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Rapid analysis of pharmaceuticals and personal care products in fish plasma micro-aliquots using liquid chromatography tandem mass spectrometry

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ABSTRACT

A sensitive analytical method based on liquid–liquid extraction (LLE) and liquid chromatography tandem mass spectrometry (LC–MS/MS) was developed for rapid analysis of 11 pharmaceuticals and personal care products (PPCPs) in fish plasma micro-aliquots (~20 μL). Target PPCPs included, bisphenol A, carbamazepine, diclofenac, fluoxetine, gemfibrozil, ibuprofen, naproxen, risperidone, sertraline, simvastatin and triclosan. A relatively quicker and cheaper LLE procedure exhibited comparable analyte recoveries with solid-phase extraction. Rapid separation and analysis of target compounds in fish plasma extracts was achieved by employing a high efficiency C-18 HPLC column (Agilent Poroshell 120 SB-C18, 2.1 mm × 50 mm, 2.7 μm) and fast polarity switching, enabling effective monitoring of positive and negative ions in a single 9 min run. With the exception of bisphenol A, which exhibited relatively high background contamination, method detection limits of individual PPCPs ranged between 0.15 and 0.69 pg/μL, while method quantification limits were between 0.05 and 2.3 pg/μL. Mean matrix effect (ME) values ranged between 65 and 156% for the various target analytes. Isotope dilution quantification using isotopically labelled internal surrogates was utilized to correct for signal suppression or enhancement and analyte losses during sample preparation. The method was evaluated by analysis of 20 μL plasma micro-aliquots collected from zebrafish (*Danio rerio*) from a laboratory bioaccumulation study, which included control group fish (no exposure), as well as fish exposed to environmentally relevant concentrations of PPCPs. Using the developed LC–MS/MS based method, concentrations of the studied PPCPs were consistently detected in the low pg/μL (ppb) range. The method may be useful for investigations requiring fast, reliable concentration measurements of PPCPs in fish plasma. In particular, the method may be applicable for in situ contaminant biomonitoring, as well as bioaccumulation and toxicology studies employing small fishes with low blood compartment volumes.

1. Introduction

The occurrence of pharmaceuticals and personal care products (PPCPs) in the environment has received increasing attention in recent years. PPCPs enter into the environment through individual human activity and as residues from agriculture, manufacturing, veterinary use, as well as community and hospital use [1]. Numerous studies have reported PPCP residues in waste water treatment plant (WWTP) influents and effluents, as well as surface waters, with concentrations generally ranging between 0.001 and 1 μg/L [2–9].

PPCPs can accumulate in tissues of aquatic organisms [10–14] and toxicity tests indicate many of these compounds can cause chronic low-level effects [15–20]. Paroxetine and fluoxetine, commonly prescribed selective serotonin reuptake inhibitor (SSRI) antidepressants, have been detected in fish muscle tissue from Hamilton Harbour, Canada, at concentrations of 0.58 ng/g and 1.02 ng/g, respectively [12]. A recent national pilot study in the United States to assess the accumulation of PPCPs in fish demonstrated the presence of norfluoxetine, sertraline, diphenhydramine, diltiazem, and carbamazepine at ng/g concentrations in fillet composites from effluent-dominated surface waters [21]. The survey showed the anticonvulsant, carbamazepine, was present at a concentration of 2.3 ng/g in fish fillets in Chicago, USA. Sertraline was detected at concentrations as high as 19 and 545 ng/g in fish fillets and liver, respectively.

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While these and other studies have focused on assessing the occurrence and levels of PPCPs in fish muscle and liver tissue, there are relatively few studies of these contaminants of emerging concern in fish plasma. Previously, carbamazepine and ibuprofen residues were detected at 0.3 pg/μL and 102 pg/μL, respectively, in plasma of fish exposed to treated sewage effluents in Sweden [13]. Tanoue et al. [14] reported concentrations of several PPCPs in plasma of cyprinoid fish from an effluent-dominated stream in Japan, with concentrations ranging between 0.03 and 110 pg/μL.

The concentration of xenobiotic compounds in blood plasma is a key parameter for comparative toxicology and pharmacology studies. Plasma concentrations can provide important information regarding recent exposure conditions [21,22]. The "Fish Plasma Model" has been proposed for prioritizing pharmaceuticals for in-depth ecological risk assessment [22,23]. The model utilizes estimated drug concentrations in fish plasma and human therapeutic plasma concentration data to assess exposure risks in fish. Implementation of this approach will require fast, reliable measurements of PPCP residue concentrations in fish plasma.

Currently, there are relatively few multi-residue analytical methods for trace quantification of PPCPs in fish plasma. Previous methods [13,14] have employed sample preparation techniques such as ultrasound assisted extraction (UAE), gel-permeation chromatography (GPC) and solid-phase extraction (SPE), prior to analysis by liquid chromatography tandem mass spectrometry (LC–MS/MS). These methods require relatively large amounts of plasma for analysis (0.5–2 mL). In many cases however, only microliter volumes of plasma can be attained from fish. This is especially true for small fish species.

The objective of the present study was to develop a multi-residue analytical method for rapid analysis of PPCPs in fish plasma micro-aliquots (20 μL). Target analytes included 11 key PPCPs that are commonly detected in environmental and biological samples, including bisphenol A (BPA), carbamazepine, diclofenac, fluoxetine, gemfibrozil, ibuprofen, naproxen, risperidone, sertraline, simvastatin and triclosan. The developed method was evaluated and further applied for the analysis of these contaminants of emerging concern in plasma micro-aliquots of zebrafish (*Danio rerio*) subjected to environmentally relevant (parts per billion) levels.

