THYROID HORMONE EXTRACTION FROM PLASMA: EXPLORING pH AND SOLVENT CHOICE

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Introduction

Thyroid hormones (THs) regulate many biological process, including the regulation of skeletal[1] and brain growth[2], amphibian metamorphosis[3], osmotic balance[4], reproduction[5] and metabolism in vertebrates [6]. An increasing number of environmental contaminants are known to disrupt the thyroid system[7,8], and as a result, there is increased emphasis on the development of reliable techniques to assess thyroid function. Liquid chromatography coupled with mass spectrometry provides the necessary specificity for thyroid hormone analysis. Methods reported in the literature often use a protein precipitation / solvent extraction step [9,10,11], which is often followed by solid phase extraction (SPE)[12,13,14,15,16].

Here we describe the modification of an existing method[16] involving protein precipitation, extraction, and application to a SPE cartridge, followed by elution. We found for fish plasma, the majority of the matrix is eluted from the cartridge along with the thyroid hormones. That is, conditions necessary to elute thyroid hormones also tend to elute proteins. Thus we felt that SPE provided no advantage in terms of sample clean-up for fish plasma, and thus developed a modified method without SPE cartridges. We have previously determined that pH is important for sex hormone extraction (unpubl. observ.), and so a similar approach was investigated in the current study. In addition to pH, we also examine the effect of extraction solvent choice on the recovery of ^{13}C-labelled thyroxine (^{13}C-T4), triiodothyronine (^{13}C-T3), reverse-triiodothyronine (^{13}C-rT3), and diiodothyronine (^{13}C-T2). The method we describe is suitable for routine determinations of T4, T3, rT3 and T2 in fish plasma.

Materials and methods

Blood was obtained from the caudal blood vessels of adult brown trout (*Salmo trutta*) that were anesthetized and sacrificed in a solution of tricane (MS-222). Plasma was separated from the red blood cells by centrifugation for 10 min at 6000xG, and a large pooled sample derived from 7 fish (5 male, 2 female) was created to ensure a homogenous matrix for the extraction trials. Aliquots of the pooled plasma were kept at -80°C prior to extraction.

3,3'-diiodothyronine (T2), 3,3',5-triiodothyronine (T3), 3,3',5'-triiodothyronine (rT3), L-thyroxine (T4), 3,3'-diiodothyronine-(phenoxy-$^{13}C_6$) (^{13}C-T2), 3,3',5-triiodothyronine-diiodophenyl-$^{13}C_6$-hydrochloride (^{13}C-T3), 3,3',5'-triiodothyronine-diiodophenyl-$^{13}C_6$-hydrochloride (^{13}C-rT3), and L-thyroxine-(ring-$^{13}C_6$) (^{13}C-T4) were obtained from Sigma-Aldrich Canada (Oakville, Ontario). A spiking solution containing all of the $^{13}C_6$-labelled thyroid hormones was prepared in methanol at concentrations of 500 pg/µL for ^{13}C-T2, ^{13}C-T3, and ^{13}C-rT3, and 2 ng/µL for ^{13}C-T4. Selected extracts were spiked with native T2 just prior to analysis in order to assess matrix effects.

This extraction is based on a method by Wang and Stapleton (2010)[16]. Briefly, 150 µL of plasma was added to a glass test tube along with 30 µL each of ascorbic and citric acid solutions (each at 50 g/L in water) to limit interconversion of TH. After vortexing for 15 seconds, ammonium hydroxide was added to adjust the pH appropriately. At each full pH interval from 4 to10, 5 samples of plasma were extracted, for a total of 35 samples for each extraction solvent (acetone, acetonitrile, ethyl acetate, isopropanol, methanol, and methyl-tert-butyl ether (MTBE)). For extraction, 1 mL of solvent was added to each sample, followed by vortexing for 30 seconds, and then the sample was allowed to stand for 30 minutes at room temperature to permit deproteination. The samples were spiked with 10 µL of a mixture of ^{13}C-labelled THs as described above, vortexed, and centrifuged at 3200xg for 5 minutes. The supernatant was decanted into a clean test tube, and the extraction process was repeated using a fresh aliquot of solvent. Recoveries were calculated by comparing chromatographic peak areas from the samples to those from a ^{13}C-TH spike in an equivalent volume of methanol.

