Use of LC-MS and GC-MS Methods to Measure Emerging Contaminants Pharmaceutical and Personal Care Products (PPCPs) in Fish

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Abstract

Aquatic ecosystems are continuously contaminated by manufactured pharmaceutical and personal care products (PPCPs). Non-regulated, multi-purpose PPCP contaminants enter aquatic systems through sewage/wastewater treatment plants after consumption and use by humans and animals. These micro-pollutants receive increased attention worldwide since significant levels of contamination have been found in various environmental compartments and organisms. Highly sophisticated equipment such as liquid chromatography-mass spectrometry (LC-MS) and gas chromatography-mass spectrometry (GC-MS) are reliable ways to determine PPCPs at sub-ppb levels from air, waters, sediments, effluents, aquatic organisms and human body fluids. Although the consequences of these pollutants are gradually becoming visible, their potential impacts on aquatic ecosystems and organisms are poorly known. Some studies have suggested that PPCPs are persistent and have bioaccumulation potential leading to ecological effects and abnormalities in fish. Other findings illustrate that PPCPs can impair swimming behavior in fathead minnow (Pimpephales promelas), interfere with thyroid axis in the zebra fish (Danio rerio), or form adducts with hemoglobin and/or suitable protein breakdown PPCPs products. Thus, this review focuses on PPCPs emerging contaminants concern with regards to sources, occurrences, analytical methods, fate and biological transformation.

Keywords: PPCPs; Micro-pollutants; Emerging contaminants; Biomarkers; Aquatic organisms; LC-MS and GC-MS

Introduction

In modern life, one of the most important issues in the world is the exposure to man-made chemicals that cause interference of regular activities such as reproduction and development of different organisms in the environment [1,2]. Some of them are hazardous and present potential or actual threat to human health, wildlife, aquatic organisms and/or soundings [2]. Newer analytical techniques have made it possible to identify these compounds at extremely low level of the order of sub-ng/g. These are frequently detected in different environmental compartments including surface waters, wastewaters, air, wildlife and fish, and had not been recognized previously at such low levels. These compounds are often referred to as “emerging contaminants” (ECs) because adequate information associated with their presence, occurrence, fate, transport and mechanisms are not available to assess their risk to human health and the ecosystem [3]. ECs are used daily in homes, farms, businesses and industry as detergents, fragrances, prescription and non-prescription drugs, disinfectants, and pesticides etc. Some ECs have been commonly found in water resources around the world and across the USA [4-6].

PPCPs as Endocrine Disruptors

Emerging evidence from wildlife and laboratory studies indicates that some chemicals may interfere with the endocrine system. Compounds identified include pesticides, polychlorinated biphenyls, dioxins, furans, alkyl phenols, and steroid hormones. These chemicals are routed to ecosystems through wastewater treatment plants. Several studies reported that many ECs present in municipal wastewater effluent can act as endocrine disruptors at concentrations capable of inducing fish feminization [7,8]. The feminization has been linked to exposure to compounds that mimic estrogen activity. However, it has also been determined that thousands of the compounds have the potential to interact with components of the endocrine system, altering the natural action of hormones [9,10] in both freshwater and marine fish species [4,8,11-13]. The occurrence of some ECs correlates with ecological effects and sexual abnormalities in fish [14-16]. In other studies, complex mixtures of ECs at environmentally relevant concentrations were reported to inhibit the growth of human embryonic cells [17,18]. Other evidence suggests that some ECs are persistent in the environment and survive through conventional water treatment plant and ultimately reaching the aquatic organisms [18]. Overall, an important concern, posed by ECs, is the interference of reproduction and development of aquatic organisms and wildlife [1,2,19].

Release of PPCPs into the Environment

Pharmaceuticals and personal care products (PPCPs) are a major class of ECs commonly used in human and animal applications. PPCPs are many chemical compounds with a variety of chemical structures, conformations, functional groups, polarities and characteristics. PPCPs include prescription and non-prescription drugs together with fragrances, cosmetic ingredients, diagnostic agents, biopharmaceuticals, and growth enhancing constituents used in livestock operations. Tons of these chemicals are produced annually worldwide [20]. After consumption, PPCPs are released into ecosystems via urine, feces or residues as either parent compounds or their metabolites. PPCPs enter the environmental system through effluent discharge from wastewater/sewage treatment plants, inappropriate disposal of expired or unused

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While Wilson [59] reported that TCS may be toxic to certain algae species. Specifically, Orvos et al. [58] found other research groups have shown that TCS in surface water may as one of the major pathways of degradation in surface waters [57]. TCS is quite stable to hydrolysis; however its photolysis was identified [56]. Still, the fate and chemistry of TCS are not fully understood.

