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Simultaneous quantification of T4, T3, rT3, 3,5-T2 and 3,3'-T2 in larval zebrafish (*Danio rerio*) as a model to study exposure to polychlorinated biphenyls

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Abstract

Environmental toxicants that interfere with thyroid hormone (TH) signaling can impact growth and development in animals and humans. Zebrafish represent a model to study chemically-induced TH disruption, prompting the need for sensitive detection of THs. Simultaneous quantification of 3,3',5-triiodo-L-thyronine (T3), thyroxine (T4), 3,3',5'-triiodo-L-thyronine (rT3), 3,5-diiodo-L-thyronine (3,5-T2), and 3,3'-diiodo-L-thyronine (3,3'-T2) in zebrafish larvae was achieved by ultra-performance liquid chromatography–tandem mass spectrometry in positive ion mode. Solid phase extraction with SampliQ cartridges and derivatization with 3N hydrochloric acid in n-butanol reduced matrix effects. Derivatized compounds were separated on an ACQUITY UPLC BEH C18 column with mobile phases consisting of 0.1% acetic acid in deionized water and 0.1% acetic acid in methanol. The limits of detection ranged from 0.5–0.6 pg injected on column. The method was validated by evaluating recovery (77.1–117.2%), accuracy (87.3–123.9%) and precision (0.5–12.4%) using diluted homogenized zebrafish embryos spiked with all target compounds. This method was then applied to zebrafish larvae collected after 114 hour exposure to polychlorinated biphenyls (PCBs) including PCB 28, PCB 66, PCB 95, or the technical mixture Aroclor 1254. Exposure to PCB 28 and PCB 95 increased the T4:T3 ratio and decreased the T3:rT3 ratio, demonstrating that this method can effectively detect PCB-induced alterations in THs.

Keywords

thyroid hormones; zebrafish; derivatization; UPLC-MS/MS; PCBs

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Conflict of Interest

The authors declare that they have no conflict of interest.

1. Introduction

L-thyroxine (T4, $C_{15}H_{11}I_4NO_4$, MW 776.87, Fig 1) is synthesized in the thyroid gland and carried in the bloodstream to target tissues where it is biotransformed by deiodinase type I or II (dio1, dio2) to the active hormone, 3,3',5-triiodo-L-thyronine (T3, $C_{15}H_{12}I_3NO_4$, MW 650.97, Fig 1) (Bianco et al., 2006). T4 and T3 are essential for regulating numerous biological processes, including oxygen consumption, carbohydrate metabolism, protein synthesis, growth and neurodevelopment (Bianco et al., 2002). Biotransformation of T4 by deiodinase type III (dio3) also produces the metabolite 3,5',3'-triidothyronine (rT3, $C_{15}H_{12}I_3NO_4$, MW 650.97, Fig 1) (Bianco et al., 2002), a competitive inhibitor of T3 (Kelly, 2000; Kohrle et al., 1988). Further degradation of T3 and rT3 produces two distinct diiodothyronines: 3,5-diiodo-L-thyronine (3,5-T2, $C_{15}H_{13}I_2NO_4$, MW 525.08, Fig 1) and 3,3'-diiodo-L-thyronine (3,3'-T2, $C_{15}H_{13}I_2NO_4$, MW 525.08, Fig 1) (Kelly, 2000). Some studies suggest that the deiodination of T4 and T3 does not result in complete loss of biological activity. For example, the isomer 3,5-T2 can suppress thyroid stimulating hormone levels and result in increased resting metabolic rate in brown adipose tissue (Kelly, 2000). In addition, both rT3 and 3,3'-T2 can stimulate TH-regulated signaling by acting on nuclear TH receptors, similar to the actions that mediate T3 signaling (Papavasiliou et al., 1977). Thus, regulating the circulating and cellular concentrations of T4, T3, rT3, 3,5-T2 and 3,3'-T2, as well as their relative ratios, is essential for TH homeostasis and cellular action.

A number of structurally diverse environmental chemicals can disrupt TH signaling at multiple levels of hormone action (Howdeshell, 2002; Schnitzler et al., 2008). Evaluating a causal link between TH disruption and developmental neurotoxicity is a major research focus, especially for established neurotoxicants, such as polychlorinated biphenyls (PCBs), which have also been demonstrated to alter TH levels and/or signaling. For example, the coplanar congener, PCB 77, increased hepatic T4 outer ring deiodination activity and decreased plasma T3 levels in American plaice (*Hippoglossoides platessoides*) (Adams et al., 2000). PCB 126, another coplanar PCB, increased T4 levels in plasma and muscle coincident with a decline in T3 levels in growing lake trout (*Salvelinus namaycush*) and juvenile rainbow trout (*Oncorhynchus mykiss*) (Brown et al., 2004; Brown et al., 2002). Exposure of juvenile Japanese flounder (*Paralichthys olivaceus*) to Aroclor 1254, a commercial mixture of coplanar and non-coplanar PCBs, significantly decreased plasma T4 and T3 levels (Dong et al., 2014). In general, coplanar PCBs have been linked to TH disruption (Bager et al., 1995; S. Safe, 1984; S. H. Safe, 1994). In contrast, limited data is available regarding the influence of non-coplanar congeners on TH concentrations.

