A Method for Analysis of Thyroid Hormones in Perchlorate-administered Rats by Liquid Chromatography-Tandem Mass Spectrometry: Potential Application to Samples Stored in *es*-Bank of Ehime University

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Abstract—Perchlorate can competitively inhibit iodide uptake by the thyroid gland (TG) via the sodium/iodide symporter (NIS), consequently reducing the production of thyroid hormones (TH). Until recently, the effects of perchlorate on TH homeostasis have been examined through measurement of serum levels of THs, by immunoassay (IA)-based methods. IA methods are fast, but for TH analysis, they are compromised by the lack of adequate specificity. 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 or control water. THs in TGs were extracted by pronase digestion and then analyzed by LC-MS/MS. 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 TGs. The LC-MS/MS method developed here can be useful to measure THs in various animal species stored in *es*-Bank.

Keywords: thyroid hormones, thyroid gland, perchlorate, iodide deficiency, LC-MS/MS

INTRODUCTION

It is well known that THs are essential for regulation of biological processes such

as growth, metabolism, neurodevelopment, and protein synthesis. T_4, synthesized in the 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, T_3 (Bianco and Kim, 2006). T_4 and T_3 measurements in the blood can supply clinically important information on TH homeostasis. Thus far, 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. In recent years, analytical methods using LC-MS/MS have been developed to measure T_4 and T_3 in human serum (Soldin *et al*., 2004; Soukhova *et al*., 2004). The LC-MS/MS methods are, in general, shown to be more accurate and reliable than the IA methods (Soldin *et al*., 2004).

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 (Charnley, 2008). Perchlorate can competitively inhibit iodide uptake by the TG via NIS, reducing the downstream synthesis and secretion of T_4 and T_3 (Wolff, 1998). An *in vitro* study found that the relative potency of perchlorate, in inhibition of $^{125}I^-$ uptake, was 30 times higher than the potency of iodide (Tonacchera *et al*., 2004). In experimental studies using rats, perchlorate exposure decreased serum levels of T_4 and T_3 and increased thyroid stimulating hormone (TSH) levels (Siglin *et al*., 2000; York *et al*., 2005). 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 (Siglin *et al*., 2000). Nevertheless, documentation of TH perturbations in the TG following perchlorate exposure is not available.

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. Using this method, we also investigated any differences in TH levels in TGs from rats exposed to perchlorate in drinking water, and fed either an iodide-deficient diet or an iodide-sufficient diet.

MATERIALS AND METHODS

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.

Experimental design and sample collection

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. Male Sprague Dawley rats (*n* = 48) were

separated into two groups and were fed either with regular diet or iodide-deficient diet 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 and whole TGs ($n = 48$) were harvested. Twelve additional TG samples were collected from rats provided with regular research diet and plain water for refinement of the extraction procedure for THs from the TG, by the pronase-digestion method described below.

Sample preparation

Because it was necessary to hydrolyze the TGs, to dissociate THs from thyroglobulin, we used pronase (protease from *Streptomyces griseus*). 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 microcentrifuge tubes. 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. 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 for 10 min. 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 was similar, when the gland was divided for analysis, two TG lobes weighing 8.4 mg and 8.8 mg from two normal rats were each subdivided into two near-equal portions. 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.

LC-MS/MS conditions and procedure

An API 2000 electrospray triple quadrupole mass spectrometer (ESI-MS/MS) interfaced with an Agilent 1100 Series HPLC system 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. MS/MS parameters were optimized for every TH standard, by infusion of 1 μg/ml-standard solution.

Twenty microliters of TG extract were injected onto an Agilent ZORBAX Extend-C18 (150 mm length $\times$ 2.1 mm internal diameter, 5 μm particle diameter) chromatographic column serially connected with a Thermo guard column (20 $\times$ 2.1 mm, 5 μm), at a flow rate of 500 μl/min. The mobile phase and gradient parameters are shown in Table 1.

Table 1. 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.

Data analysis

The analytes were quantified from an external calibration curve prepared at TH concentrations ranging from 1 to 200 ng/ml. Data processing was performed with the Analyst 1.4.1 software package. Statistical analyses were conducted with Statistica V. 06J.