Most PPCPs/ECs ultimately end up in the aquatic environment. Consequently, these compounds frequently appear in ecosystems and are frequently detected in different environmental compartments and organisms at different concentration levels [25]. PPCPs have been frequently detected in different environmental matrices such as air, waters, sediments, sewage sludge, humans and fish [26-45]. The high rate of occurrence of PPCPs is due to the fact that they are easily dissolved in aquatic environment and do not evaporate at normal temperature and pressures. Moreover some PPCPs appear to show low biodegradation rate and high lipophilicity, and have high bioaccumulation potential as environmental contaminants [21]. Several studies have been published that further discuss the sources of PPCPs and their transportation from personal usages to waters and aquatic organisms [3,18,21,22-27].

Until recently, many around the world were unaware that a new environmental health concern had emerged. Now, regulatory authorities, health agencies, and professional organizations, all over the globe are informed of the growing PPCP problem which drives research on the presence, occurrence, fate of the PPCPs and metabolites [26,27]. Today, the U. S. Environmental Protection Agency (EPA) and other organizations are working together to improve its understanding of a number of ECs, particularly PPCPs.

**Occurrence and Effects of PPCPs on Organisms**

PPCPs and their metabolites have been detected in aquatic and terrestrial organisms [28,29], surface water [30], air [31], sewage effluent [32], lake Michigan water and sediments [33], industrial sewage sludge [34], municipal effluents [35], marine sediments [36], marine mammals [37], effluent-dominated river water fish [38,39], Pecan Creek fish [40] and German fish specimen bank [41], fish-eating birds and fish [42], receiving marine waters and marine bivalves [43]. PPCPs and metabolites have also been identified in human milk [44], and human blood [45]. More importantly, multiple studies have indicated that PPCPs are not only accumulated but also subsequently metabolized to reactive intermediates that form covalently-bound protein adducts in human [46] and aquatic organisms such as fish [47,48].

Studies indicate that many PPCPs are environmentally persistent, bioactive, and have bioaccumulation potential [49-52]. For example, the PPCPs known astriclosan (TCS), an antimicrobial agent, has been widely used in dental care products, disinfectants, hand soaps, footwears, skin care creams and textiles. TCS and its methyl metabolites were detected in surface waters [53], biosolids [54], fish [55], and algae [56]. Still, the fate and chemistry of TCS are not fully understood. TCS is quite stable to hydrolysis; however its photolysis was identified as one of the major pathways of degradation in surface waters [57]. Other research groups have shown that TCS in surface water may be toxic to certain algae species. Specifically, Orvos et al. [58] found no observer-effect concentration (72-h growth) at 500 ng/L for algae Scenedesmus subspicatus while Wilson [59] reported that TCS may cause significant increase in Synedra algae and a substantial reduction of the rare genus Chlamydomonas algae at 15 ng/L and 150 ng/L. Levy et al. [60] demonstrated that TCS can block bacterial lipid biosynthesis inhibiting the enzyme enoyl-acyl carrier protein reductase, which leads to a possible development of bacterial resistance to TCS. Recent studies have shown that TCS impaired swimming behavior and altered expression of excitation-contraction coupling proteins in fathead minnow (Pimephales promelas) [61] and interfered with thyroid axis in the zebra fish (Danio rerio) [62].

**Analytical Methods**

Modern equipment has made it possible to detect PPCPs from different matrices at sub-ng/g levels. The main advances in PPCPs analysis have been made using liquid chromatography-mass spectrometry (LC-MS) and gas chromatography-mass spectrometry (GC-MS) techniques. Pharmaceuticals comprised of polar compounds are easily dissolved in water or polar solvents, which is special advantage for LC-MS analysis. Employing isocratic or gradient elution in LC method, complex composites/mixtures of sample can be separated using different polarities (polar or medium polar or mixed polar) mobile phases with an analytical column such as C18. The separated compounds are characterized with MS detection. LC - tandem mass spectrometry (LC-MS/MS) with positive- and or negative modes of operations using electrospary ionization (ESI) and atmospheric pressure chemical ionization (APCI) are able to detect PPCPs up to sub-pb level. The presence of unknown compounds are confirmed and identified by comparing the mass signals and retention times of unknown samples to known standards.