Zebrafish (*Danio rerio*) are increasingly used as a relevant model for studying the mechanisms regulating TH activity, both physiologically and following exposure to chemicals that disrupt TH signaling (Scholz et al., 2008). Both thyroid gland function and mechanisms of neurodevelopment are highly conserved between zebrafish and humans, and zebrafish response to chemical exposure is similar to that of other vertebrate models (Porazzi et al., 2009; Raldua et al., 2012). Zebrafish studies of TH-signaling and disruption by chemical exposure routinely measure T4 and T3 concentrations by automated immunoassay methods. However, there is significant variability in data collected by this method, with

reported concentrations ranging from 0.45 to 21 ng/g tissue for T4 and from 0.1 to 1.6 ng/g tissue for T3 in zebrafish depending on sample preparation and immunoassay kit (Chang et al., 2012; Liu et al., 2011; Yan et al., 2012). Despite high sensitivity, immunoassays (IA) often lack of adequate specificity because of factors such as endogenous antibodies, free fatty acids, abnormal protein binding and binding competitors (Fritz et al., 2007; Murthy et al., 1998; Palmer-Toy et al., 2005; Sapin et al., 2003; Steele et al., 2003). This is confirmed by variation in assay results for T4 and T3 with the use of different antibodies as reported by the College of American Pathologists (O. P. Soldin et al., 2004; Soukhova et al., 2004). It is also important to note that commercial immunoassays are generally validated for TH analysis of human serum (Gnanasekar et al., 2010), but not for other matrices. In the past decade, UPLC-MS/MS has been successfully implemented to overcome some of the analytical difficulties recognized with immunoassay approaches (S. J. Soldin et al., 2005). Several methods have been validated to measure T3 and T4 in serum by LC-MS (Hansen et al., 2016; Kunisue et al., 2011a; Tai et al., 2004; Tai et al., 2002; Wang et al., 2010) and GC-MS (Hantson et al., 2004; Thienpont et al., 1999), and for measuring T2 and T3 levels in fish muscle (Little et al., 2013). There are no published methods assessing T4, T3, rT3, 3,5-T2 and 3,3'-T2 simultaneously in zebrafish larvae. In the present work, we describe the development and application of a method using solid phase extraction (SPE) extraction of zebrafish larvae, coupled with UPLC-ESI/MS/MS analysis after derivatization for detection and quantification of T4, T3, rT3, 3,5-T2 and 3,3'-T2. This method was applied to the detection of THs in zebrafish larvae exposed to PCB 95 (2,2’,3,5’,6-pentachlorobiphenyl), PCB 28 (2,4,4'-trichlorobiphenyl), PCB 66 (2,3',4,4'-tetrachlorobiphenyl) and Aroclor 1254. Our findings confirm that this method provides a sensitive and reliable approach for quantifying thyroid hormones in zebrafish larvae to complement and enhance the use of zebrafish as a vertebrate model of TH signaling and disruption during development.

2. Materials and methods

2.1. Chemicals and materials

Unlabeled thyroid hormones (T4 >98%, T3 >95%, rT3 >97%, 3,5-T2 >99%, 3,3'-T2 >98%), L-ascorbic acid (pharmaceutical secondary standard), dithiothreitol (for molecular biology, minimum 99% titration), L-glutathione (minimum 99%), *N*-phenylthiourea (analytical standard, minimum 99%), and pronase (> 3.5 units/mg solid, protease from *Streptomyces griseus*) were purchased from Sigma-Aldrich (St. Louis, MO, USA). $^{13}C_6$-labeled T3 ($^{13}C_6$-T3, >95%) was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). $^{13}C_6$-labeled T4 ($^{13}C_6$-T4, >95%) was purchased from Toronto Research Chemicals, Inc. (Toronto, Canada). Polychlorinated biphenyls (PCB 28, PCB 66, PCB 95) and Aroclor 1254 technical PCB mixture were purchased from AccuStandard, Inc. (New Haven, CT, USA). Citric acid (monohydrate, 99.5%, for analysis), tris(hydroxymethyl)aminomethane (molecular biology grade), triton X-100 (electrophoresis), acetic acid (analytical grade), and methanol (analytical grade) were purchased from Fisher Scientific (Pittsburgh, PA, USA). Ammonium hydroxide (ACS) was purchased from EMD Millipore (Gibbstowm, NJ, USA). The reagent 3N hydrochloric acid in n-butanol was purchased from Regis Technologies, Inc. (Morton Grove, IL, USA). SampliQ SPE cartridges (3 mL, 60 mg of optimized polymer technology polymer) were purchased from Agilent Technologies (Santa Clara, CA, USA).

Acetone (ACS) was purchased from VWR Analytical (Radnor, PA, USA). Ultrapure water (418 mΩ) was obtained using a Milli-Q system (Millipore, Billerica, MA, USA).

2.2. Preparation of stock standards solution, antioxidant solution, and digestion buffer

Stock standard solutions of target analytes and internal standards were prepared in methanol with 25% (v/v) ammonium hydroxide. Working solutions of 10 and 100 ng/mL were prepared in methanol. The internal standard working solutions containing 2 and 50 ng/mL each of $^{13}C_6$-T3 and $^{13}C_6$-T4 were prepared in methanol. An antioxidant solution consisting of ascorbic acid, dithiothreitol, and citric acid at concentrations of 25 mg/mL each in water was prepared to prevent degradation of THs during sample preparation. A digestion buffer to dissolve pronase was prepared containing 153.65 mg of L-glutathione, 42.55 mg of *N*-phenylthiourea, 1.21 g of tris(hydroxymethyl)aminomethane, and 1 mL of Triton X-100 in 100 mL of deionized water. PCB and Aroclor 1254 stock solutions were prepared in anhydrous DMSO and stored at 10 mM.

