Developing analytical approaches to explore the connection between endocrine-active pharmaceuticals in water to effects in fish

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Abstract

The emphasis of this research project was to develop, and optimize, a solid-phase extraction method and high performance liquid chromatography-electrospray ionization- mass spectrometry method, such that a linkage between the detection of endocrine active pharmaceuticals (EAPs) in the aquatic environment and subsequent effects on fish populations could eventually be studied.

Four EAPs were studied: tamoxifen (TAM), exemestane (EXE), letrozole (LET), anastrozole (ANA); and three TAM metabolites; 4-hydroxytamoxifen, e/z endoxifen, and n-desmethyl tamoxifen. In aqueous matrices the use of isotopically labeled standards for the EAPs allowed for the generation of good recoveries, greater than 80%, and low relative standard deviations (% RSDs) (3% to 27%). TAM metabolites had lower recoveries in the spiked water matrices: 35% to 93% in waste/source water, compared to 58% to 110% in DI water. The precision in DI water was acceptable ranging from, 8%-38% RSD. However, the precision in real environmental wastewaters could be poor, ranging from 15% to 120% RSD, dependent upon unique matrix effects. In plasma the overall recoveries of the EAPs were acceptable: 88% to 110%; with %RSDs of 6% to 18 %, Table 3. The spiked recoveries of the TAM metabolites from plasma were good, ranging from 77% to 120%, with %RSDs ranging from 27% to 32%.

Two of the TAM metabolites, 4-hydroxytamoxifen and n-desmethyl tamoxifen, were confirmed in most of the environmental aqueous samples. The discovery of TAM metabolites demonstrates that the source of the TAM metabolites, TAM, is constant, introducing a pseudo-persistence of this chemical into the environment.

KEYWORDS

Aromatase inhibitors, tamoxifen, tamoxifen metabolites, LC-MS/MS, environmental chemistry

Introduction

Since the 1990's there have been thousands of publications presented in peer-reviewed literature addressing the detection of pharmaceuticals and personal care products (PPCPs) in the environment [1]. Specifically, PPCPs that are inadvertently released into the environment, as well as their potential to cause undesirable effects upon living organisms in the environment, are the main focus of concern. For example, a class of PPCPs, endocrine active pharmaceuticals (EAPs), are designed to target the endocrine system through hormonal modes of action. EAPs may be prescribed for medicinal purposes in humans and other animals, however, they can potentially be released into the aquatic environment through sewage and wastewater treatment plants, thereby impacting aquatic organisms who were not the intended target.

As an example, tamoxifen (TAM), a selective estrogen receptor modulator (SERM), is widely-used in the treatment of certain medical issues, such as; breast cancer, infertility, gynecomastia, and bipolar disorder [2-4]. Therefore there is a potential for environmental release of TAM, and subsequent inadvertent aquatic exposure through sewage and wastewater effluents. The potential for harm from TAM in the aquatic environment was demonstrated in a 2007 fish study that showed that Japanese medaka were detrimentally affected when exposed to TAM [5], by affecting their hatchability, fertility and fecundity. Also, TAM and its metabolite 4-hydroxytamoxifen (4HTAM), were shown to inhibit the respiratory activity of bacterial protoplasts, as well as, inhibit mitochondrial membrane potential in cell lines [6]. In a very early paper Furr and Jordan [7] report that 4-hydroxytamoxifen is more potent than TAM in its affinity to bind to estrogen receptors. Recent research has also demonstrated that TAM, when in combination with two other common drugs found in hospital wastewaters (i.e., ciprofloxacin and cyclophosphamide), can cause DNA breaks and inhibit algae growth at very low doses [8].

Other EAPs belong in the chemical class of aromatase inhibitors (AIs). Aromatase is the steroidogenic enzyme responsible for estrogen biosynthesis in vertebrates and is critical to normal reproductive processes. If exogenous EAPs are introduced, then the reproduction development of the animal exposed may be affected. For example, anastrozole (ANA, trade name *Arimidex*^{TM}), like TAM, is used in the treatment of breast cancer, to delay precocious puberty in boys [9], and reduce gynecomastia in boys [10], thereby increasing its potential for release into the aquatic environment. Another AI, letrozole (LET), was shown in a study by Sun et al. [11], to have an adverse effect on fish reproduction, fecundity

and endocrine function in Japanese medaka (*Oryzias latipes*). Eggs, larvae and breeding adults were exposed to various levels of LET, and dose-dependent effects were seen with regards to fertility and fecundity through inhibition of oocyte growth and maturation. Although no detrimental morphological changes were observed, trans-generational effects were observed with regards to egg hatchability and time to hatching [11]. Other researchers have demonstrated that LET can induce sex reversal in European pond turtles (*Emys orbicularis*) [12]. About 22% of the turtles showed mixed gonadal tissue (i.e., ovotestes that did not differentiate into ovaries), and this condition persisted up to a year after exposure [12]. Another study, by Liao et al. [13], demonstrated that medaka (*Oryzias latipes*) when exposed to LET at environmentally relevant concentrations showed endocrine disruption through interruption of gene expression in the adults; trans-generational effects were also observed.

The emphasis of our study was to develop, and optimize, a solid-phase extraction (SPE) method and high performance liquid chromatography-electrospray ionization- mass spectrometry/mass spectrometry (LC-MS/MS) method, such that a linkage between the detection of EAPs in the aquatic environment and subsequent effects on fish populations could eventually be studied. The chemicals under investigation in our study were narrowed to four EAPs: tamoxifen (TAM), exemestane (EXE), letrozole (LET), anastrozole (ANA); and three TAM metabolites; 4-hydroxytamoxifen, e/z endoxifen, and n-desmethyl tamoxifen. The four EAPs were chosen because of their use in the treatment of human disorders, potential for environmental release through sewage effluent [14,15], and inadvertent exposure of aquatic organisms. In addition, the information available in the literature was limited with regards to the environmental occurrence of the four EAPs, and the TAM metabolites. Nearly all of the environmentally-relevant literature, published-to-date, on the EAPs that are the focus of our study, were focused solely on TAM in water samples [16,14,17-24,15,25-27]. One article detected TAM in fresh-water fish tissue (perch) [15], there were two publications regarding TAM detected in sediments [28,29], and two papers were found regarding the detection of the TAM metabolites in the environment [22,25]. There was only one environmentally-relevant article by Liu et al., [14] regarding methods for the detection of the other three EAPs (i.e., EXE, ANA, and LET) in hospital wastewater effluents.

Finally, an *in situ* time-weighted sampler, the Polar Organic Chemical Integrative Sampler (POCIS), was assessed as an alternative to grab sampling of aqueous environmental samples.

Materials and Methods

Statement of human and animal rights

This paper does not raise any concern regarding human and animal rights. The fish used in this study were treated respectfully, and each individual fish was anesthetized using tricaine methane sulfonate (MS-222) before sacrifice.

Chemicals. TAM, and the tamoxifen metabolites (4-hydroxytamoxifen, n-desmethyl tamoxifen, and e/z-endoxifen), were obtained from Sigma-Aldrich (St. Louis, MO, USA). LET was obtained from BDG Synthesis (Wellington, New Zealand) and Cerilliant (Round Rock, Texas, USA). ANA was obtained from US Pharmacopeia (Rockville, Maryland, USA). EXE was obtained from BetaPharma (Branford, Connecticut, USA). All labeled standards: d5-TAM, d4-LET, d12-ANA and d3-EXE; were obtained either from Toronto Research Chemicals (Ontario, Canada), or Sigma-Aldrich (St. Louis, Missouri, USA).

HPLC-grade methanol was obtained from multiple sources [e.g., Burdick and Jackson (Muskegon, MI); EK Industries (Joliet, IL); JT Baker (Phillipsburg, NJ)]. Reagent grade acetic acid (glacial) and HPLC-grade methyl tert butyl ether (MTBE) were obtained from VWR (West Chester, PA). HPLC-grade acetonitrile (ACN) was obtained from Burdick and Jackson (Muskegon, MI). ACS reagent grade formic acid and ammonium hydroxide (28%) were obtained from Anachemia (Rouses Point, NY). Deionized water (DI) was produced on-site using a NANOpure^{TM} filtration system (Barnstead, Dubuque, Iowa, USA).

Stock standard solutions were individually prepared in HPLC-grade ACN, from the neat compound, and stored in darkness at < 4^{o}C. A high-level standard mix, used for spiking and calibration standards, was prepared bimonthly in ACN, at concentrations of 10 to 20 ng μL^{-1}, and stored in darkness at < 4^{o}C until use. A mass spectrometric calibration standard was prepared weekly in 99% ACN:1% acetic acid from the high-level standard mix, at concentrations ranging from 0.25 ng μL^{-1} to 2 ng μL^{-1}.

