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Measurement of thyroxine and its glucuronide in municipal wastewater and solids using weak anion exchange solid phase extraction and ultrahigh performance liquid chromatography-tandem mass spectrometry

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ABSTRACT

A solids extraction method, using sonication in combination with weak anion exchange solid phase extraction, was created to extract thyroxine (T4) and thyroxine-*O*-β-D-glucuronide (T4-Glc) simultaneously from wastewaters and sludges, and to quantify these compounds via reversed-phase ultra-high performance liquid chromatography-tandem mass spectrometry. The method limits of quantification were all in the low ng/g (dry weight solids) range for both T4 and T4-Glc: 2.13 and 2.63 ng/g respectively in primary wastewater, 4.3 and 28.3 ng/g for primary suspended solids, for 1.1 and 3.7 ng/g for return activated sludge. Precision for measurements of T4 and T4-Glc were 2.6 and 6.5% (intraday) and 9.6 and 5.7% (interday) respectively, while linearity was 0.9967 and 0.9943 respectively. Overall recoveries for T4 and T4-Glc in primary suspended solids were 94% and 95%, and 86 and 101% in primary wastewater, respectively. Extraction efficiency tests using primary sludge determined that one methanol aliquot was sufficient during the extraction process as opposed to 2 or 3 aliquots. Mass loadings at the North Main Wastewater Treatment Plant in Winnipeg, Canada showed 316%, 714%, and 714% greater T4-Glc than T4 associated with the suspended solids of the primary, secondary, and final effluent respectively, yet 765% more T4 than T4-Glc associated with the solids of the mixed liquor. Moreover, 26% of T4 and 49% of T4-Glc were associated with the suspended solids during the treatment process. This method demonstrates the need to assess accurately both metabolite conjugates of contaminants of emerging concern, as well as the sorbed levels of particle-reactive analytes such as T4 in the aquatic environment.

1. Introduction

Thyroxine ((S)-2-amino-3-[4-(4-hydroxy-3,5diiodophenoxy)-3,5-diiodophenyl]propanoic acid); (T4) is an essential hormonal regulator of several vertebrate metabolic processes. The follicular cells of the thyroid gland produce the total exclusive amount of T4 and approximately 20% of triiodothyronine (T3) in the human body, with the remainder of T3 generated by peripheral tissues (e.g. brain, placenta, muscle, pituitary) [1,2]. T4 is converted into T3 most likely through a mono-deiodination process via deiodinases [3–5]. In normal euthyroid individuals, approximately 8 and 90 μg/day respectively of T3 and T4 are secreted [6,7], with approximately 42% of secreted T4 converted to T3, suggesting that mono-deiodination is an obligatory step in peripheral metabolism of T4 [3].

In developed countries, approximately 10–15% of the total population has clinical or sub-clinical hypothyroidism [1]. The aggregate sum of the various brands of thyroxine are the third most prescribed pharmaceutical in Canada, with the majority to women aged 6–79 between 2007 and 2011 at 1.08 million prescriptions. This represented 9.8% of the total scripts during that time frame [8]; moreover, more than 400 different formulations are distributed worldwide [2]. Thus, not only is endogenous T4 metabolised and secreted, but also non-trivial amounts of peripheral T4 derived from prescription medicines such as Synthroid® and Levothyroxine® [2] contributing to T4 in the environment due to incomplete removal in wastewater treatment [9]. Thyroxine-binding globulin (TBG) is a

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high-affinity, low concentration binding protein that binds approximately 80% of T3 and 75% of T4; the remainder are associated with other lower-affinity proteins that circulate in the human body [1]. Only 0.02% T4 and 0.04% T3 are considered bioavailable in circulation [1]. Thus, it is likely that T4 would be excreted in the sorbed form to proteins found in the feces.

T4-Glc is a conjugate of T4 and glucuronic acid, and is known to be a major metabolite to inactivate and clear T4 from the body [10,11]. It is important to note that bacteria in the lower intestine (jejunum and ileum) possess glucuronidases, which results in very little T4-Glc being excreted via the feces [12]. In addition to the intestine, the kidney is also a major site of T4-Glc deconjugation in rats [12]. Thus, T4-Glc potentially found in a wastewater environment would most likely be due to the sorption of T4-Glc to suspended particulate matter from the aqueous phase; and an analytical method needs to be developed that can account for this sorbed fraction of analyte.

The potential environmental concern for thyroid hormones being released into receiving waters is two-fold. First, as aforementioned T4 and the associated metabolites have the potential to elicit physiological effects on different tissues, and elicit biological effects involved in cell signaling [13,14]. Second, given that T4 and T4-Glc bind to proteins within the human body for transport and storage, these compounds have the potential to be recalcitrant to hydrolysis, photolysis [13], or biotic transformation due to the lack of availability in the sorbed state. Levels of T4-Glc are also important to gauge, as biota (e.g. mammals and teleosts) can deconjugate and de-iodinate T4-Glc to active forms and elicit these aforementioned effects [15]. If much of the environmental load of T4 or T4-Glc is associated with particulate matter, then this proportion of the total inventory has the potential to persist environmentally, especially farther downstream from the WWTP where there is lesser potential for degradation or deconjugation via coliforms typically found throughout the wastewater treatment process [16].