2.3. Zebrafish husbandry, exposure, and sample collection

All procedures involving zebrafish were approved by the Institutional Animal Care and Use committee at the University of California Davis. Adult zebrafish (wildtype Tropical 5D, bred in house) were raised under controlled conditions at 28.5 ± 0.5 °C, pH 7.2 ±0.4, and conductivity at 700 μS ± 100 in fish water consisting of reverse osmosis water supplemented with salt (Instant Ocean, Aquatic Ecosystems, Apopka, FL, USA) under a 14 h light/10 h dark photoperiod. Adult fish were fed a combination of *Artemia nauplii* (INVE Aquaculture, Inc., Salt Lake City, UT) and commercial flake foods (a combination of Zeigler Zebrafish Granule (Zeigler Bros, Inc. Gardners, PA), Spirulina Flake (Zeigler Bros, Inc.), Cyclopeeze (Argent Aquaculture, Redmond, WA) and Golden Pearl (Brine Shrimp Direct, Ogden, UT) twice daily. Adult zebrafish were spawned in groups of 8–10, and embryos were collected and staged following fertilization (Kimmel et al., 1995). To generate zebrafish embryo matrix for spiked calibration samples, embryos were collected and pooled immediately following fertilization, homogenized and later diluted 10 times with deionized water. For PCB exposures, zebrafish at 6 hours post fertilization (hpf) were transferred to 6 well tissue culture dishes, 30 embryos per well, in 3 mL embryo media (15 mM NaCl, 0.5 mM KCl, 1.0 mM $MgSO_4$, 150 μM KH_2PO_4, 50 μM Na_2HPO_4, 1.0 mM $CaCl_2$, 0.7 mM $NaHCO_3$; (Westerfield, 2000), and treated with PCB 28, PCB 66, PCB 95, or Aroclor 1254 (final concentration 10 μM). Controls were exposed to the same experimental conditions consisting of 0.2% DMSO in embryo media. Exposures were static from 6 hpf to 120 hpf (5 days post fertilization). Exposure plates were covered with parafilm to minimize evaporation and kept in a 28.5 °C incubator with a 14 h light/10 h dark photoperiod until sample collection at 120 hpf. At 120 hpf, larvae were removed from exposure plates and washed 6 times in PBS. Groups of 150 larvae were pooled in 2 mL Eppendorf tubes, excess water was removed, and samples were stored at −20 °C. Samples were later thawed and homogenized using a Virtis Virsonic 100 ultrasonic cell disrupter (SP Industries, Warminster, PA, USA) in 200 μL Ultrapure distilled water (Invitrogen, Carlsbad, CA, USA). Samples were re-frozen at −20 °C until extracted for TH analyses.

2.4. Preparation of zebrafish sample

To extract THs, frozen fish samples were first hydrolyzed with pronase (Kunisue et al., 2011b). One hundred and fifty homogenized fish larvae were placed in a 15 mL polypropylene (PP) tube. Ten μL of $^{13}C_6$-T3 (2 ng/mL) and $^{13}C_6$-T4 (50 ng/mL) were added as internal standards. Then, 20 mg pronase was dissolved in 1 mL digestion buffer and added to the PP tube. After vortexing, the fish samples suspended in pronase buffer were incubated at 37 °C for 16 h. After the incubation, 120 μL of antioxidant solution was added to prevent deiodination of THs during sample preparation, and the combination vigorously mixed. After addition of 1 mL of cold acetone with subsequent vortexing, the mixture was kept at 4 °C for 1 h. The mixture was centrifuged at 3000 × g for 15 min, and the supernatant collected into a glass tube. The residue was extracted once by adding 0.5 mL of cold acetone and 0.5 mL deionized water, vortexing, and centrifuging at 3000 × g for 15 min. After combination of all supernatants, acetone was evaporated to a final volume of approximately 2 mL under a gentle stream of nitrogen. The SampliQ SPE cartridges were preconditioned sequentially with 3.0 mL of methanol and 5.0 mL of distilled water before loading of extracts. The cartridges were washed with 3 mL of 10% MeOH in deionized water that contained 0.01% acetic acid (MeOH : water : acetic acid 10 : 89.99 : 0.01 by volume) and eluted with 3 mL of MeOH. The eluent was collected and evaporated to dryness under a gentle nitrogen stream. For derivatization, 400 μL of 3N hydrochloric acid in n-butanol were added to the dried residue, followed by 2 h incubation at 60 °C. Following incubation, any excess derivatizing agent was removed by evaporation under a gentle stream of nitrogen, before reconstituting the residue in 100 μL of MeOH. The solution was filtered through a 0.22 μm membrane, and 5 μL of each filtrate was injected into the UPLC-MS/MS system.

To evaluate the digestion efficiency of pronase, we tested three different enzyme amounts at 5, 10, or 20 mg per sample. Pronase digestion efficiency was also compared to an acid-digestion method using trichloroacetic acid (TCA). For acid digestion, 120 μL volume of antioxidant solution was added to 150 homogenized zebrafish larvae in a 15 mL PP tube. After vortexing, 1 mL of TCA solution (0.1 g of TCA/mL of deionized water) was added, and the solution was kept at 4 °C for 1 h. Subsequently, 1 mL volume of cold acetone was added and all steps as listed above after the pronase digestion were followed.

2.5. UPLC-MS/MS analysis

The ultra-performance liquid chromatography–electrospray mass spectrometry (UPLC-ESI/MS/MS) system, which consisted of a Bruker Advance UPLC system coupled to the Bruker EVOQ Elite MS/MS triple quadrupole mass spectrometer, was used for the analysis (Bruker Corp, Freemont, CA, USA). The chromatograph was fitted with a 100 mm × 2.1 mm i.d. 1.7 μm ACQUITY BEH C18 column (Waters, Milford, MA). Mobile phases consisted of 0.1% acetic acid in deionized water (channel A) and 0.1% acetic acid in methanol (channel B). Initially, 10% B was increased to 100% B over 8 min, which was held for 1 min and then returned to 10% B within 0.5 min and held for 1 min for equilibration. The column was maintained at 25 °C and the flow rate was 0.2 mL/min. Analytes were monitored by multiple reaction monitoring (MRM) in positive mode. Table 1 provides MS/MS parameters and MRM transitions in positive mode. For each analyte, the three most

abundant product ions were chosen for quantification and confirmation. Nitrogen gas was used as the nebulizer, collision gas. Other source parameters were as follows: capillary voltage of 4000 V, cone temperature of 300 °C, cone gas flow of 20 a.u., heated probe temperature of 350 °C, probe gas flow of 35 a.u., nebulizer gas flow of 45 a.u. The injection volume was 5 μL. Bruker MSWS 8.1 software was used for all data acquisition and processing.