Sample collection

Water samples were collected from sites in Missouri, Nevada, and Ohio, using either grab sampling, or the passive time-weighted sampling technique, POCIS [30]. Passive samplers were deployed for approximately 30 to 60 days at certain collection sites, and collected in conjunction with the grab sampling collection dates to allow for the comparison to grab sampling [31].

Grab sampling. A pre-cleaned (i.e., acid washed, rinsed with methanol and de-ionized water, then baked at 105ºC until dry) 4-L amber glass bottle was submerged under water until filled. The grab samples were placed in a cooler, on ice, transported overnight to the laboratory, and stored at < 4ºC until extraction. Extractions usually occurred on the date of receipt of the samples. Extracted samples were analyzed by LC-MS/MS [31].

Passive sampling - POCIS. Passive sampling devices were used to obtain a time-weighted average (TWA) concentration of dissolved organic contaminants at select sites. The POCIS was chosen for this study because it is designed to sample organic chemicals ranging from hydrophilic to moderately hydrophobic [30]. The EAPs pass through the microporous membrane of the POCIS and are trapped onto a solid-phase sorbent. After the POCIS devices are recovered from the field site, and brought back to the laboratory, they are gently cleaned and the sorbents from each POCIS are transferred into empty SPE cartridges (25 mL capacity) for extraction. The sequestered chemicals are then recovered from the sorbent in the laboratory using a simple organic solvent extraction [32].

Experimental fish plasma

All fish experimentation was performed in collaboration with USEPA's Atlantic Ecology Division, National Health and Environmental Effects Research Laboratory (NHEERL) in Narragansett, Rhode Island.

Adult cunner (*Tautogolabrus adspersus*), between 11-15 cm length and 20-45 grams weight, were collected from Narragansett Bay in southern Jamestown, Rhode Island during the fall of 2008 and overwintered in the laboratory in large holding tanks. In the spring, spawning cunner (one male and two female per tank) were moved to 80-cm tall, 114-L high-density polyethylene experimental tanks with clear Plexiglas™ cover, and 1 L min^{-1} flow-through of seawater (18°-20°C). The fish were exposed to a photoperiod of 15 hr light and 9 hr dark, with simulated dawn and dusk light dimming, and fed blue mussels *ad libitum* every day.

Fish in each tank were randomly assigned to one of three treatments: control (matrix only), low dose (0.075 mg kg^{-1}: EXE, LET, ANA; 0.5 mg kg^{-1}: TAM); or high dose (0.750 mg kg^{-1}: EXE, LET, ANA; 5 mg kg^{-1}: TAM). There were six replicate tanks for each treatment. All treatments were delivered to each fish by oral gavage and renewed every four days. The individual test chemicals (i.e., TAM, LET, ANA or EXE) were delivered in a suspension (matrix) of methyl cellulose and DI water. Control animals were given methyl cellulose in DI water only. Each fish received a gavage treatment a total of five times during the course of the experiment. For gavage, fish were lightly anaesthetized with MS-222 and treatment was delivered using a 1-ml glass syringe with a 4-cm piece of very thin, flexible silastic tubing attached to a blunted 18-gauge needle. The tubing was carefully inserted through the mouth of each fish and the treatment was extruded into the stomach. The volume (mL) of solution administered to each animal was equal to its body weight in grams multiplied by 0.005. Treatments were delivered on days 0, 4, 8, 12 and 16 of the experiment. All fish were sacrificed on day 17. Individual fish were anesthetized using MS-222, and as much blood as possible was drawn from the caudal vein (usually 0.1 - 0.4 ml) using a heparin-coated 1 mL tuberculin syringe with a 22-gauge needle. Blood was kept on ice in pre-chilled microcentrifuge tubes containing heparin and aprotinin until centrifuged to separate plasma. Chilled blood was centrifuged for five minutes at 5000 revolutions-per-minute (rpm) to separate plasma. Plasma samples were rapidly

frozen in liquid nitrogen and stored at -80°C, until shipped on dry ice to the USEPA, National Exposure Research Laboratory-Las Vegas laboratory, for extraction and analysis by LC-MS/MS.

Fish plasma extraction.

A manual extraction procedure for fish plasma was optimized first using rat plasma, Supplemental Table 2a, since fish plasma was not as abundantly available as rat plasma, see see Electronic Supplemental Material, Table 1a and Figures 1a and 1b for optimization raw data and graphs,. Plasma samples were thawed and pipetted from the original microcentrifuge collection tubes into tared test tubes and the weight was recorded. Depending upon the sample size, 10 to 15 μLs of labeled standards (0.5 μg g^{-1} to 1 μg g^{-1}) were spiked directly into the plasma, and the samples were vortexed for 30 sec. After adding 5 mL of 90% MTBE/10% methanol, the sample was vortexed for another 30 sec, then centrifuged for 2 minutes at 1675 rpm, followed by 2 minutes at 670 rpm. The supernatant was carefully pipetted into 50mL TurboVap™ tubes and reduced in volume to 0.5 mL using a TurboVap™ evaporator (Biotage Corp., formerly Caliper Life Sciences, Hopkington, MA), set to approximately 10 psi N_2, at 25ºC, solvent exchanging with ACN/1% acetic acid, transferred to 2-mL clear glass vials, and stored in a refrigerator until analysis by LC-MS/MS.

Aqueous grab sample extractions.

A solid phase extraction procedure was optimized first in DI water, and then in waste and source waters, see Electronic Supplemental Material for optimization raw data and graphs, Tables and Figures 2a-d. The final procedure is as follows: 500 mL of water was poured into a 500 mL volumetric flask, labeled standards were spiked into each sample, pH was adjusted to > pH 9 using ammonium hydroxide (NaOH, 28%), three grams of NaCl was added to each sample, and then the flask was shaken and placed onto a previously prepared automated solid-phase extractor (AutoTrace, ThermoDionex Corporation, Sunnyvale, CA). Solid-phase extraction (SPE) cartridges (Oasis MCX 6cc cartridges, Waters Corp.) were pre-conditioned with: 5 mL methanol, 5 mL DI water, and 5 mL 95% water/5% methanol at a flow rate of 1 mL min^{-1}. The 500 mL water samples were loaded directly from the sample flasks through the SPE cartridges at a flow rate of 7 mL min^{-1}. After the sample loading finished, the cartridges were dried for 40

min using nitrogen and the analytes were subsequently eluted at a flow rate of 1 mL min^{-1} using 5 mL 90% MTBE/10% methanol, followed by 10 mL 95% methanol/5% NH_4OH. The extracts were qualitatively transferred into TurboVap™ tubes and reduced in volume to 0.5 mL using a TurboVap™ evaporator, set to approximately 10 psi nitrogen, at 23°C, solvent exchanging with ACN/1% acetic acid. The final extracts were transferred to 2-mL clear glass vials and stored (< 4°C) until analysis by LC-MS/MS.

LC-MS/MS.

A Varian 500MS (Agilent Corp., formerly Varian Inc, Walnut Creek, CA) ion trap mass spectrometer, configured with an electrospray ion source, and a Varian 212-liquid chromatograph, was used for all analyses [31]. Due to potentially interfering materials co-extracted with the EAPs, the analyses were performed using the collision induced dissociation (CID) mode, referred to as LC-MS/MS, for both identification and quantitation of the analytes of interest [31]. Two to three product ions were used for identification, and the most intense product ions were chosen for quantification. The product ions used to identify the EAPs and their limits-of-detection (LOD) are shown in Table 1.

The 500MS was operated in the positive ionization mode under the following conditions: ES needle, 5 kV; drying gas, set at 20 psi and 350° C; housing chamber, 50° C; nebulizer gas, 40 psi; and spray shield, 600 V. Capillary voltage and percent radio frequency (%RF, on the hexapoles) were set dependent upon the optimized response of the precursor and product ions of interest [31].

Liquid chromatographic separations were performed using either a Phenomenex Fusion-RP, 4 µm, 150 mm x 2.1 mm column, or a Sigma-Aldrich Ascentis C_{18} , 2.7 µm, 100 mm x 2.1 mm column, coupled with a Varian guard column (MetaGuard Pursuit XRs C_{18}, 3 µm, 2.0 mm). Compositions of the mobile phases were as follows: (A) DI water/0.5% formic acid, and (B): 82% methanol/18% ACN/0.5% formic acid. The flow rate through the column was 300 µL min^{-1}, with the following gradient elution conditions: mobile phase A 100%, hold for 2 min; 3 min gradient to 30% A:70% B, hold for 5 min; 3 min gradient to 100% A, hold for 2 min; end run, 5 min equilibration time between analyses [31].

LOD/LOQ.

