, 2.5-, or 10-times the mass of TG, while the highest level of T_4 was found in the sample treated with pronase at 7.5 times the mass of TG (Fig. 1, test 1). However, when 7 mg of TG were used for extraction, TH concentrations tended to decrease with the increase in pronase level (Fig. 1, test 2). TH levels in all of the 7-mg TG samples were lower than those in the 5-mg TG samples treated with pronase at 2.5-, 5.0-, and 7.5-times the weight of TG (Fig. 1, test 1). This result may be due to the ionization suppression effect of the LC–MS/MS at greater levels of TG and pronase. The above results indicate that the use of approximately 5 mg-TG and pronase mass five times the weight of TG is appropriate for the analysis of THs.

Since the weight of almost all TGs harvested from rats in this study was above 5 mg, the TGs needed to be cut into 5-mg pieces for extraction. Accordingly, we conducted a test to examine the homogeneity of the distribution of THs in the TG, across two portions of the TG from two normal rats. Each TG was divided into two portions, and THs in each were analyzed by pronase added at five times the weight of each portion. In both of the TG portions tested, similar concentrations of rT_3, T_3, and T_4 (3-T_1, 3,3′-T_2, and 3,5-T_2 were not detected) were found between the paired subsamples (Table 3), suggesting the homogeneity in the distribution of THs in TG.

Based on the above tests, we chose 5 mg of TG as the extraction amount and a value for the pronase mass used for extraction set at five times the weight of the TG sample. This method was then applied for the analysis of THs in 48 rat TG samples obtained from a perchlorate-iodine exposure study.

Table 3
Distribution test for thyroid hormones in two portions of the thyroid gland from two normal rats.

	Sample 1 (8.4 mg)		Sample 2 (8.8 mg)	
	Portion 1 (3.8 mg)	Portion 2 (4.6 mg)	Portion 1 (5.0 mg)	Portion 2 (3.8 mg)
rT_3 (ng/mg)	2.1	1.94	1.74	1.84
T_3 (ng/mg)	28.4	26	14.3	14.4
T_4 (ng/mg)	95.4	90.7	53.5	51.7

Prior to the analysis of rat TG samples, a recovery test was conducted, through the spiking of either of two concentrations of each of six THs (10 and 100 ppb) into the pronase solution, with subsequent passage through the entire analytical procedure. The recoveries of the six THs were between 88.4 and 105%, and the coefficient of variation (CV) of triplicate analyses was between 1.1 and 4.1% (Table 4), suggesting that this method provides adequate accuracy and precision. No TH peaks were detected in procedural blanks, which consisted of solvents and reagents passed through the entire analytical procedure, without TG present.

3.3. THs in TGs from rats exposed to perchlorate

Typical MRM chromatograms of THs detected in rat TG samples and standard solutions are shown in Fig. 2. rT_3, T_3, and T_4 were detected in almost all samples of rat TG, whereas 3,3′-T_2 and 3,5-T_2 were below the LOQ in all samples. 3-T_1 was found in only one TG sample, from the day 14-RW group. Trace levels (<LOQ) of 3,3′-T_2 and 3,5-T_2 were found in some samples (Fig. 2), suggesting the presence of T_2, a rarely studied TH in rat TG. To our knowledge, this is the first study to detect T_1, and trace levels of T_2, from rat TG. Although the physiological roles of T_1 and T_2 are not well understood, it has been recently reported that 3,5-T_2 stimulates mitochondrial fatty acid oxidation, through activation of several metabolic pathways [34].

Concentrations of rT_3, T_3, and T_4 detected in rat TG are summarized for each group representing four conditions (IDP: iodide deficient diet + perchlorate-treated water, IDW: iodide deficient diet + water, RP: regular diet + perchlorate-treated water, RW: regular diet + water) in Table 5. After 24 h perchlorate exposure, no significant difference in concentrations of three THs was observed between IDP and IDW groups, or between RP and RW groups, although mean values for the rats exposed to perchlorate were slightly lower than those in unexposed rats. In comparison with rats given regular diet (*i.e.*, RP and RW), significantly diminished TH

Table 4
Recoveries (%) of thyroid hormones through the entire analytical procedure, after spiking either of two concentrations (10 and 100 ppb) of thyroid hormones into the pronase solution (n = 3), without thyroid gland.