RESULTS AND DISCUSSION

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 excellent linearity ($r > 0.99$). The limits of detection (LOD) and quantification (LOQ) in the samples analyzed in this study were determined to be 0.08 and 0.25 ng/mg TG for $3\text{-}T_1$; 0.10 and 0.33 ng/mg TG for both $3,3'\text{-}T_2$ and $3,5\text{-}T_2$; and 0.16 and 0.52 ng/mg TG for each of rT_3, T_3, and T_4.

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. $3\text{-}T_1$, $3,3'\text{-}T_2$, and $3,5\text{-}T_2$ were not detected in rat TG, by our 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). 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 5 times the weight of TG is appropriate for the analysis of THs.

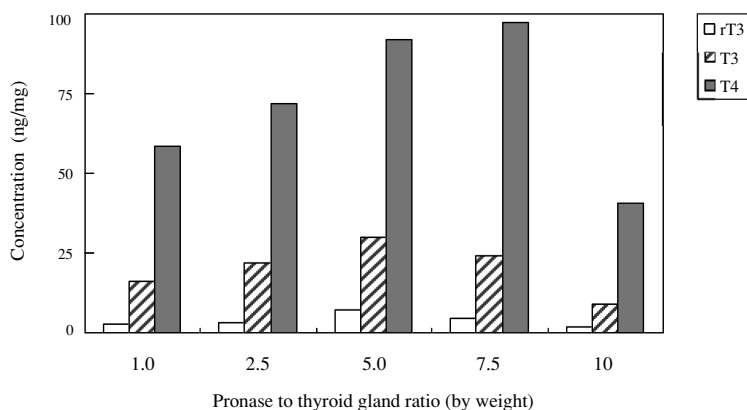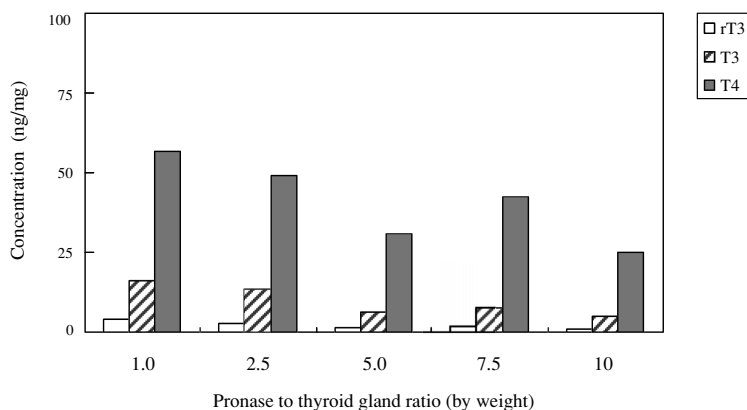Since the weight of almost all TGs harvested from rats in this study was

Test 1: 5 mg of thyroid gland

Test 2: 7 mg of thyroid gland

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.

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. 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 (Fig. 2), suggesting the homogeneity in the distribution of THs in the TG.

Based on the above tests, we chose 5 mg of TG for the extraction amount and a value for the pronase mass used for extraction was set at 5 times the weight of
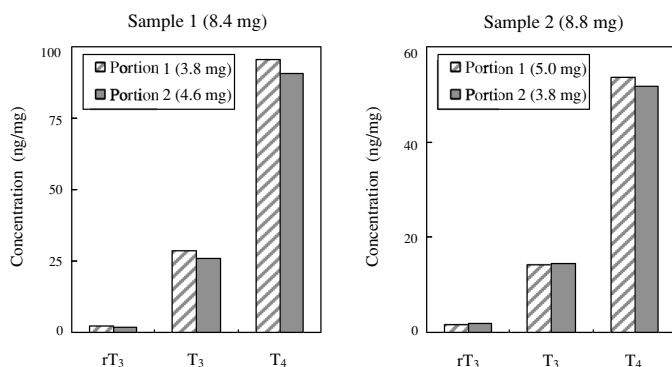
Fig. 2. Distribution test for thyroid hormones in the thyroid gland from two normal rats.

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.