The LOD was determined by analyzing a 4-point standard calibration curve, and a solvent blank,

for each compound four times [33]. Using linear regression, and the slope of the line (passing through zero), the LOD (in units of ng on-column) was calculated from the calibration curve and 3 times the standard deviation (3σ) of the blank area counts, Equation 1. The LOD is further defined for the extraction and detection method by the volume injected on-column (10 µL), the volume of sample extracted (500 mL for aqueous media), and the final extract volume (500 µL), Equation 2. The limit-of-quantitation (LOQ), defined as 10σ, was calculated by substituting 10 for 3 in Equation 1. The results are shown in Table 1. The standard calibration curves were linear, > 70% of the compounds had r^2 > 0.99, and all had r^2 > 0.97.

Equation 1.

$$y = [[(3 * s) + x)]/m] * b$$

y = LOD (in ng on-column)

s = standard deviation of area counts

x = average of area counts

b = microliters of standard injected (10 µL)

m = slope of calibration curve [(area counts)/(ng/µL)]

Equation 2.

$$Y = [(y/b)*c]/d * 1000 \text{ (mL } L^{-1})$$

y = LOD (ng)

b = microliters of standard injected (10 µL)

c = amount of final extract (500 µL)

d = amount of volume of aqueous media extracted (500 mL)

Y = final method LOD (ng L^{-1})

Quality Control.

Precision and accuracy of calibration standards and sample spikes (in all matrices) were determined over the course of the study. Trip blanks (water and POCIS) and control blanks (fish and rat plasma) were analyzed alongside each batch of extractions. Spiking the sample matrices with surrogates (i.e., deuterated EAP standards: d5-TAM, d12-ANA, d3-EXE, and d4-LET), and the analytes of interest before extraction, and then comparing the amount detected with the amount spiked, determined the recoveries of the EAPs and TAM metabolites, see Table 1.

Results and discussion

It was found that the EAPs had limited stability in methanol that was initially used to make the stock standards. Teunissen et al. [34] suggested that TAM and TAM metabolites are stable in methanol at a minimum of 2 hours at room temperature, but suggested storing them at -70^{o}C. Teunissen et al. (2011), also discovered that n-desmethyl tamoxifen is sensitive to light and temperature [34]. However, in our study, ACN was found to be the preferred solvent medium used to formulate stock standards, calibration standards, as well as the solvent medium for the SPE extracts. The stock standard solvents, and the final extract solvents, were switched from methanol to ACN in order to prevent deuterium exchange from occurring with the deuterated EAP surrogates (isotopically labeled standards: d5-TAM, d12-ANA, d3-EXE, and d4-LET). Also, there was mass spectrometric evidence of hydrogen and hydroxyl exchanges occurring between the EAP standards and the methanol.

Quantitation of the four EAPs, except for the TAM metabolites, was based on an isotope dilution quantitation method as established in EPA Water Method 1694 [35], whereby isotopically labeled standards are used on a one-to-one basis for each of the four EAPs (i.e., d5-TAM, d12-ANA, d3-EXE, and d4-LET). Determination of the EAPs was accurate, using a minimum of two product ions for confirmation (only the major product ions are listed in Table 1), as well as, a retention time window of ± 30 sec from the standard retention time. An internal standard quantitation method, as established by USEPA Water Method 1694 [35], was used to quantitate the amounts of the TAM metabolites present. The metabolites proved

difficult to quantitate due to unexpected multiple, and inconsistent, fragmentation processes occurring in the ion trap of the LC-MS/MS. The precursor ion for the metabolites, the $(M+H)^+$ ion, remained intact when analyzing these compounds in the full-scan mode, but when a slight voltage was applied during CID analysis multiple fragment ions were produced, sometimes at different ratios than previous analyses. Eventually, optimized LC-MS/MS source parameters allowed for unique fragment ions to be chosen for identification and quantitation of the TAM metabolites, Table 1.

In heavily polluted environmental matrices the chromatography of these compounds was prone to difficulties. The EAPs, and TAM metabolites, were poorly resolved one from another, and their mass chromatograms showed degradation quickly on the LC column after just very few analyses. The solution to this problem necessitated clean-up of the mass spectrometer's source, column re-conditioning after a few injections, then re-analysis of standards and re-analysis of sample extracts.

LOD and LOQ.

The LODs and LOQs obtained for the four EAPs, and three TAM metabolites, were in the parts-per-trillion (ppt) range, ranging from 10 ng L^{-1}(ANA) to 65 ng L^{-1} (TAM). These LODs are comparable to other ECs detected in the environment, such as macrolide antibiotics [31]. As an example, the LOD for TAM was 65 ng L^{-1}, and the LOD for azithromycin (a widely prescribed antibiotic in the US) was 2.5 ng L^{-1} [31]. In comparison to other methods reported for TAM, the LOD was approximately 76-fold lower than that reported by Teunissen et al. [34], 65 ng L^{-1} vs. 5000 ng L^{-1}, but were one to two orders of magnitude higher than those obtained by Negreira et al. [22] and Lopez-Serna et al. [23], 0.3 ng L^{-1} and 0.01 ng L^{-1}, respectively. The difference in LOD levels obtained in different laboratories might be partly attributed to the differences in instrumentation, and the LODs and LOQs are expected to continue to improve assuming that hybrid high-performance high mass resolution mass spectrometers will continue to become more available and affordable.

Extraction method performance.

Water. An optimized SPE extraction method for the recovery of the four EAPs, and TAM metabolites, was tested in DI water, wastewater, and source waters. The EAPs, and TAM

metabolites, were spiked at varying concentrations, ranging from 0.2 µg L^{-1} to 2 µg L^{-1}, and extracted from spiked samples during the study, Table 2. All EAP recoveries were better than 80%, with most being 90%, or greater, in all aqueous matrices. The use of isotopically labeled standards for the EAPs (i.e., d5-TAM, d12-ANA, d3-EXE, and d4-LET) in the method allowed for the generation of good recoveries, 82% and greater, and low relative standard deviations (% RSDs) (3% to 27%). These recoveries are better than those reported by Zhou et al. [20], wherein their recoveries of TAM ranged from 52% in river water, to 55% in WWTP effluents.

In this study the TAM metabolites had lower recoveries in the spiked water matrices, 35% to 93% in waste/source water, and 58% to 110% in DI water. The precision in DI water was generally good ranging from, 8%-38% RSD. However, the precision in real environmental waste/source waters could be poor, ranging from 15% to 120% RSD in a wastewater sample. Some of the wastewater samples contained large amounts of interferents (e.g., surfactants) that interfered with, or masked, the recovery of the TAM metabolites; to the point that the spiked TAM metabolites were not recovered at all in some samples. The internal standard quantitation method [35], that was used for quantifying the metabolites (d5-TAM as the internal standard), was not as accurate as the isotopically labeled quantitation method used to quantify the EAPs. Nor can an internal standard quantitation method, unlike an isotopically labeled quantitation method, fully account for the irregularities that can occur during the electrospray ionization process. However, isotopically labeled standards for the TAM metabolites are expensive, or non-existent, and were therefore not considered for this study.

Plasma. Fish plasma (< 0.2 g) were spiked with 150 ng to 500 ng of each of the four EAPs, equivalent to 750 to 2500 ng g^{-1}. Overall, the recoveries of the EAPs were acceptable, ranging from 88% to 110%, as was the reproducibility, with %RSDs of 6% to 18 %, Table 3. One gram of rat plasma, spiked with 500 ng g^{-1} of TAM metabolites, was used for the extraction studies of the TAM metabolites. Overall, the recoveries of the TAM metabolites were good, ranging from 77% to 120%, with %RSDs ranging from 27% to 32%.

Fish dosing data.

Adult cunner fish, (*Tautogolabrus adspersus*), were dosed over a series of days, with each of the individual four EAPs. ANA was detected in the fish plasma only in the higher dosed fish, 0.75 mg g^{-1}. LET was detected in the fish plasma from both the low (0.075 mg g^{-1}) and high level (0.75 mg g^{-1}) dosed fish. However, EXE was not detected in either the low (0.075 mg g^{-1}) or high (0.75 mg g^{-1}) dosed fish plasma. The results from those dosing experiments are shown in Table 4.

In the TAM-dosed fish, TAM was not detected in any fish plasma samples. However, two of the three metabolites of TAM, 4-hydroxytamoxifen and n-desmethyl tamoxifen, were detected in the TAM-dosed fish. 4-hydroxytamoxifen was detected at 370 ng g^{-1} in one high dosed (5 mg kg-1) fish, 500 ng g^{-1} in one low dosed (0.5 mg kg-1) fish, and at 270 ng g^{-1} in one pooled control fish plasma sample. The other TAM metabolite, n-desmethyl tamoxifen, was detected in all the fish plasma samples, including the fish plasma control samples, as well as in the rat plasma controls, Table 4. Through mass spectrometric analyses, it was determined that the n-desmethyl tamoxifen detected in the rat plasma controls was from the degradation of the labeled internal standard, d5-TAM, partially converting to n-desmethyl tamoxifen through deuterium exchange, and degradation of d5-TAM to n-desmethyl tamoxifen through demethylation. Therefore, all of the plasma (i.e., fish and rat) data were background corrected for n-desmethyl tamoxifen. Following the background correction, a substantial amount of n-desmethyl tamoxifen was still determined to be present in the fish plasma samples.