While T4 and its metabolites have been analysed numerous times from biological tissues and standards [18,22–25], there is only one published report of T4 in wastewater [17]. As well, the only studies found that quantified T4-Glc was via analysing various radioisotopes [11,12]. To date, no studies have measured T4-Glc either in environmental waters or wastewater. Both provide analytical challenges, given likely low concentrations (ng/L and ng/g solids range) and potential matrix effects from the presence of copious amounts of organic matter (Table S1).

This study highlights the importance of extracting both the parent hormone T4 and its metabolite conjugate T4-Glc using a single solid phase extraction (SPE) sorbent, and quantifying in a single chromatographic run, using identical mass spectrometric parameters. This is valuable because a single sorbent and single column using identical solvents reduces preparatory and analytical time. Additionally, this reduces costs on consumable materials for additional procedures, thus making this procedure universally cost-effective and yields the potential for higher throughput of quantifying both the parent and conjugate simultaneously. It was important to build upon the framework we previously developed [18] that utilised the weak anion exchange (WAX) SPE protocol for extracting parent and conjugate pharmaceuticals. It was originally postulated that the anionic characteristics of transformation products such as conjugates (e.g. glucuronides, sulfates) would have predisposed these compounds to be found in greater proportions in the aqueous phase. However, compounds that associate with proteinaceous materials (i.e. T4 and T4-Glc) for biological storage and transport were hypothesised to exist at non-trivial levels in the particulate phases within the wastewater treatment regimen due to moderate $\log K_{OW}$ values (Table S1). Thus, a solids extraction process that was compatible with the previous framework was essential to develop before comprehensive quantification of T4 and T4-Glc occurred.

2. Materials and methods

2.1. Chemicals and consumables

Methanol, formic acid, ammonium hydroxide (28.9% v/v), and isopropanol (for sterilization) were obtained from Fisher Scientific (Fair Lawn, NJ, USA), while acetonitrile was purchased from Fisher and EMD Millipore; all organic solvents were HPLC-grade. Ultrapure Milli-Q (18 MΩ-cm) water was produced from a Synergy™ Milli-Q purification system from Millipore (Billerica, MA, USA). Nitrocellulose filter paper (0.45 μm) was obtained from Merck (Ireland), and 13 mm, 0.22 μm white PTFE luer lock inlet syringe filters was purchased from Restek (Bellefonte, PA, USA). Syringe filters were attached to an Agilent 1.0 mL glass syringe (Australia). All WAX solid phase extraction cartridges were Oasis 3 cc, 60 mg from Waters Corporation (Milford, MA, USA), Nalgene® 250 mL white HDPE bottles were purchased from Thermo Fisher (Rockwood, Tennessee, USA). Centrifuge bottles (50 mL) were purchased from VWR (Mississauga, ON, Canada). Glassware was pre-cleaned by ashing at 450 °C for 1 h to destroy organic materials unless otherwise indicated. PEEK tubing (Fisher Scientific, Toronto, ON, Canada) was used in the syphoning of environmental matrices through SPE cartridges.

2.2. Chemical standards

Standards of T4 (chemical purity 94.16%) and T4-*O*-β-D-Glc (chemical purity 98%); and matching isotopically-labeled standards T4-$^{13}C_6$ (chemical purity 98%, isotope purity 98.8%) and T4-$^{13}C_6$-*O*-β-D-Glc (chemical purity 96%, isotope purity 98.6%) (Toronto Research Chemicals, Toronto, ON) were obtained as neat powders (Table S1, Fig. 1). Methanolic stock solutions (40 mg/L) were made and stored at −20 °C. Calibration curve standard solutions (0.1, 0.5, 1, 5, 10, 50, 100, 500, 750 μg/L) for quantitative assessments were prepared from stock solutions in 50/50 (v/v) Milli-Q water:methanol and also stored at −20 °C.

2.3. Ultrahigh performance liquid chromatography-tandem mass spectrometry (UHPLC–MS/MS) methods

Chromatography was performed with a Agilent 1200 UHPLC, with separation using a Waters Acquity HSS $T3C_{18}$ column (2.1 mm × 50 mm, 1.8 μm dp), coupled to a Waters Acquity HSS $T3C_{18}$ guard column (2.1 mm × 5 mm) at 42°C at 0.4 mL/min. Injection volumes were 2 μL during optimisation and 10 μL during analysis. Precursor and product ion fragments, collision energy, abundance, and fragmentation energy for both parent and conjugate compounds were determined using Agilent's Mass Hunter Optimizer software. Optimizer results were derived from single injections of isolated analyte standards measured in positive mode ESI. Mobile phase A1 was 0.05% formic acid (FA) in Milli-Q water, B1 was acetonitrile with 0.05% formic acid, A2 was 95/5 (v/v) Milli-Q water:methanol, and B2 was 100% acetonitrile. A binary gradient elution using both channels A1 and B1 was performed as follows: 0–3.00 min linear ramp from 5% B1 to 70% B1, 3.01–5.00 hold at 70% B1, then re-equilibrated from 5.01–12.00 min at 5% B1. Upon completion of all analytical runs the columns were flushed with a binary combination of solvents of A2 and B2 for 20 min, which consisted of 10% B2, then 25 min of 95% B2 to eliminate formic acid residues for column storage.

Qualitative assessment and quantification was performed through multiple reaction monitoring (MRM) on an Agilent 6410 triple quadrupole mass spectrometer in positive electrospray ionisation mode (ESI+), a capillary voltage of 4000 V, and a source