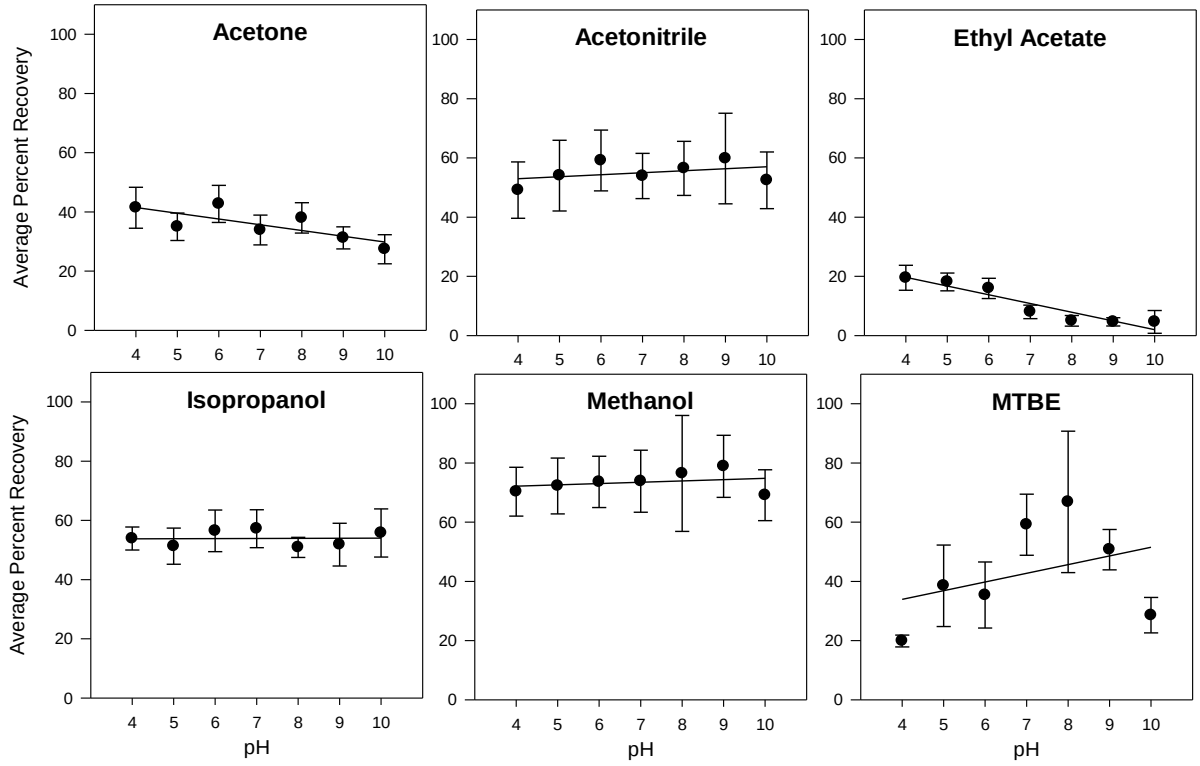
All analyses were performed on a Agilent 1100 high performance liquid chromatograph (HPLC) coupled to an Applied Biosystems API 2000 tandem mass spectrometer (MS/MS) system. Ionization was achieved using TurboSpray in the negative ionization mode.

Results and discussion:

^{13}C-Labelled Thyroid Hormone Recoveries

Overall recovery of spiked standards is the sum of the efficiency of the extraction process itself, and the ion suppression or enhancement that may occur in the ion source. Average percent recoveries were calculated at each pH, and these data are shown in Figure 1.

Figure 1: Average percent recoveries of ^{13}C-thyroid hormones from pH 4 to 10. Error bars represent one standard deviation above and below the data point. Regression lines represent the trend in recovery with respect to pH.



Ethyl acetate was evaluated because of its low water solubility (8.7 % w/w)[17] and potential for co-extracting less material from the plasma than the other solvents. This solvent was more effective at pH < 6, but recoveries were less than 10 percent above pH 6 and recoveries were never more than 30 percent. MTBE also has a low water solubility (4.8 % w/w)[17], but produced higher recoveries than ethyl acetate. ^{13}C-T2, ^{13}C-T3, and ^{13}C-T4 exhibited adequate recoveries at pH 8 (70-85 %), but ^{13}C-rT3 was low (35 %) at pH 8, as well as over the rest of the pH range. ^{13}C-T4 recoveries also showed high variability. Acetonitrile and isopropanol produced similar recoveries of between 50 and 60 percent, and did not show any variability related to pH. Acetonitrile was used in two of the studies[12,14], but in both cases SPE cartridges were used and the sample matrix was human serum, so TH recoveries are not directly comparable.