Finally, the TAM metabolite e/z endoxifen was not detected in any of the fish plasma samples.

Environmental data.

In our study, twenty-three individual grab samples were taken from sites in Missouri, Nevada, and Ohio, an overview of the results are shown in Table 5. Only two EAPs were identified: letrozole was spectrally confirmed at one Nevada sampling site, NV WWTP, but the level detected was below the LOD; and TAM was detected, spectrally confirmed, at one site in Ohio, OH WWTP-3. When this study was first undertaken the metabolite standards were not available. Therefore, when the sample from OH WWTP-3 site was initially extracted (within 4 days from receipt) and analyzed (within 18 days from extraction), and

TAM was detected, this sample (and other OH samples) was re-extracted at a later date to screen for the TAM metabolites. During the subsequent re-extraction (6 months after receipt of the initial sample) and re-analysis, TAM was not detected in the same sample, however, the TAM metabolite, 4-hydroxytamoxifen, was detected at 430 ng L^{-1}, Electronic Supplemental Material, Table 3. While the original samples were stored in a walk-in refrigerator at < 4°C, and at neutral pH 7, it is probable that the TAM, initially measured in the water sample, had transformed over time to the metabolite 4-hydroxytamoxifen. There have been reports in the literature regarding the instability of the TAM metabolites and TAM. For example, Morin et al. [36] reported highly variable concentrations of TAM in a controlled dosing study, which was attributed to TAM's poor stability in water.

Initially, the results in our study seemed inconsistent with what others had reported globally; detailed concentrations are presented in Electronic Supplemental Material, Table 3. For example, Zhou et al. [20] reported detecting TAM in 100% of their WWTP effluent samples in the United Kingdom (UK), ranging from 0.1 – 1.3 ng L^{-1}. The Swedish Environmental Research Institute (SERI) reported detecting TAM in Swedish WWTP effluents ranging from not detected (ND) to 210 ng L^{-1} [15]. However, SERI also reported that the removal efficiencies of TAM, from the various WWTPs in their study, ranged from -22% to 74% [15]. So the fact that TAM was not detected in any of the samples, except one, demonstrates that there are a range of possibilities regarding WWTPs and removal efficiencies. Only one other study to date has been published that looked at the environmental concentrations of TAM metabolites in raw wastewater, Negreira et al. [22]. In their study, none of the TAM metabolites were detected, and TAM was detected ranging from ND to 17.2 ng L^{-1} [22]. In our study, at various sampling sites, the TAM metabolites, 4-hydroxytamoxifen and n-desmethyl tamoxifen, were detected in water samples. The concentrations detected ranged from ND to 1250 ng L^{-1} for 4-hydroxytamoxifen; ND to 1000 ng L^{-1} for n-desmethyl tamoxifen; and e/z endoxifen was not detected in any environmental samples, Electronic Supplemental Material, Table 3.

Comparison of POCIS (time-weighted sampling) vs. Grab sampling.

As a proof of concept determination for the variables measured in the laboratory, the POCIS were deployed in the field for periods of 30-60 days at multiple locations receiving WWTP effluents in Missouri and Nevada. At each of these sites, sets of POCIS were deployed in, or as close as possible to, the effluent stream. Table 6 shows the three sites where POCIS and grab samples were simultaneously deployed, and a grab sample collected during the deployment of the POCIS. No EAPs were detected at any of the three sites in either the POCIS or grab samples. TAM metabolites were detected, but at only one site (the Missouri wetlands site) could the TAM metabolite data be compared, due to the lack of the TAM metabolite standards early on in the study. The TAM metabolite, 4-hydroxytamoxifen, was detected in both the POCIS and grab samples. Although the concentrations from the POCIS were calculated at about one-half of the grab sample concentrations, this limited data shows the potential of using a passive sampling technique such as the POCIS for monitoring TAM metabolites. Direct comparison of the estimated and measured concentrations is not reasonable as it should not be expected to see an exact match between a single point-in-time measurement (i.e., grab sampling) and an integrated sampler (i.e., POCIS) over 30 to 60 days. The time-weighted average water concentrations of 4-hydroxytamoxifen from the POCIS were determined using a theoretical estimation model in which the molar volume of a chemical, a theoretical diffusion coefficient for the chemical, and an average thickness of the water boundary layer at the membrane surface are used to determine a sampling rate [37]. This model tends to slightly underestimate the chemical-specific sampling rate, resulting in the overestimation of the chemical concentration in the water. However, the estimates are considered to be within a factor of 2 or 3 of the actual value (D. Alvarez, USGS, personal communication). Further development of the POCIS by experimentally deriving the uptake rates of the TAM metabolites would be required to use the POCIS as a quantitative sampling technique.

Conclusions

From the environmental data at least two of the TAM metabolites, 4-hydroxytamoxifen and n-desmethyl tamoxifen, are present (spectral and retention time confirmed) in a substantial number of environmental aqueous samples. The discovery of the TAM metabolites demonstrates that the source of the TAM metabolites, TAM, is constant; thereby, introducing a pseudo-persistence of these chemicals in

the environment [38]. While the findings of the individual TAM metabolites from non-detect to ppb concentrations might not imply acute toxic effects upon aquatic organisms at the levels detected, the reality is that no chemical is found by itself. The probability that aquatic organisms are constantly exposed to low concentrations of multiple ECs, and subsequent hormesis response, should be taken into consideration when performing environmental risk assessments. One might expect that these effects will become more pronounced during continual release of AIs into the environment.

The understanding of environmental concentrations and ultimately the ability to conduct accurate and sensible risk assessments is a complex one. A very recent paper by Rand-Weaver et al. [39] suggests that the models and risk assessments that utilize the read-across hypothesis may well in fact give false endpoints; thereby, giving a false sense of security in approaching current methodologies for undertaking environmental risk assessments. Rand-Weaver et al. [39] suggests that studies where we tried to follow the Adverse Outcome Pathway framework as outlined by Ankley et al. [40], would be the better path to follow for more accurate risk assessments.

NOTICE: The United States Environmental Protection Agency through its Office of Research and Development funded and managed the research described here. It has been subjected to Agency's administrative review and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

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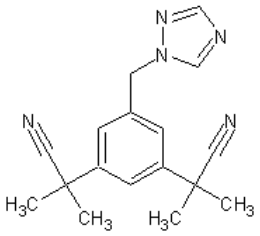
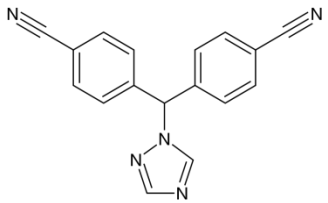
Figure 1. Chemical structures of four EAPs and three TAM metabolites

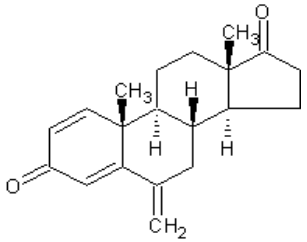
Anastrozole



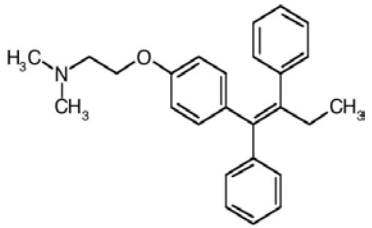
Letrozole



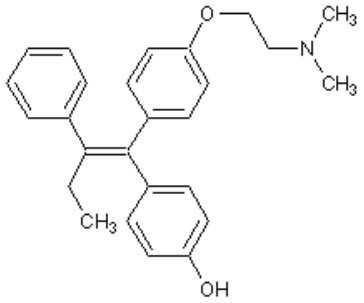
Exemestane



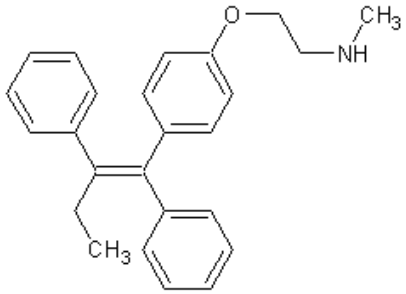
Tamoxifen



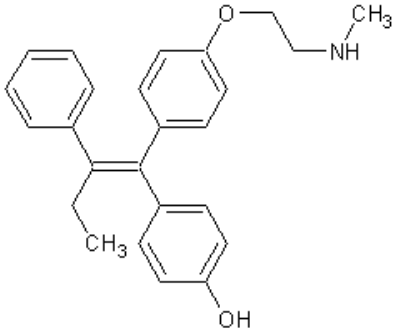
4-hydroxytamoxifen



n-desmethyltamoxifen



e/z- endoxifen



Figures were drawn using ACD Labs Phys/Chem History program.