	Amount spiked (ng/ml)	Mean value	(SD)	Recovery (%)	CV (%)
3-T_1	10	9.21	(0.17)	92.1	1.8
	100	92.0	(1.13)	92.0	1.2
3,3′-T_2	10	8.66	(0.17)	86.6	2.0
	100	91.7	(0.97)	91.7	1.1
3,5-T_2	10	9.58	(0.39)	93.6	4.1
	100	91.5	(1.94)	91.5	2.1
rT_3	10	9.03	(0.35)	90.3	3.9
	100	90.4	(1.44)	90.4	1.6
T_3	10	10.53	(0.40)	105.3	3.8
	100	96.3	(2.34)	96.3	2.4
T_4	10	8.84	(0.33)	88.4	3.7
	100	91.9	(1.05)	91.9	1.1

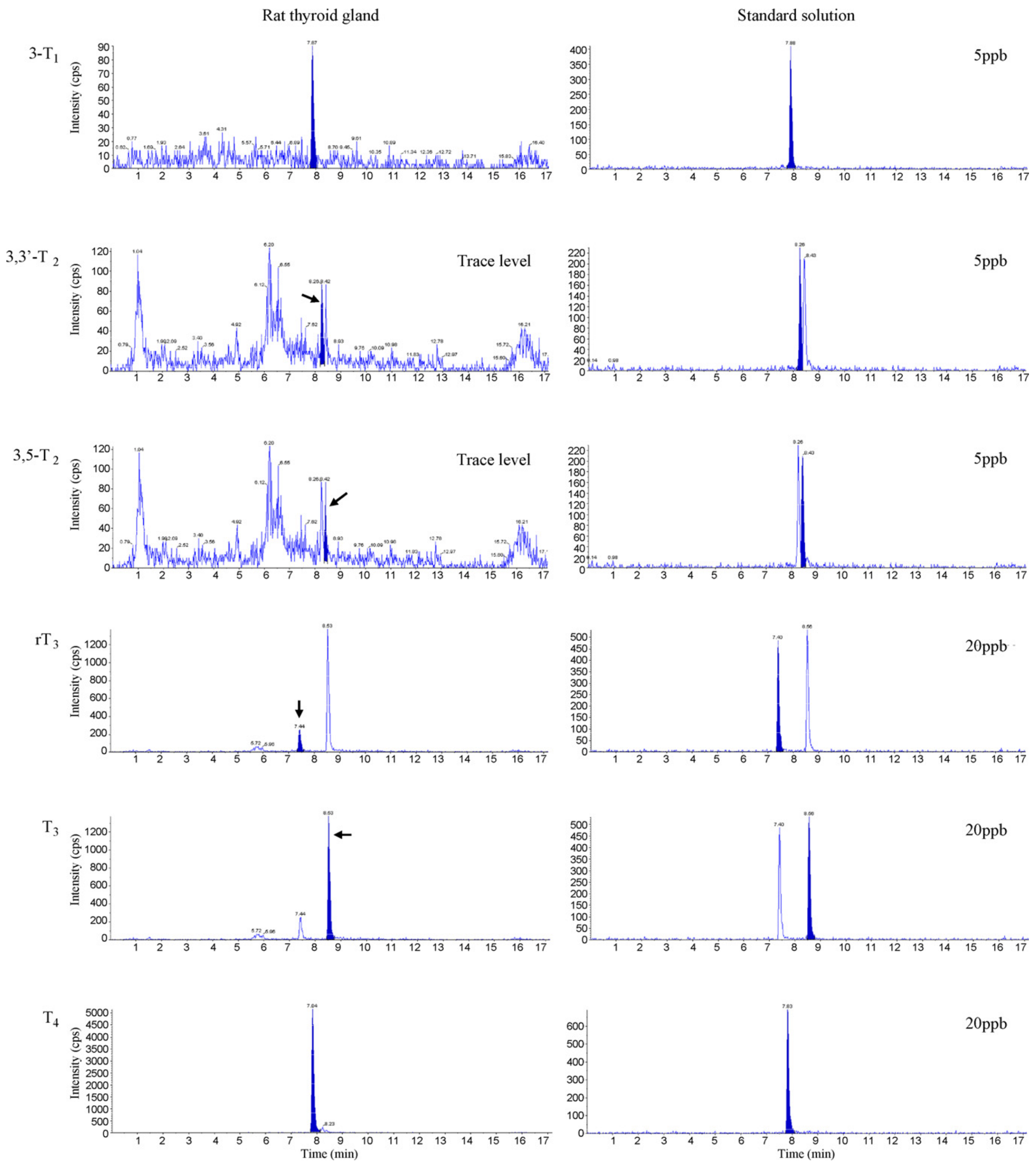

Fig. 2. MRM chromatograms for thyroid hormones detected in rat thyroid gland and in standard solution.

levels were found in rats given iodide-deficient diet (*i.e.*, IDP and IDW) ($p < 0.05$), suggesting that the production of the THs in TG of rat given the iodide-deficient diet for 2 months was suppressed.