Methanol had the highest average recoveries, ranging from 72 % for ^{13}C-T4 to 89 % for ^{13}C-rT3. These recoveries are considerably greater than the 30 % T4 recovery noted by Hopley et al.[13] for their simple ethanol

extraction with human serum. In trials prior to the current study, we found ethanol recoveries to be not significantly different from those of methanol.

Overall recoveries of TH using acetone were low and ranged from 25-51% with the majority of recoveries below 40 percent. There was also a notable decrease in recovery with increasing pH. Wang et al.[16] used acetone to precipitate protein in their samples, followed by 1:1 acetone:water extractions for bovine serum. We tried this approach with our fish serum, but it seemed to re-dissolve rather than precipitate the suspended protein, adding to the co-extracted material in the extracts. The relatively high boiling point of water makes for a time-consuming evaporation in order to do a solvent exchange into methanol. It is possible that the bovine serum used by Wang et al.[16] is a less complex matrix (i.e. possibly less protein) than brown trout plasma, a factor that could account for the low recoveries we obtained with acetone.

Matrix Effects: Suppression or Enhancement

Matrix effects comprise a variety of theoretical explanations for the increase or decrease in analyte recoveries from a biological sample compared with solvent. Ion suppression is typically the more common phenomenon [18,19], and this is largely the case in the current study. The data in Table 3 are averages of five extracts at the pH with maximum average ^{13}C-standard recovery. ^{13}C-T2 was spiked at the beginning of the extraction, giving an estimate of the relative analyte amount detected after extraction and analysis, whereas the native T2 was spiked just prior to analysis, isolating the matrix effects from the extraction losses.

The T2 recoveries of acetone, acetonitrile, isopropanol, and methanol were all <100 %, indicating that some form of ion suppression occurs. Ethyl acetate produced a T2 recovery of 102 %, which indicates a slight ion enhancement. This may be due to there being less visible co-extracted material in the ethyl acetate extracts. With an apparent T2 recovery of 143 %, MTBE exhibits significant ion enhancement.

A calculation of T2 recovery - ^{13}C -T2 recovery gives an estimate of recovery losses that occur during the extraction phase. Acetone, ethyl acetate, methanol, and MTBE exhibit extraction losses ranging from 14 % (methanol) to 88 % (ethyl acetate). For ethyl acetate, the high number indicates that there are minimal matrix effects and poor recoveries during the extraction phase. Acetonitrile and isopropanol have (T2 - ^{13}C-T2) numbers of ~0 %, which translates into good extraction efficiency, but relatively high ion suppression (44 – 48 %). Methanol has a low difference between the T2 and ^{13}C-T2 recoveries (13.5 %), indicating relatively small losses during the extraction processes. Methanol extracts have an ion suppression rate of ~10 %, which is considered acceptable. Ideally, extractions methods should have consistently high extraction efficiency as well as minimal ion suppression or enhancement. Of the solvents and pHs tested, methanol extraction at pH 9 provides the appropriate balance between extraction efficiency and reduced matrix effects.

Table 3: Average percent recoveries of spiked T2 and ^{13}C-T2. T2 was spiked into the vial insert just prior to analysis, and provides an assessment of ion enhancement or suppression occurring in the LC-MS source. ^{13}C-T2 was spiked into the sample at the beginning of the extraction, and provides an assessment of all recovery losses.

	T2	**^{13}C-T2**	**(T2 - ^{13}C-T2)**
Acetone (pH 6)	64.1	40.0	24.1
Acetonitrile (pH 9)	51.7	55.1	-3.3
Ethyl Acetate (pH 4)	101.9	13.6	88.3
Isopropanol (pH 7)	55.6	57.4	-1.8
Methanol (pH 9)	89.7	76.2	13.5
MTBE (pH 8)	143.2	78.2	65.0

We have presented here a simplified method for the extraction of thyroid hormones from plasma, without the added cost and prep time of solid-phase extraction cartridges. For a batch of 25 samples, we estimate that the removal of SPE decreases extraction time by approximately 30 percent. Methanol represents the best compromise in terms of maximizing overall recovery, consistency and limiting matrix effects.

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