Table 1. Chemical and experimental MS data for compounds of study

Compound	Formula	CAS #	Molecular weight Da	log D*ow*[1] pH 7	log D*ow*[1] pH 8	pK_a[1]	Precursor ion	Product ion	LOD† method ng L^{-1}	LOQ method ng L^{-1}
Tamoxifen	$C_{26}H_{29}NO$	10540-29-1	371.53	3.45	4.36	8.69 (MB)	372 $(M+H)^+$	327.3 $(M-H-N(CH_3)_2)^+$	65	130
Tamoxifen-d5	$C_{26}H_{24}D_5NO$	None available	376.55	NA	NA	NA	377 $(M+H)^+$	332.3 $(M-H-N(CH_3)_2)^+$	NA	NA
4-hydroxy tamoxifen	$C_{26}H_{29}NO_2$	68047-06-03 (68392-35-8)	387.51	NA		NA	388 $(M+H)^+$	166.3	30	80
e/z-endoxifen	$C_{25}H_{27}NO_2$	None available	373.49				374 $(M+H)^+$	152.3	10	25
n-desmethyl tamoxifen	$C_{25}H_{27}NO$	15917-65-4 (HCl)	357.49				358 $(M+H)^+$	207.3+209.3	50	110
Exemestane	$C_{20}H_{24}O_2$	107868-30-4	296.40	2.43	2.43	NA	297 $(M+H)^+$	279.3 $(MH-H_2O)^+$	30	75
Exemestane-19-d3	$C_{20}H_{21}D_3O_2$	None available	299.42	NA	NA	NA	300 $(M+H)^+$	282.3 $(MH-H_2O)^+$		
Anastrozole	$C_{17}H_{19}N_5$	120511-73-1	293.37	0.29	0.29	2.62 (MB)	294 $(M+H)^+$	225.3 $(M-H-C_2H_7N_3)^+$	10	20
Anastrozole-d12	$C_{17}H_{19}D_{12}N_5$	None available	305.44	NA	NA	NA	306 $(M+H)^+$	237.3 $(M-H-C_2H_7N_3)^+$		
Letrozole	$C_{17}H_{11}N_5$	112809-51-5	285.30	0.43	0.43	1.52 (MB)	286 $(M+H)^+$	217.3 $(M-H-C_2H_2N_3)^+$	15	30
Letrozole-d4	$C_{17}H_{11}D_4N_5$	None available	289.33	NA	NA	NA	290 $(M+H)^+$	221.3$(M-H-C_2H_2N_3)^+$		

[1] log D*ow* and pK_a values were calculated using ACD Labs Phys/Chem History program. NA= not available; MB = mostly basic; †This is for an aqueous sample, as determined using MacDougall et al. guidelines [33]. Based on 10 µL injections from the linear regression analyses (based on the average between the calculations of LOD through the slope through zero) of a 4-point standard curve, and based upon the analysis of that 10 µL injection from 500 µL of an extract, which is from 500 mLs of aqueous sample.

Table 2. Method Performance: a) Spike recoveries of EAPs from waste/source water and DI water, and b) Spike recoveries of tamoxifen metabolites from waste/source water and DI water

a)

	DI water (n = 4) 1 µg L^{-1}		Waste/source water (n = 16) 1 µg L^{-1}		Waste/source water (n = 3) 0.7 µg L^{-1}		DI Water (n = 3) 0.5 µg L^{-1}		DI Water (n = 4) 0.2 µg L^{-1}	
Analyte	% Recovery	% RSD	% Recovery	% RSD	% Recovery	% RSD	% Recovery	% RSD	% Recovery	% RSD
Tamoxifen	92	13	100	19	114	16	96	9	120	14
Anastrozole	90	4	100	15	111	15	100	6	88	13
Letrozole*	95	3	87	27	89	24	98	11	82	10
Exemestane	130	17	100	17	103	18	170	8	90	21

*Letrozole spike amount is 2 x the other AI spiked amount, i.e., 2 x 1 µg L^{-1} = 2 µg L^{-1}.

b)

Tamoxifen metabolites	DI water (n = 4) 1 µg L^{-1}		DI water (n = 3) 2 µg L^{-1}		Waste/source water (n = 3) 1 µg L^{-1}		Waste/source water (n = 4) 2 µg L^{-1}	
	% Recovery	% RSD	% Recovery	% RSD	% Recovery	% RSD	% Recovery	% RSD
4-hydroxy tamoxifen	110	21	67	38	93	47	35	120
e/z-endoxifen	96	8	61	26	77	15	44	36
n-desmethyl tamoxifen	94	19	58	36	89	39	62	69

Table 3. Method Performance a) Spike recoveries 150 ng of EAPs in fish plasma; b) Spike recoveries 250 ng of EAPs in fish plasma; and c) Spike recoveries 500 ng of tamoxifen metabolites in rat plasma

a) Spike recoveries 150 ng of EAPs in fish plasma

Compounds	pH > 9	
	% recovery (n = 3)	% RSD
Tamoxifen	100	18
Exemestane	88	16
Anastrozole	97	7
Letrozole	110	12

b) Spike recoveries 250 ng of EAPs in fish plasma

Compounds	pH > 9	
	% recovery (n = 3)	% RSD
Tamoxifen	100	15
Exemestane	94	6
Anastrozole	99	9
Letrozole	110	10

c) Spike recoveries 500 ng of tamoxifen metabolites in rat plasma

Compounds	% recovery (n = 4)	% RSD
4-hydroxytamoxifen	110	29
n-desmethyltamoxifen	77	27
e/z endoxifen	120	32

Table 4. Fish plasma data from fish gavage studies: a) Tamoxifen dosing study;

b) Letrozole, Anastrozole, and Exemestane

a)

Sample ID	Tamoxifen	4-hydroxy tamoxifen	N-desmethyl tamoxifen	e/z endoxifen
	ng g^{-1} detected			
TAM CTL	ND	270	250	ND
TAM 5 mg kg^{-1}	ND	ND	280	ND
TAM 0.5 mg kg^{-1} ‡	ND	500	280	ND
TAM CTL	ND	ND	310	ND
TAM 5 mg kg^{-}	ND	370	250	ND
TAM 0.5 mg kg^{-1}	ND	ND	430	ND
rat plasma blank	ND	ND	64*	ND

CTL = control; ND = not detected; TAM = tamoxifen; ***** breakdown from Internal Standard;
‡ Anastrozle was mass spectrally confirmed at < LOD in this sample

b)

Sample ID	Anastrozole	Letrozole	Exemestane
	ng g^{-1} detected		
ANA CTL	ND	ND	ND
ANA 0.075 mg kg^{-1}	ND	ND	ND
ANA 0.075 mg kg^{-1}	ND	ND	ND
ANA 0.75 mg kg^{-1}	66	ND	ND
ANA 0.75 mg kg^{-1}	16	ND	ND
LET CTL	ND	ND	ND
LET 0.075 mg kg^{-1}	ND	240	ND
LET 0.075 mg kg^{-1}	ND	160	ND
LET 0.75 mg kg^{-1}	ND	540	ND
LET 0.75 mg kg^{-1}	ND	580	ND
EXE CTL	ND	ND	ND
EXE 0.075 mg kg^{-1}	ND	ND	ND
EXE 0.075 mg kg^{-1}	ND	ND	ND
EXE 0.75 mg kg^{-1}	ND	ND	ND
EXE 0.75 mg kg^{-1}	ND	ND	ND

CTL = control; ND = not detected; ANA=anastrozole; LET=Letrozole; EXE=exemestane

Table 5. Overview of environmental data from Arizona, Missouri, Nevada and Ohio.

site	tamoxifen	letrozole	anastrozole	exemestane	4-hydroxy tamoxifen	n-desmethyl tamoxifen	e/z-endoxifen
OH-1	-	-	-	-	-	+	-
OH-2	-	-	-	-	+	+	-
OH-3	+	-	-	-	+	+	-
OH-4	-	-	-	-	-	+	-
NV-1	-	+	-	-			
NV-2	-	-	-	-			
NV-3	-	-	-	-			
AZ-1	-	-	-	-	-	-	-
AZ-2	-	-	-	-	-	-	-
AZ-3	-	-	-	-	-	-	-
AZ-4	-	-	-	-	-	-	-
MO-1	-	-	-	-	+	-	-
MO-2	-	-	-	-	+	-	-