2. Experimental

2.1. Materials

High purity (>97%) standards of diclofenac, naproxen, triclosan, ibuprofen, gemfibrozil, sertraline, risperidone, simvastatin were obtained from Sigma–Aldrich Co. LLC. (St. Louis, USA). Bisphenol A was obtained from Merck (Hohenbrunn, Germany). Carbamazepine was purchased from TCI-EP (Tokyo, Japan). Fluoxetine was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Isotopically labelled compounds, propanolol-d_7, ibuprofen-d_3, fluoxetine-d_5, carbamazepine-d_{10} and warfarin-d_5, were purchased from CDN Isotopes Inc. (Pointe-Claire, Quebec, Canada). Bisphenol A-d_6, $^{13}C_6$-2,4,5-trichlorophenoxyacetic acid ($^{13}C_6$-TCPAA) and gemfibrozil-d_6 were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). $^{13}C_{12}$-Triclosan was purchased from Wellington Laboratories Inc. (Ontario, Canada). HPLC grade Acetone, methanol and acetonitrile were purchased from Fisher Scientific Inc. (Loughborough, UK). All standards were prepared in methanol and stored at −20 °C.

2.2. Collection of fish plasma micro-aliquots

Adult zebrafish were purchased from a local fish farm in Singapore (Mainland Tropical Fish Farm, Singapore). A flow-through experimental system was employed to expose fish to the studied PPCPs. Fish were separated into three groups, a low dose group, a high dose group and a control group. The low dose fish were exposed to a mixture of the studied PPCPs, with water concentrations ranging between 0.01 and 5 μg/L. Concentrations of individual PPCPs in water for the high dose group ranged between 0.04 and 12 μg/L. Control fish were subjected to the same conditions but without any exposure to the PPCPs. During the exposure period all fish were fed a diet consisting of artemia, twice daily. At the end of exposure, zebrafish were euthanized by submersion in ice water for 5 min. It cannot be submersed for too long otherwise blood clots will occur. The tail of the fish was cut off using sterilized spring scissors to expose caudal vein and blood was collected via pipette (Eppendorf Research, Germany). To maximize blood collection efficiency, we used 200 μL Prot/Elec tips with an elongated capillary tip of 10 μL (#223-9915, Bio-Rad, USA). Tips were fully filled with 18 mg/mL of EDTA (E5134, Sigma–Aldrich) and immersed in the same solution for at least 24 h, completely dried at 60 °C, then cooled to room temperature. The anticoagulant coated tips were prepared within 1 week prior to use. It was found that approximately 10–20 μL of whole blood can be collected from one adult zebrafish. The whole blood was placed in a BD microtainer lithium heparin tube (#422-365971, BD, USA). The plasma was separated by centrifugation at 3000 × *g* for 10 min at 4 °C (Eppendorf Centrifuge 5417R). Then plasma was transferred into a 2 mL centrifuge tube and stored at −80 °C prior to extraction.

2.3. Sample preparation

For extraction of target analytes from the fish plasma micro-aliquots (20 μL), we tested and evaluated two different protocols, including LLE and SPE. To check for background contamination, a procedural blank consisting of 20 μL of Milli-Q water was processed with every batch of 10 samples.

For LLE, 20 μL of thawed plasma (pooled from five individual fish) was added to a 2 mL centrifuge tube and spiked with eight isotopically labelled internal surrogate (IS) compounds (propanolol-d_7, ibuprofen-d_3, fluoxetine-d_5, carbamazepine-d_{10}, warfarin-d_5, bisphenol A-d_6, gemfibrozil-d_6 and $^{13}C_{12}$-triclosan). For six of the 11 target analytes, a matching mass labelled compound was available, while five of the target compounds were assigned a labelled compound exhibiting similar physicochemical properties, matrix effect and method recovery. A list of the corresponding internal surrogate compounds used to quantify the various target analytes is shown in Table 1. The amount of internal surrogate compounds spiked varied between 7.5 and 256.8 ng, depending on analyte sensitivity (Table 1). 1 mL of ice-cold acetone was added to extract target compounds and to precipitate proteins. Centrifuge tubes were sonicated for 3 min, then centrifuged at 12,000 rpm under 4 °C for 5 min. Extraction was repeated a second time with an additional 1 mL of acetone. The combined supernatant was evaporated under a gentle nitrogen stream to dryness. The test tube was reconstituted with 120 μL of methanol:water (1:4, v/v). Final extracts were transferred into LC vials and spiked with 5 μL of the injection internal standard, $^{13}C_6$-TCPAA (5 mg/L). The main purpose of the injection internal standard, $^{13}C_6$-TCPAA, is to determine the absolute recovery of internal surrogate compounds spiked prior to extraction [24]. All extracts were stored in darkness at 4 °C prior to LC–MS/MS analysis.

For SPE, 1.5 mL of 1% formic acid was added to 20 μL plasma micro-aliquots, then spiked with internal surrogate standards. The mixture was diluted to 5 mL with Milli-Q water before SPE. SPE was performed using Phenomenex X-33u 200 mg SPE cartridges. The conditioning of the SPE cartridges was performed with 5 mL of methanol followed by 5 mL of Milli-Q water. A vacuum manifold system was employed. The 5 mL samples were passed through the cartridges at a flow rate of 5 mL/min. The cartridges were