2.6. Method performance and application

The validation of the LC-MS/MS method for the determination of T4, T3, rT3, 3,5-T2 and 3,3'-T2 in zebrafish larvae was performed in accordance with the International Conference on Harmonisation Validation of Analytical Procedures (Guideline, 2005). The method was validated by testing linearity, intra- and inter-batch precision and accuracy, sensitivity, selectivity, recovery, limits of quantification and limits of detection, carryover and stability. The diluted homogenized zebrafish embryos collected immediately after fertilization (zero day) was used as a matrix. To prepare the calibration standards, working solutions of target analytes and internal standards were added to diluted embryo matrix following the sample preparation method. A set of six levels of calibration standards at concentrations of 0.2, 0.5, 1, 5, 10 and 20 ng/mL were included. Three quality control (QC) samples at concentrations of 0.5, 2 and 10 ng/mL were prepared in the identical way as the calibration standards. QC samples were analyzed in parallel with samples. To evaluate accuracy, precision, stability, absolute recovery and matrix effects, the diluted embryo matrix was spiked with five THs at 0.05, 0.2, and 1 ng yielding a set of three levels at 0.5, 2, and 10 ng/mL. At each fortification level, quadruple experiments were performed following the sample preparation method. The injection volume was 5 μL. Blanks of diluted homogenized zero day zebrafish embryos were analyzed to determine the background and showed no interference. The method was also applied to measure THs in zebrafish larvae exposed to PCBs. A procedural blank consisting of methanol was run with every batch of samples. No contamination was detected when analyzing these samples by UPLC-MS/MS.

2.7. Data analysis

Statistical analyses of TH concentrations in 120 hpf zebrafish exposed to PCBs were performed using GraphPad Prism, version 7.0 (GraphPad Software, San Diego, CA). Concentrations of T4, T3, rT3, 3,5-T2 and 3,3'-T2, were calculated as pg/larvae in each sample with 150 pooled larvae. Five pooled samples were collected for DMSO controls and 3 pooled samples were collected for each of the PCB exposure. Concentrations of T4, T3, and rT3 in pg/larvae, as well as the T4:T3 and T3:rT3 ratio for each experimental group, were compared to DMSO controls using unpaired Student’s t-test.

3. Results and discussion

3.1. Optimization of derivatization conditions

The effect of derivatization was directly evaluated by comparing samples prepared as described above to samples subjected to the same procedure with no final derivatization step. Two samples consisting of 100 homogenized zero day zebrafish embryos were spiked with each of the 5 THs at 0.6 ng per sample. Representative results obtained from embryo

samples are shown in Fig 2. Derivatization yielded better separation for 3,3'-T2 and overall improvement in both peak shape and intensity for T4, T3, rT3, 3,5-T2 and 3,3'-T2. As for T4, derivatization resulted in a 10 fold higher intensity compared to underivatized sample.

To identify the optimal conditions for esterification, we tested the effect of changes in the following variables: amount of derivatizing agent (100, 200 and 400 μL of 3N hydrochloric acid in n-butanol), incubation temperature (40, 50 and 60 °C), and incubation time (30, 60 and 120 min). The yield was determined by measuring the amount of underivatized hormones remaining in the sample after the esterification reaction. The most suitable parameters (400 μL, 60 °C, 120 min) provided an esterification yield of 98.6, 97.3, 97.6, 98.8 and 96.3% for T4, T3, rT3, 3,5-T2 and 3,3'-T2, respectively.

3.2. Optimization of digestion conditions

The digestion efficiencies of pronase enzyme used at 5, 10, or 20 mg per sample and the acid-digestion method with TCA for the extraction of THs from 120 hpf larvae were compared. 3,5-T2 and 3,3′-T2 were not detected in the 120 hpf larvae. T4 was found at 3.3 fold higher concentrations in samples that had been digested with 20 mg of pronase compared to samples treated with TCA. T3 and rT3 were detected in the enzyme digestion sample but not in the TCA-treated sample. These results suggest that pronase digestion is more efficient than treatment with TCA in fish larvae for the dissociation of T4, T3 and rT3 from protein. When comparing the effect of varying enzyme concentrations, a slightly higher T3 concentration was found when samples were treated with 20 mg pronase compared to 10 mg pronase, while no differences were seen in the T4 and rT3 concentrations. On the basis of these results, we selected 20 mg of pronase in 1 mL of buffer solution for the extraction of THs, and applied this procedure to all subsequent analyses.

3.3. Method performance

Isotopically labeled standards were used as internal standards (IS) to allow for correction of potential loss of analytes during extraction and to be able to compensate for variations in instrument response between injections. $^{13}C_6$-T4 was used as the internal standard for T4 and $^{13}C_6$-T3 for T3, rT3, 3,5-T2 and 3,3'-T2 because of the different concentration ranges of T4 and T3, rT3, 3,5-T2, 3,3'-T2. Calibration curves were created by plotting IS corrected peak area ratios of the analyte derivative against the analyte concentrations and fitting these data with linear regression. The linear calibration range studied was 0.2–20 ng/mL with coefficients of determination greater than 0.99 (Table 2). Quantitative results were calculated using the corresponding calibration curve.

For the three fortification levels, the mean (n = 4) absolute recoveries for each of the target analytes ranged from 77.1% to 117.2%. The accuracy of the method was assessed using the recovery percent of the amount of target compounds added to the sample and calculating the mean of four samples analyzed in one batch. The intra-batch precision was based on four samples analyzed in one batch, while the inter-batch precision was based on the means of four samples in each of three batches. As shown in Table 3, the intra-batch accuracy for the three spiked levels of samples was 87.3–113.1%, 96.8–123.9% and 93.4–118.2% while inter-batch accuracy was 87.8–119.0%, 92.9–123.5% and 90.3–119.6%. The intra-batch

precision was 3.6–11.0%, 5.2–12.4% and 1.9–11.0% for the three concentrations, respectively. The inter-batch precision was 0.5–11.7%, 1.6–5.3% and 2.0–8.3% for the three concentrations, respectively.