+ = present
- = absent
= samples were not analyzed for tamoxifen metabolites

72 water samples were analyzed: including travel blanks, system blanks, duplicates, and spikes.
Of the 72 samples 3 tested positive for an AI, and 21 tested positive for tamoxifen metabolites

17 Table 6. Comparison of POCIS vs Grab sampling at two sites

Sampling Site		Tamoxifen	Anastrozole	Letrozole	Exemestane	4-hydroxy tamoxifen	n-desmethyl tamoxifen	e/z endoxifen
		ng L^{-1}						
MO site 2 wetlands outflow	POCIS	ND	ND	ND	ND	650*	ND	ND
MO site 2 wetlands outflow	GRAB	ND	ND	ND	ND	1000	ND	ND
MO site 2 wetlands outflow (dup)	GRAB	ND	ND	ND	ND	1250	ND	ND
NV site 2 WWTP	POCIS	ND	ND	ND	ND	ND	ND	YES in field blank
NV site 2 WWTP	GRAB	ND	ND	ND	ND	NA	NA	NA
NV site 2 WWTP (dup)	GRAB	ND	ND	ND	ND	NA	NA	NA
NV site 1 wetlands stream	POCIS	ND	ND	ND	ND	830*	ND	ND
NV site 1 wetlands stream	GRAB	ND	ND	ND	ND	NA	NA	NA

18 ND = not detected; NA = not applicable (metabolites were not analyzed in this sample); *Calculated using theoretical estimation technique.

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The following tables and graphs are for illustration of optimization of both the fish and rat plasma extraction methods.

Table 1a. Rat plasma optimization raw data.

base extractions using 4%NH4OH and a simple shake/rotor method

	% recovery rat plasma 0.5 g			
	Tamoxifen	Anastrozole	Letrozole	Exemestane
Rat plasma #				
lvrcp013	32	36	43	40
lvrcp013	39	46	53	41

Acidic ASE extractions	% recovery rat plasma 1.0 g ASE			
Rat plasma #	Tamoxifen	Anastrozole	Letrozole	Exemestane
spiked on top of plasma in hydromatrix boat				
lvrcp003	44	73	58	33
lvrcp004	51	78	67	37
meoh/1% acetic spiked directly into plastic vial of rat plasma				
lvrcp001	64	76	65	41
lvrcp002	65	83	64	62

Base ASE extractions		% recovery rat plasma 1.0 g ASE			
Rat plasma #	Date analyzed	Tamoxifen*	Anastrozole	Letrozole	Exemestane
spiked directly into plastic vial of rp					
lvrcp006		NA	74	9	74
lvrcp007		NA	69	11	98
lvrcp008		NA	68	12	94

*missing from aromatase mix

All samples were vortexed w/ 90%MTBE/10%MeOH.

			neg mode	
	Tamoxifen	Anastrozole	Letrozole	Exemestane
1000 ng/g	75	80	44	105
1000 ng/g	53	67	40	95
1000 ng/g	80	85	70	98
1000 ng/g	86	84	69	79
500 ng/g	101	105	76	110
500 ng/g	93	87	73	104
avg	81	85	62	99
std dev	17	12	16	11
% rsd	20	14	25	11

Figure 1a. Rat plasma optimization graph.

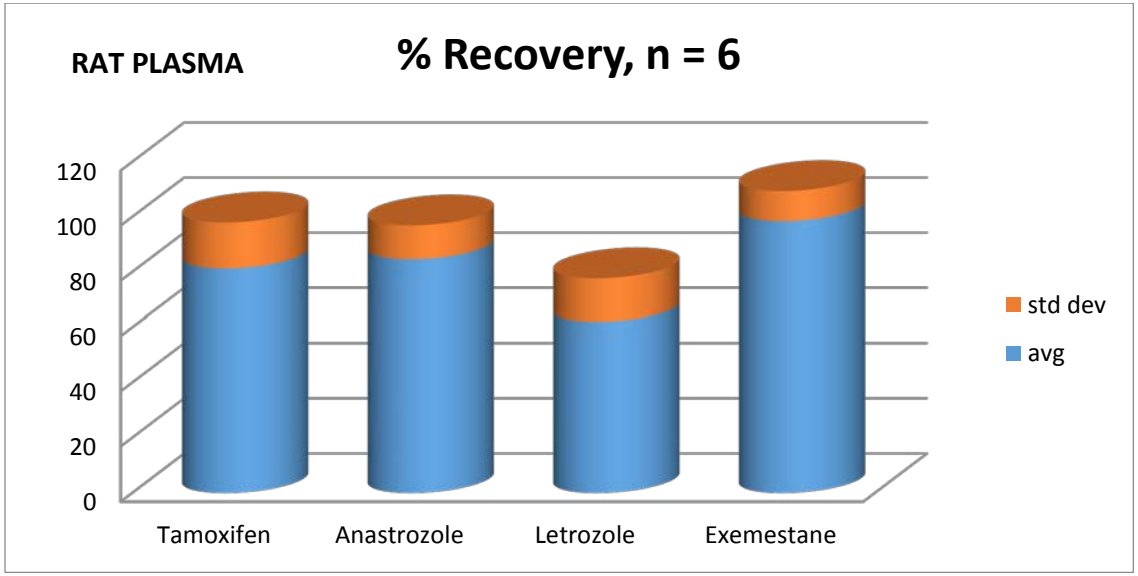
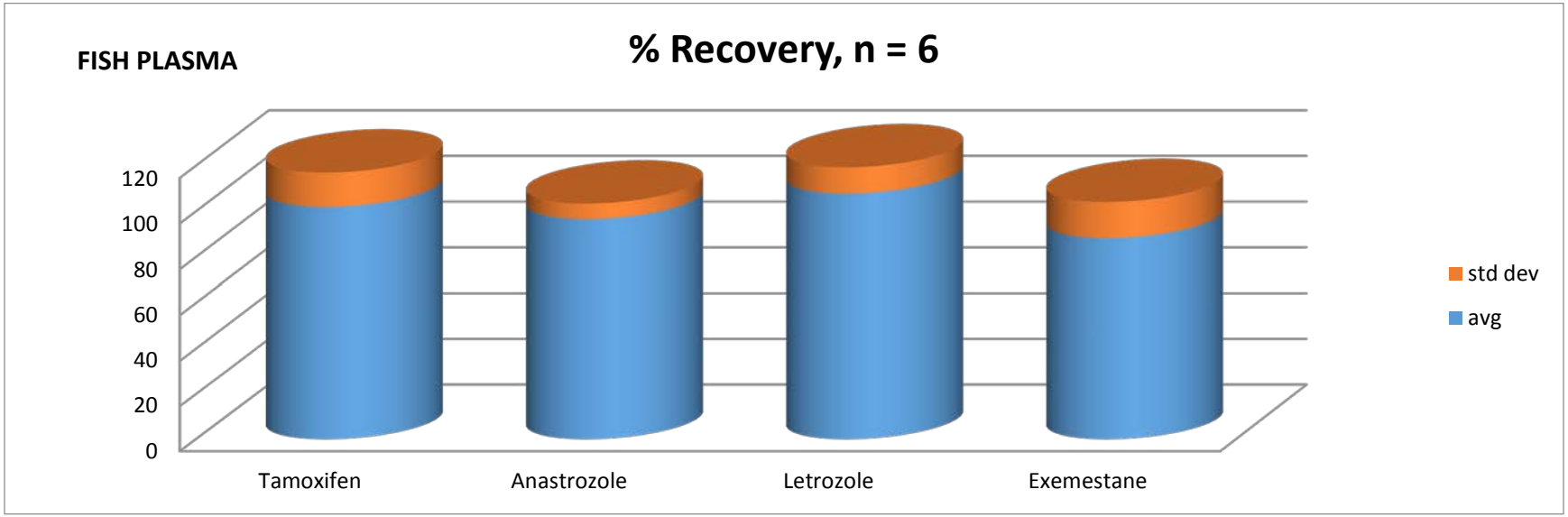


Figure 1b. Fish plasma optimization graph.



The following tables and graphs are for illustration of optimization of the aqueous extraction method.

Table 2a. Aqueous sample extraction method development raw data.