After 14 days of exposure to perchlorate, significantly reduced concentrations of rT_3, T_3, and T_4 were found between IDP and IDW groups, or RP and RW groups (Table 5). In the IDP group, T_3 was detected in only one of the samples, and T_4 in only two samples; concentrations were slightly above the LOQ. Levels of rT_3 were below the LOQ in all samples. These results support the hypothesis that perchlorate can competitively inhibit iodide uptake by the TG via NIS, and can subsequently reduce the production of THs. Although it has been reported that oral administration of perchlorate to rats via drinking water decreased serum T_4 and T_3, not only in dams but also in fetuses and neonates [27–29,35], the results of our study suggest that, instead, the secretion of active THs into the bloodstream is reduced due to reduced TH production in the TG. Furthermore, our results suggest that adverse effects of perchlorate on TH homeostasis are more pronounced under iodide-deficient conditions. A recent study on humans reported that perchlorate was a significant negative predictor of serum T_4, and a positive

Table 5
Concentrations (ng/mg) of thyroid hormones in the thyroid gland from iodide-perchlorate exposed and control rats ($n = 6$ per group; totally $n = 48$).

	24-h exposure			14-day exposure		
	rT_3 Mean (SD)	T_3 Mean (SD)	T_4 Mean (SD)	rT_3 Mean (SD)	T_3 Mean (SD)	T_4 Mean (SD)
Rat groups given iodide-deficient diet						
IDP	1.1 (0.84)	5.4 (2.2)	16 (6.7)	0.26[a,b,**]	0.30 (0.10)[b,**]	0.38 (0.18)[b,**]
IDW	1.3 (0.67)	6.6 (2.7)	20 (7.5)	1.2 (0.48)	4.6 (1.6)	7.8 (3.6)
Rat groups given iodide-sufficient diet						
RP	2.1 (1.0)	11 (3.2)	63 (28)	0.35 (0.22)[c,**]	1.5(1.4)[c,**]	6.7 (8.3)[c,*]
RW	3.7 (1.8)	16 (6.4)	81 (30)	3.6 (2.6)	13 (9.6)	41 (31)

IDP: iodine deficient diet and perchlorate-treated water; IDW: iodine deficient diet and water; RP: regular diet and perchlorate-treated water; RW: regular diet and water. The concentration below LOQ was set at half the value of the LOQ (0.26), for calculation of mean and SD values and for performance of statistical analyses.
[a] Concentration in all of the samples was below LOQ.
[b] Significantly lower concentrations than IDW, $^{**}p < 0.01$.
[c] Significantly lower concentrations than RW, $^{*}p < 0.05$, $^{**}p < 0.01$.

predictor of serum TSH, for women with low urinary-iodide status (<100 μg/l) [19]. Given that significantly lower serum levels of T_4 and T_3 have been observed in rats treated at perchlorate levels below 10 mg/kg/day than in control rats [27,28,35], the effects of perchlorate exposure can be elicited in iodide-deficient rats at much lower levels of the anion.

4. Conclusions

We have developed a reliable LC–MS/MS method for the measurement of THs in rat TG, and we have demonstrated a significantly reduced production of rT_3, T_3, and T_4 in rats exposed to perchlorate, especially when an iodide-deficient diet was co-administered. The LC–MS/MS method is useful to measure THs in TG of various animal species, and alleviates the need to use species-specific antibodies used in the IA methods. In addition, the enzymatic extraction by pronase can be applied to measure THs in other organs and tissues including brain.

Acknowledgements

We thank Dr. T. Isobe (CMES and Senior Research Fellow Center, Ehime University) for his advice on the LC–MS/MS column. This study was supported by US EPA STAR Cooperative Agreement R832134 and by a biomonitoring grant (1U38EH000464-01) from the Centers for Disease Control and Prevention (CDC), Atlanta, GA, USA.

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