The present study is the first to use 3N hydrochloric acid in n-butanol derivatization to analyze rT3, 3,5-T2 and 3,3'-T2. The method limits of detection were based on a signal-to noise ratio of 3:1 in a diluted homogenized zero day embryo samples spiked with 10 pg of each TH standard. LODs were 0.1 ng/mL (0.2 ng/g larva, 0.5 pg on column) for T4, rT3, 3,5-T2, 3,3'-T2 and 0.12 ng/mL (0.24 ng/g larva, 0.6 pg on column) for T3 (Table 3). The LODs of THs observed in this study were 2.1 and 2.5 fold more sensitive for T3 and T2 compared to a previously published LC-MS method for zebrafish muscle (Little et al., 2013). The significance of this method is also demonstrated by the small sample size required to achieve low parts-per-trillion detection limits. In a direct comparison to a previously published method for quantitative analysis of THs in zebrafish larvae, our method requires 150 larvae for sample preparation, as opposed to 200–400 larvae required for the immunoassay methods (Chen et al., 2012; Liu et al., 2011).

The extent of matrix effects was evaluated by spiking extracts of diluted, homogenized zero day embryos. At 0.5, 2, and 10 ng/mL, the peak areas of the analytes were compared with the peak areas in the standard solution at the same concentration. In the matrix, the percentage of matrix effect for T4 ranged from 79.9–103.1% and from 40.5% to 59.0% for T3, rT3, 3,5-T2 and 3,3'-T2. While there are some matrix effects, the use of internal standards and the preparation of calibration curves in a similar matrix could rectify this issue and correct quantification. Blanks of pure water (50 μL) were analyzed to determine the contribution of background throughout the entire procedure. No significant interfering peaks were detected in the blanks and no carryover was observed between runs.

To check for bench-top stability, the spiked, diluted homogenized zero day embryo sample was processed, and then stored at room temperature for 7 d. The concentrations of target analytes were quantified throughout the 7 d period, and the relative standard deviation (RSD) was less than 10.9%. These findings suggest that suggests the derivatives are stable over a 7 d period at room temperature. To check for freeze and thaw stability, the spiked, diluted homogenized zero day embryo samples were subject to three freeze-thaw cycles prior to analysis. The concentration recoveries ranged from 79.6 to 112.5% illustrating stability of THs over the course of multiple freeze-thaw cycles.

3.4. Application of the assay for TH determination in zebrafish

The UPLC-MS/MS method was applied to the analysis of TH in zebrafish larvae treated with PCB 28, 66, or 95 or the technical mixture Aroclor 1254 from 6 hpf to 120 hpf. The effects of these PCB exposures on TH levels in zebrafish larvae are shown in Fig 3. We observed T4, T3 and rT3 in control and exposed groups; however, the levels of 3,5-T2 and 3,3'-T2 were below the method detection limits. In the control group, T4 levels were 5.98 ± 0.06 pg/larvae, T3 levels were 0.44 ± 0.17 pg/larvae, and rT3 levels were 0.16 ± 0.06 pg/larvae. Compared to the control, the PCB-exposed groups exhibited changes in T4 levels ranging from 90.6% to 112.9%, changes in T3 levels ranging from 43.6% to 129.3%, and changes in rT3 levels ranging from 78.4% to 96.5%. The T4, T3, and rT3 levels were not

significantly altered by any of the PCB exposures; however, exposure to PCB 28 or 95 resulted in decreased levels of T3 that neared statistical significance ($p = 0.054$ and $p = 0.052$, respectively). In addition to TH concentrations, the ratios of T4:T3 and T3:rT3 were evaluated as indicators of TH deiodinase activity and overall TH balance. T4 is deiodinated to T3 by deiodinase 2 (dio2) or deiodinase 1 (dio1), which serve an important function in regulating intracellular T3 levels. T4 can also be directly deiodinated to rT3 by the deiodinase 3 (dio3) enzyme. The ratio of T4:T3 can serve as an indicator of dio 1 and dio 2 activity, whereas the ratio of T3:rT3 can serve as an indicator of relative dio3 activity. In addition, these parameters highlight the overall balance of the complete thyroid system including the central hypothalamic-pituitary-thyroid axis and the extra thyroidal tissues (Noyes et al., 2014). The ratio of T4:T3 was significantly increased ($p < 0.05$) while the ratio of T3:rT3 was significantly decreased in larvae treated with PCB 28 and PCB 95 ($p < 0.05$). These results suggest that PCB 28 and 95 may disrupt cellular TH homeostasis by altering deiodinase activity or deiodinase gene expression. It has been hypothesized that deiodinases may be one of many targets of PCB-mediated TH disruption, and the PCB mixture Aroclor 1254 has previously demonstrated the ability to alter activity of dio1 and dio 2 in rodent studies (Hood et al., 2000; Morse et al., 1996). The changes observed in the T4:T3 ratio and the T3:rT3 ratio highlight the importance of simultaneous quantification of THs to evaluate both changes in TH concentrations and alterations in TH balance and regulation, which have implications for cellular activity and signaling.