Sample ID	Tamoxifen	Letrozole	Anastrozole	Exemestane	MEOH	MEOH/ 4 or 5 % Acetic
lv08wat069	46	12	34	45	X	
lv08wat069	41	16	36	48	X	
lv08wat069	44	13	36	45	X	
lv09wat011	56	7	27	53	X	
lv09wat011	49	6	23	45	X	
lv11wat002	19	25	29	19		X
lv11wat008	36	ND	15	22		X
lv09wat006/007	28	ND	47	31		X
lv11wat002	32	23	33	17		X
lv11wat002 dup	26	20	27	19		X

Figure 2a. Aqueous sample extraction method development raw data graph

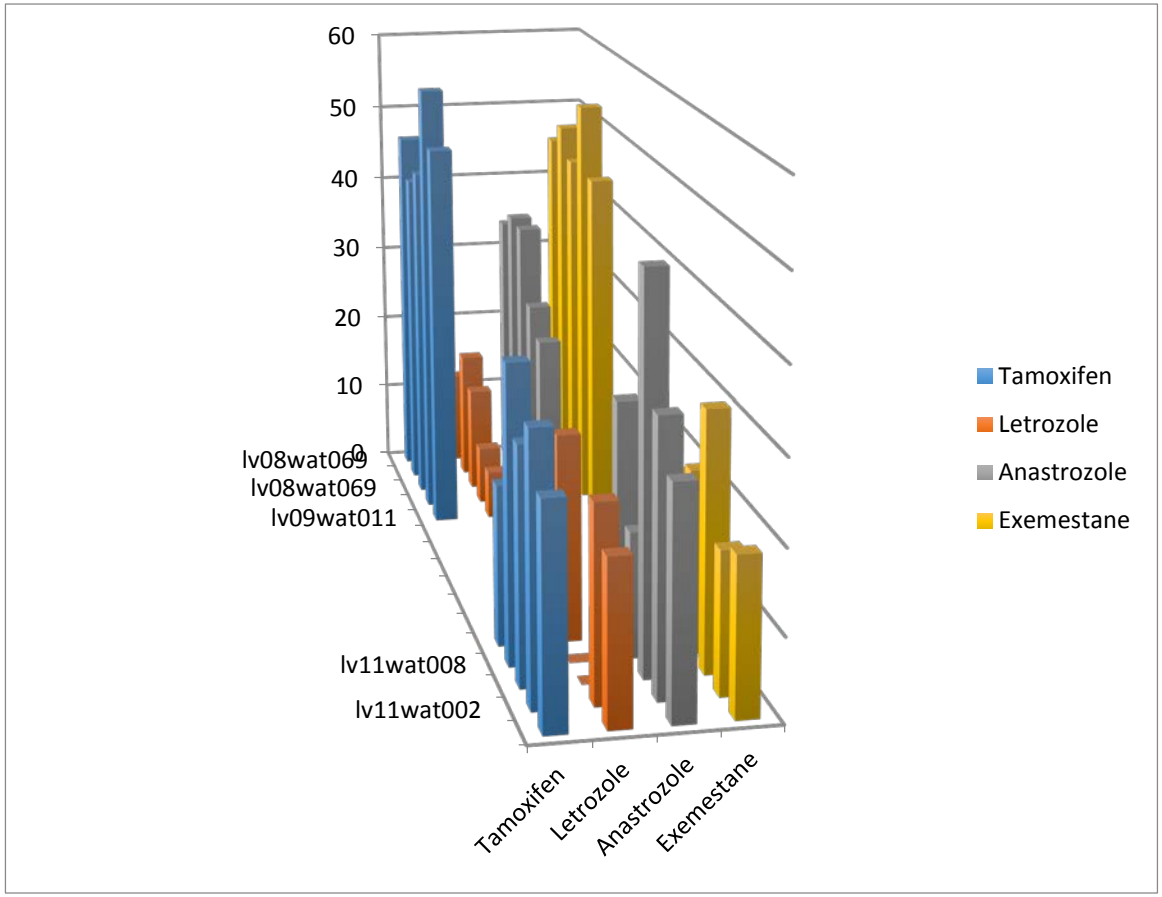


Table 2b. Aqueous sample extraction method development raw data.

all 3 g NaCl, 20 min drying time, all MEOH cond, all 3 extracts

Sample ID	Tamoxifen	Anastrozole	Exemestane	
lv09wat006/007	28	24	17	pH 3
lv09wat006/007	34	26	20	
lv09wat006/007	35	30	17	
lv09wat006/007	16	48	28	pH 9-10
lv09wat006/007	26	47	31	
lv09wat006/007	28	47	31	

Figure 2b. Aqueous sample extraction method development raw data graph

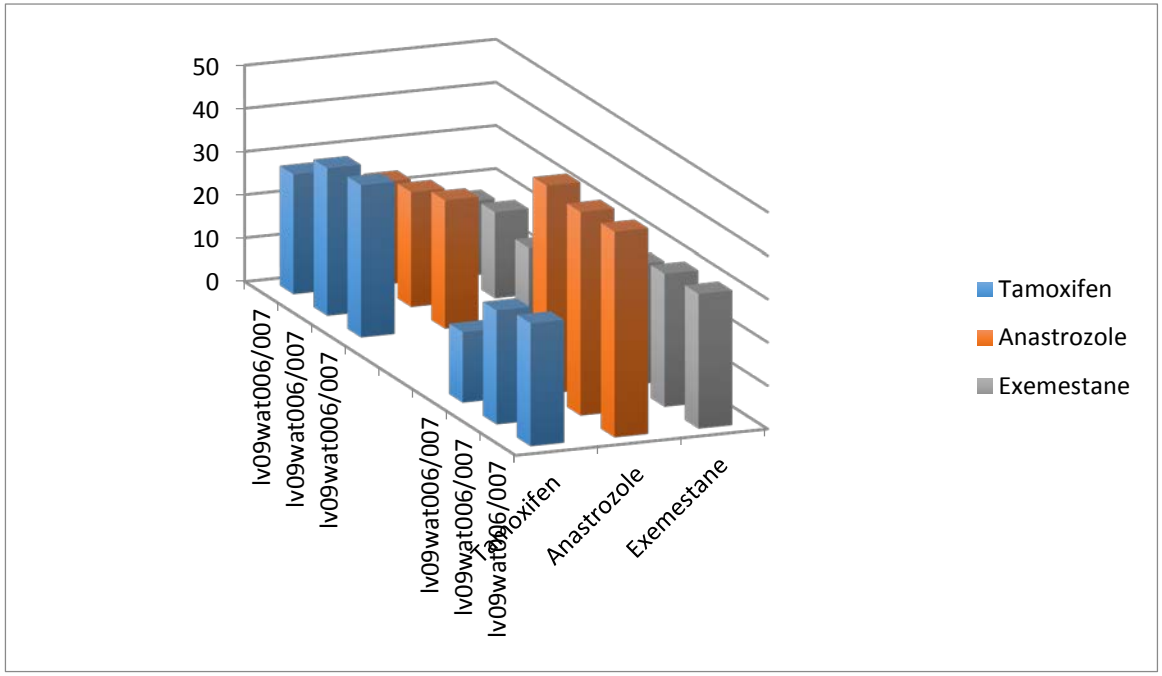


Table 2c. Aqueous sample extraction method development raw data.

		% recovery			final
	uLs NH4OH	Tamoxifen	Anastrozole	Letrozole	Exemestane	pH
lv11wat003	0	33	35	28	62	7
lv11wat003	0	42	37	28	70	7
lv11wat003	100	49	37	23	63	10
lv11wat003	100	50	39	28	70	10
lv11wat003	200	43	35	24	71	10
lv11wat003	200	46	35	26	66	11