THs in TGs from rats exposed to perchlorate

rT_3, T_3, and T_4 were detected in almost all samples of rat TG, whereas $3\text{-}T_1$, $3,3'\text{-}T_2$, and $3,5\text{-}T_2$ were below the LOQ in all samples. Concentrations of rT_3, T_3, and T_4 detected in rat TGs are summarized for each group representing four conditions (Fig. 3). 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. In comparison with rats given regular diet (i.e., RP and RW), significantly diminished TH 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 TGs of rats 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 between RP and RW groups (Fig. 3). In the IDP group, T_3 was detected in only one of the samples, and T_4 in only two samples. 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. 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 predictor of serum TSH, for women with low urinary-iodide status (<100 μg/L) (Blount *et al*., 2006). 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 (Siglin *et al*., 2000; York *et al*., 2005), the effects of perchlorate exposure can be elicited in iodide-deficient rats at much lower levels of the anion.

The LC-MS/MS method is useful to measure THs in the TG of various

24-h exposure
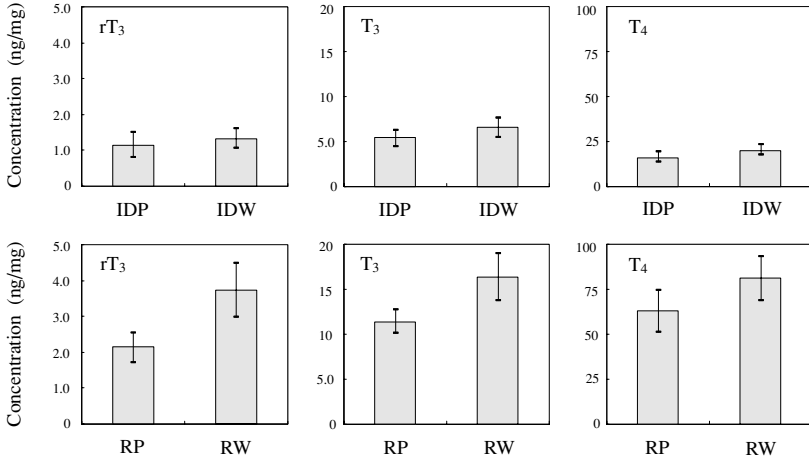
14-day exposure
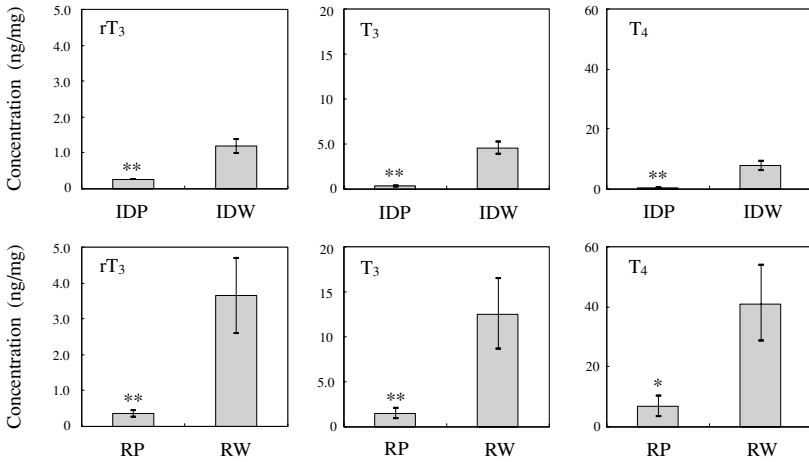
*p < 0.05, ** p < 0.01*

IDP: Iodine deficient diet and perchlorate-treated water   RP: Regular diet and perchlorate-treated water
IDW: Iodine deficient diet and water   RW: Regular diet and water

Fig. 3. Concentrations of thyroid hormones in the thyroid gland from iodide-perchlorate exposed and control rats ($n = 6$ per group; totally $n = 48$).

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. We previously found elevated concentrations of OH–PCBs, which can disturb TH homeostasis, in the blood of mammalian and avian species of samples stored in *es*-Bank of Ehime University (Kunisue and Tanabe, 2009). Using TG, brain, and serum samples stored in the *es*-Bank, it is possible to investigate the relationships between concentrations of THs and TH homeostasis-disrupting chemicals, such as perchlorate and OH–PCBs, in wildlife.

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