3.5. Method comparisons

The significance of this method is demonstrated by simultaneous detection of a set of critical THs in a small sample size while achieving low parts-per-trillion detection limits in zebrafish larvae. Radioimmunoassay kits are routinely used in clinical settings to measure free and total T3 and T4 in most clinical laboratories (Ekins, 1990; Midgley, 2001; Stockigt, 2001). Also, diiodothyronines have been detected by immunoassays (Horst et al., 1989). However, radioimmunoassay or immunoassay methods generally demand larger sample sizes, and measure THs individually, thereby limiting critical assessment of TH homeostasis (Chang et al., 2012; Liu et al., 2011; Yan et al., 2012). Another limitation is that commercial radioimmunoassays are validated over a narrow range of standards typically well above the comparatively lower levels of THs in matrices such as zebrafish larvae. Using ELISA analysis of zebrafish larvae, Yan et al. reported the LODs of T4 and T3 at 3 and 0.12 ng/mL, while Liu et al. reported 3 and 0.6 ng/mL (Liu et al., 2011; Yan et al., 2012). The LODs of our method were 0.1 and 0.12 ng/mL for T4 and T3, a 30-fold increase in sensitivity for T4. Although commercial radioimmunoassay kits are routinely used to determine THs in human serum, there are very few reports of their use in zebrafish larvae. Using ELISA kits from two different manufacturers, T4 and T3 levels in zebrafish larvae were 21.4 and 1.64 ng/g in one study, while another study reported 0.52 and 0.11 ng/g at 96 hpf (Chang et al., 2012; Yan et al., 2012). Furthermore, comparison of data using identical ELISA kits from the same manufacturer showed T4 and T3 levels in zebrafish larvae of 17.7 and 1.55 ng/g while levels were reported to be 0.58 and 0.18 ng/g at 120 hpf in another study (Chang et al., 2012; Liu et al., 2011). Since identical immunoassay methodology was used in all experiments, differences in sample preparation are more likely to have played a role. Data generated in our study are comparable to the findings reported by Yan et al. (Yan et al., 2012) and Liu et

al. (Liu et al., 2011) but differ from the results reported by Chang et al. (Chang et al., 2012), who reported T4 and T3 levels of 0.188 and 0.052 pg/ larvae, respectively. In contrast, we report T4 and T3 levels of 5.98 and 0.44 pg/ larvae at 120 hpf, approximately 8 times higher.

In studies assessing TH levels in wildlife with exposure to environmental stressors, LC-MS/MS was viewed as the more reliable and accurate method (Kunisue et al., 2011a). Several studies have evaluated the correlation between serum TH concentrations as measured by LC-MS/MS and ELISA methods using simple regression analysis (Kunisue et al., 2011a). When comparing TH concentrations in human sera measured by ELISA to LC-MS/MS data, significant correlations for T4 ($r = 0.852$) and T3 ($r = 0.676$; after elimination data from a serum sample with abnormal T3 level) were found. In contrast, low correlation for T4 ($r = 0.466$) and no correlation for T3 ($p = 0.093$) were found between ELISA and LC-MS/MS generated data in sera of Baikal seals (Kunisue et al., 2011a). Thus, mass spectrometry (MS) is considered as a superior detection method for THs with high specificity compared to radioimmunoassays or immunoassays. Herein, we demonstrate the application of UPLC-MS/MS to studies where accurate and low level quantification of THs is critical.

4. Conclusions

An analytical method for parts-per-trillion level quantitative analysis of THs in zebrafish larvae has been developed and validated. The key features of this method are the simultaneous detection of five THs in zebrafish larvae, the use of small sample size (150 larvae), and the short chromatographic run time (9 min). A quick analysis of THs in zebrafish exposed to TH disrupting PCBs using the developed method demonstrated the relevance of this method for the rapidly evolving research field of endocrine disruption in the zebrafish model. This method serves as a key method to the use of zebrafish for neurotoxicology research.

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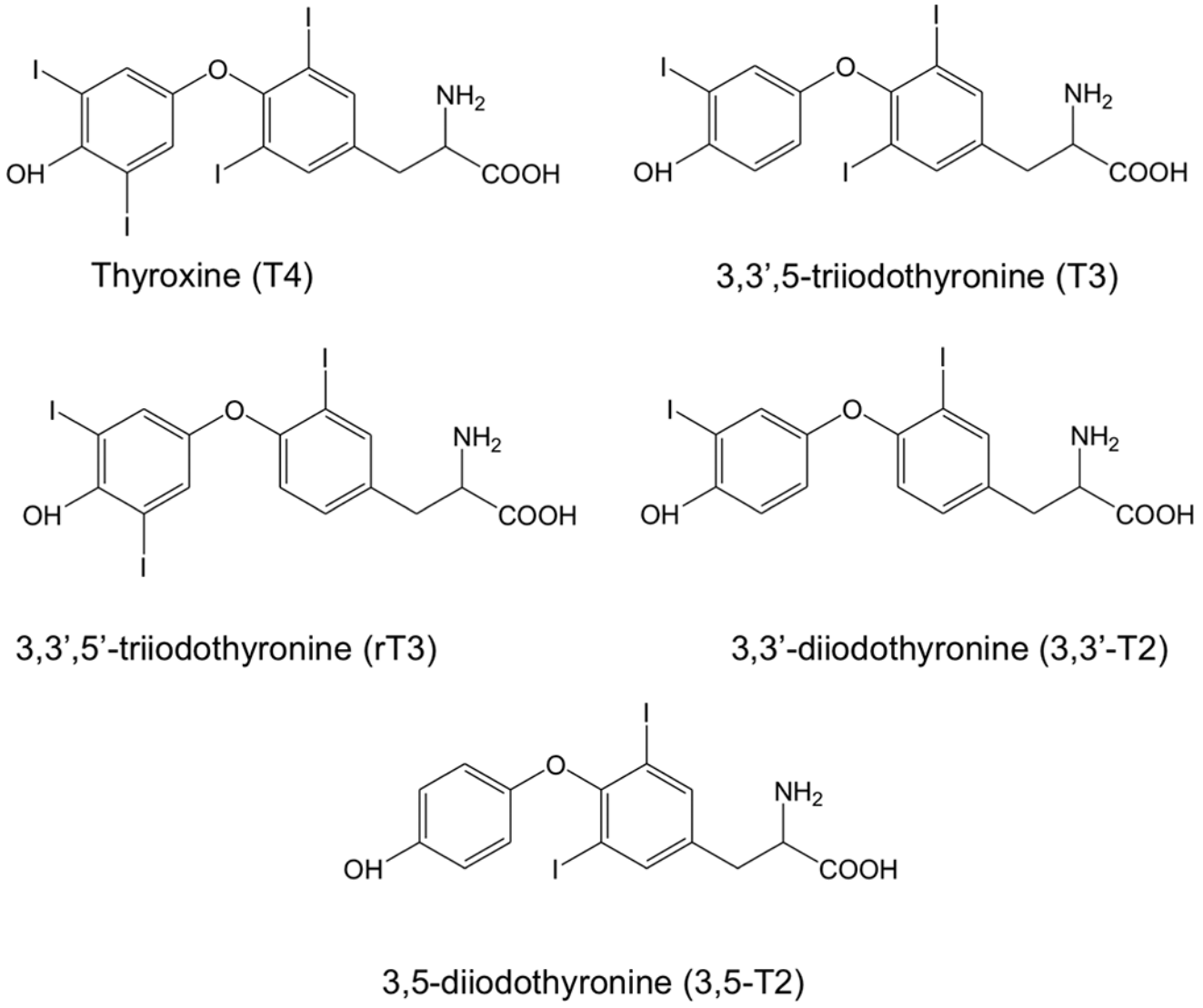