Figure 2c. Aqueous sample extraction method development raw data graph

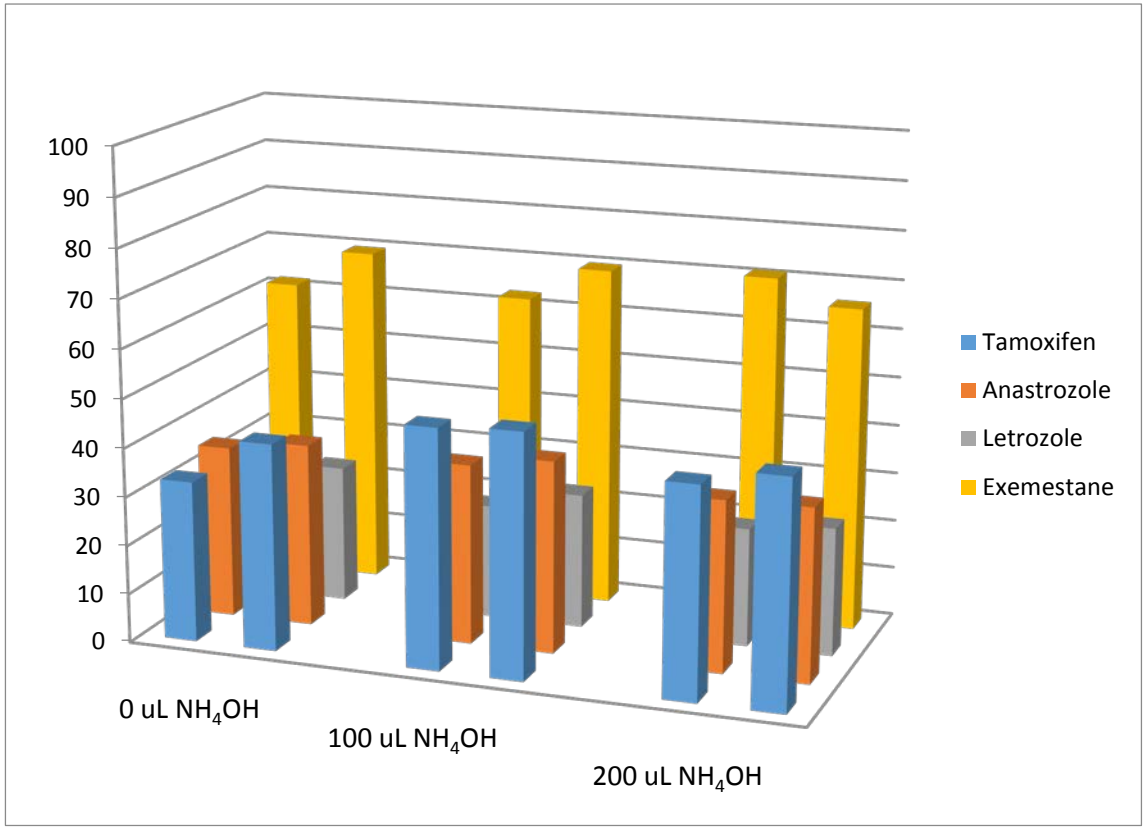
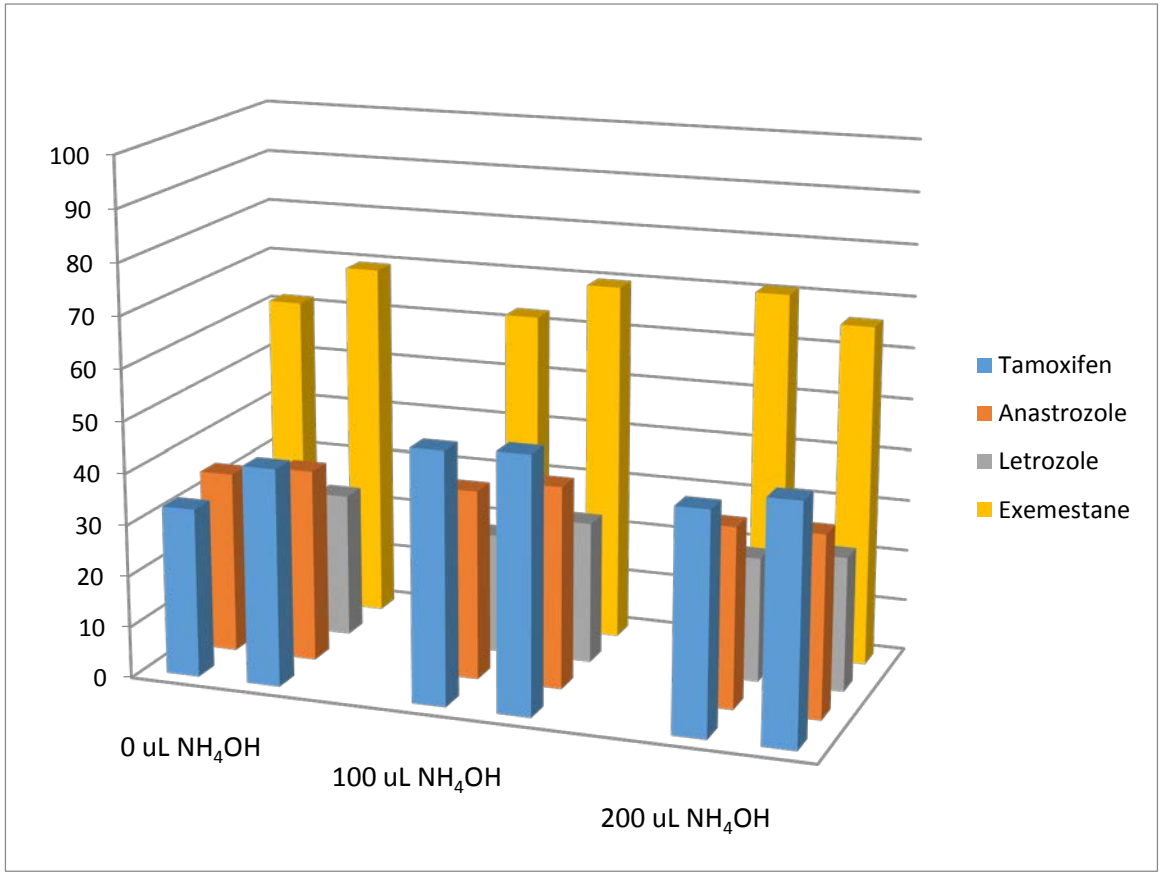


Table 2d. Aqueous sample extraction method development raw data.

	3-step extraction	% recovery				final
	uLs NH4OH	Tamoxifen	Anastrozole	Letrozole	Exemestane	pH
lv11wat006	0	27	34	16	44	7
lv11wat006	0	44	37	15	42	7
lv11wat006	100	31	36	14	41	10
lv11wat006	100	30	34	16	40	10
lv11wat006	200	42	33	13	39	11
lv11wat006	200	25	32	16	42	11

Figure 2d. Aqueous sample extraction method development raw data graph



1 Table 3. Environmental concentrations at sampling sites in Arizona, Missouri, Nevada and Ohio.

Site Description	Date Collected	Tamoxifen	4-hydroxy-tamoxifen	n-desmethyl-tamoxifen	e/z endoxifen	Letrozole	Anastrozole	Exemestane
				ng L^{-1}				
NV WWTP	06/09/11	ND	NA	NA	NA	< LOD	ND	ND
NV site 2 WWTP (P)	03/05/12	ND	NA	NA	NA	ND	ND	ND
NV site 1 wetlands stream (P)	03/05/12	ND	NA	NA	NA	ND	ND	ND
NV site 3 < 2mgd WWTP (P)	03/05/12	ND	NA	NA	NA	ND	ND	ND
OH WWTP 1 RW	06/25/12	ND	NA	NA	NA	ND	ND	ND
OH WWTP 1 E	06/25/12	ND	NA	NA	NA	ND	ND	ND
OH WWTP 2 RW	06/25/12	ND	NA	NA	NA	ND	ND	ND
OH WWTP 2 E	06/25/12	ND	NA	NA	NA	ND	ND	ND
OH WWTP 3 RW	06/25/12	ND	NA	NA	NA	ND	ND	ND
OH WWTP 3 E	06/25/12	190	NA	NA	NA	ND	ND	ND
OH WWTP 4 RW	06/25/12	ND	NA	NA	NA	ND	ND	ND
OH WWTP 1 RW‡	06/25/12	ND	ND	ND	ND	ND	ND	ND
OH WWTP 1 E‡	06/25/12	ND	ND	ND	ND	ND	ND	ND
OH WWTP 2 RW‡	06/25/12	ND	ND	ND	ND	ND	ND	ND
OH WWTP 2 E‡	06/25/12	ND	470	800	ND	ND	ND	ND

OH WWTP 3 RW‡	06/25/12	ND	ND	ND	ND	ND	ND	ND
OH WWTP 3 E‡	06/25/12	ND	430	ND	ND	ND	ND	ND
OH WWTP 4 RW‡	06/25/12	ND	ND	300	ND	ND	ND	ND
OH WWTP 3	03/27/12	ND	650	430	ND	ND	ND	ND
OH WWTP 4	03/27/12	ND	ND	900	ND	ND	ND	ND
OH WWTP 1	03/27/12	ND	ND	430	ND	ND	ND	ND
OH WWTP 2	03/27/12	ND	890	1000	ND	ND	ND	ND
lower Colorado River site 1	01/16/13	ND	ND	ND	ND	ND	ND	ND
lower Colorado River site 2	01/16/13	ND	ND	ND	ND	ND	ND	ND
lower Colorado River site 4	01/16/13	ND	ND	ND	ND	ND	ND	ND
MO site 1 wetlands intake (P)	06/26/13	ND	1000	ND	ND	ND	ND	ND
MO site 1 wetlands intake dup	06/26/13	ND	1000	ND	ND	ND	ND	ND
MO site 2 wetlands (P)	06/26/13	ND	1000	ND	ND	ND	ND	ND
MO site 2 wetlands dup	06/26/13	ND	1250	ND	ND	ND	ND	ND

2

3 E = Treated Effluent; RW = Receiving water (near the effluent discharge); dup = duplicate; ND = not detected; NA = not applicable; AZ = Arizona;

MO = Missouri; NV = Nevada; OH = Ohio; (P) = POCIS were deployed at this site.