Figure 1.
Chemical structures of thyroid hormones (Created by ChemDraw Ultra 8.0)

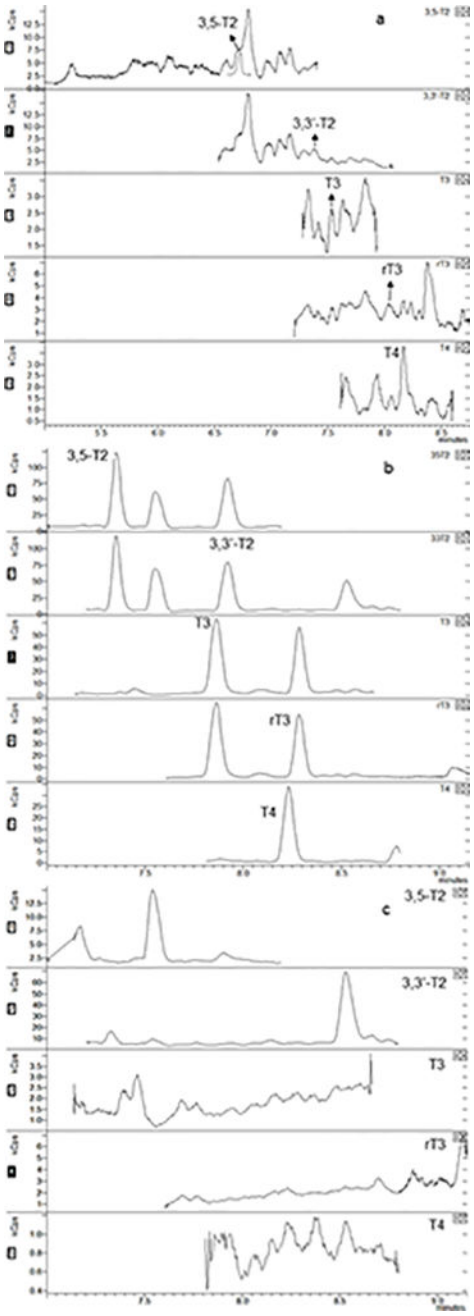

Figure 2.

MRM chromatograms of underivatized (a), derivatized (b) thyroid hormones and blank matrix (c). Underivatized and derivatized thyroid hormones are from two identically spiked samples (100 zero day zebrafish embryos spiked with five THs at 0.6 ng per sample). Blank matrix consisted of diluted homogenized zero day zebrafish embryos. (Created by Bruker MSWS 8.1 software)

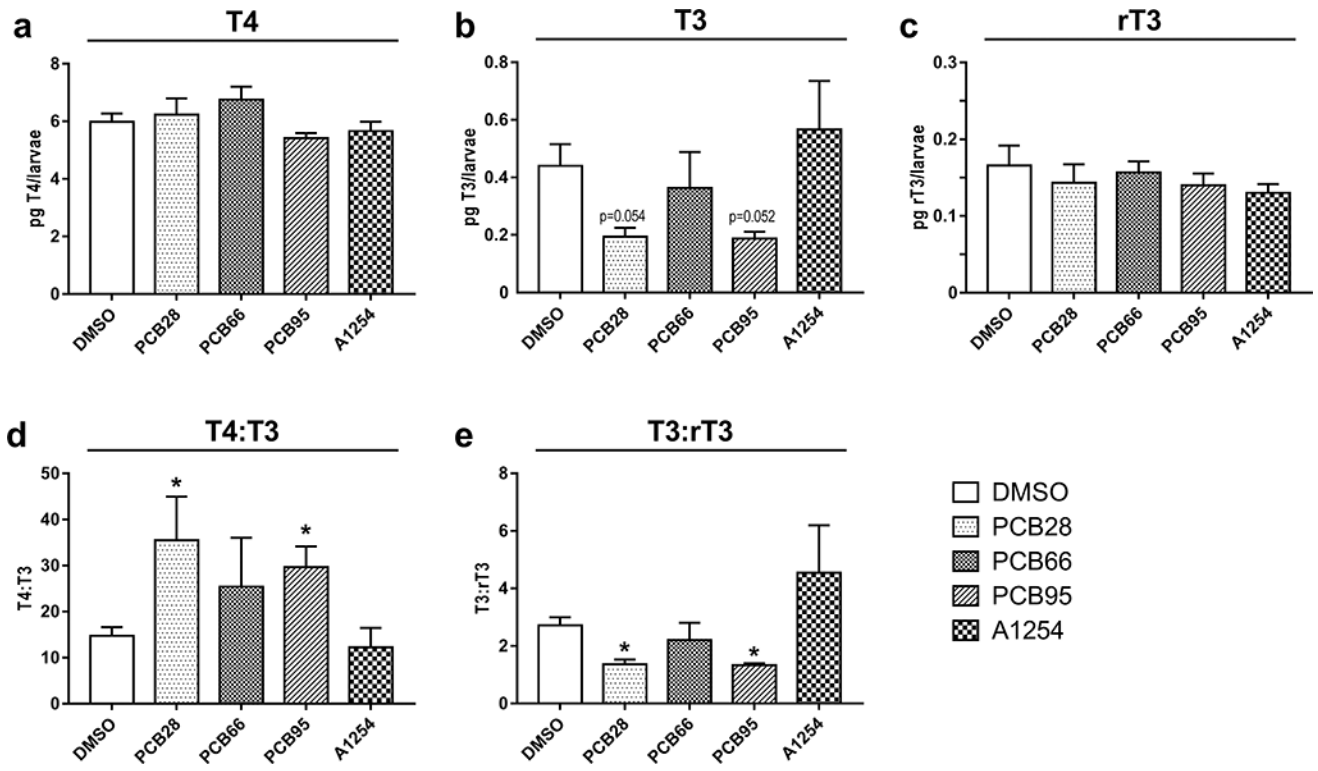

Figure 3.

Thyroid hormone concentrations at 120 hpf following static 114 h treatment with 10 μM PCB 28, PCB 66, PCB 95, Aroclor 1254 or 0.02 % DMSO (vehicle control). Concentrations of T4 (a), T3 (b), and rT3 (c) as well as the T4:T3 ration (d) and T3:rT3 ratio (e) are presented as the mean (pg TH/larvae) from pooled samples of 150 larvae at 120 hpf. DMSO controls (n=5), each PCB treatment (n=3). Errors bars are SEM. * $p < 0.05$ in unpaired t-test relative to DMSO control (Created by GraphPad Prism 7.0)

Table 1

Tandem mass spectrometry (MS/MS) parameters and multiple reaction monitoring (MRM) transitions in positive mode

Analyte	MRM transitions (m/z)	Collision energy (V)
3,5-T2	(+)581.8→479.7 (Q)	21
	(+)581.8→352.8 (C)	36
	(+)581.8→324.7 (C)	46
3,3'-T2	(+)581.8→479.7 (Q)	22
	(+)581.8→352.7 (C)	38
	(+)581.8→381.8 (C)	24
T3	(+)707.7→605.4 (Q)	26
	(+)707.7→478.6 (C)	42
	(+)707.7→197.9 (C)	63
rT3	(+)707.7→605.5 (Q)	26
	(+)707.7→478.6 (C)	45
	(+)707.7→507.6 (C)	27
T4	(+)833.6→731.3 (Q)	32
	(+)833.6→604.5 (C)	49
	(+)833.6→777.4 (C)	20
$^{13}C_6$-T3	(+)713.8→611.6 (Q)	28
	(+)713.8→484.7 (C)	47
	(+)713.8→657.6 (C)	19
$^{13}C_6$-T4	(+)839.6→737.4 (Q)	33
	(+)839.6→610.4 (C)	48
	(+)839.6→783.4 (C)	20

Q quantitation ions
C confirmation ions

Table 2

Slopes, intercepts and R^2 of calibration curves (n=7)

Analyte	Slope	Intercept	R^2
3,3'-T2	4.1069 ± 0.7638	0.1769 ± 0.1516	0.9953 ± 0.0061
3,5-T2	4.2561 ± 0.4308	0.0440 ± 0.0869	0.9930 ± 0.0056
rT3	2.5788 ± 0.8096	−0.1177 ± 0.3354	0.9966 ± 0.0039
T3	2.9713 ± 0.7719	0.0837 ± 0.4190	0.9952 ± 0.0043
T4	0.2166 ± 0.0090	0.0013 ± 0.0010	0.9980 ± 0.0013

Values are mean values ± SD.

Table 3

Intra-batch accuracy, inter-batch accuracy, intra-batch precision and inter-batch precision of UPLC–MS/MS measurement of analytes and method limit of detection (LOD)

Analyte	Concentration (ng/mL)	Intra-batch accuracy [a] (%)		Inter-batch accuracy [b] (%)		Intra-batch precision [c] (%)	Inter-batch precision [d] (%)	LOD [e] (pg)
		Mean	SD[f]	Mean	SD			
3,3'-T2	0.5	112.65	8.04	109.38	3.14	3.62	3.51	0.5
	2	109.63	6.57	112.57	3.35	9.59	3.65	
	10	102.93	6.33	110.26	7.34	5.94	8.16	
3,5-T2	0.5	105.45	8.47	100.75	4.33	10.99	5.26	0.5
	2	110.78	13.21	116.82	5.09	12.40	5.34	
	10	112.09	8.06	113.90	1.86	4.37	2.00	
rT3	0.5	100.13	5.76	112.33	10.71	9.96	11.67	0.5
	2	123.86	13.08	121.68	1.57	10.42	1.58	
	10	118.18	10.63	119.62	8.10	10.97	8.29	
T3	0.5	113.14	16.35	119.01	4.43	10.06	4.56	0.6
	2	122.15	8.21	123.51	4.04	12.35	4.01	
	10	111.60	8.96	118.36	5.96	7.02	6.17	
T4	0.5	87.30	7.86	87.83	0.38	8.97	0.53	0.5
	2	96.79	5.03	92.90	2.98	5.16	3.93	
	10	93.42	4.05	90.34	2.32	1.90	3.14	

[a] Intra-batch (intra-assay) accuracy was determined by four determinations per concentration and was expressed by the percentage of deviation between nominal and calculated mean concentrations. Samples were extracted, derivatized and analyzed in one batch.

[b] Inter-batch (inter-assay) accuracy was determined by four determinations per concentration and was expressed by the percentage of deviation between nominal and calculated mean concentrations. Samples were extracted, derivatized and analyzed in three consecutive batches.

[c] Intra-batch (intra-assay) precision (coefficient of variation) was determined by four determinations per concentration and was expressed as CV (%) for replicate measurements. Samples were extracted, derivatized and analyzed in one batch.

[d] Inter-batch (inter-assay) precision (coefficient of variation) was determined by four determinations per concentration and was expressed as CV (%) for replicate measurements. Samples were extracted, derivatized and analyzed in three consecutive batches.

[e] LOD: The method limit of detection was based on a signal to noise ratio of 3:1

f_{SD}: standard deviation