On the other hand, personal care products (PCPs) are relatively non-polar and are more easily dissolved and extracted in relatively non-polar organic solvents. After cleaned up, the samples are analyzed by GC-MS/MS or - selected ion monitoring (SIM) modes with election ionization (EI) or negative ion chemical ionization (NICI) based on sample nature. The GC-MS/MS or GC-SIM-MS methods are capable of detecting PPCPs down to sub-ng/g levels. The presence of unknown compounds are characterized establishing over 80% to 99% agreement with standard compounds of the respective samples [40,63]. Examples of some analyses performed by LC-MS and GC-MS are illustrated below.

**Analysis of PPCPs by LC-MS and GC-MS**

Many researchers have reported the identification and analysis of emerging PPCPs contaminants [39,42,64]. Our research group [39,64] developed LC-MS/MS methods for determination of pharmaceuticals and metabolites from environmental fish. Specifically, non-linear gradient elution of water and methanol solvents consisting of 0.1% (v/v) formic acid in water and 100% methanol are passed through a C18 analytical column at a flow rate 350 μL/min to achieve the separation of the complex mixture of PPCPs. An auto-sampler is used to inject 10 μL sample solution. Column effluents are monitored by MS/MS equipped with an electrospray interface (ESI). Figure 1 displays chromatographic separation of 25 target pharmaceutical drugs and metabolites, 5 surrogates and 2 internal standards that were spiked to the clean fish tissues by LC-MS/MS using electrospray ionization (ESI) positive (+) and negative (-) modes.

Employing the extraction protocol and LC-MS/MS method [64] all target compounds were analyzed from environmental fish samples that were obtained from the Pecan Creek, Denton, Texas, downstream...
from the effluent discharge. The presence and characterization of the target analytes were confirmed based on comparison of retention time and relative intensities of fragment ions observed from spiked and environmental fish specimens. Four pharmaceutical compounds were detected over method detection limits (MDLs). Figure 2 displays a typical LC-MS/MS ion chromatogram for identification and determined the concentration of diphenhydramine (0.66 - 1.32 ng/g), diltiazem (0.11 - 0.27 ng/g), carbamazepine (0.83 - 1.44 ng/g), norfluoxetine (3.49 - 5.114 ng/g) drugs [64]. Method performance associated with method detection limits (MDLs), limit of detection (LOD) and limit of quantitation (LOQ) of 25 target compounds were compared and illustrated in Table 1. It was estimated that MDLs were approximately 3 to 10 times higher than LOD for a majority of the target analytes. In the LC-ESI-MS/MS analyses, matrix influences played a critical role that was essential to consider. These matrix effects are caused by co-extracted constituents that affect analyte ionization using either ESI positive or negative modes [42]. An approach, developed by our group [64] was used to measure the matrix influence for extraction solvents that promoted recoveries. The matrix effect in analyzing the samples that influence mass signal generation, matrix-match calibration curve was proven to be excellent method that minimized the matrix effect in quantitation of analytes from the environmental samples such as fish [64].

GC-MS is a highly efficient tool widely used to analyze semi-volatile and volatile organic personal care products at extremely low levels from environmental samples. Sample nature and complexity are critical considerations in choosing the GC-MS techniques. Extraction/pre-concentration and clean-up steps are required in preparation of samples for GC-MS examination. As fish samples are complex matrices containing lipids, fat etc., a wide range of extraction and clean-up techniques are needed to handle the samples prior to analysis [40,63,65-68]. Extraction methodologies include Soxhlet extraction, microwave assisted extraction, ultrasound-solvent liquid extraction, and pressurized liquid extraction (PLE) and clean-up approaches are silica gel, Florisil, and/or gel permeation chromatography (GPC) [40,63,65-68]. Figure 3 shows a schematic diagram of fish sample extraction, pre-concentration and clean-up protocols for GC-MS analysis [63].

Our research group developed GC-SIM-MS and GC-MS/MS.
The presence of target compounds in the fish fillet extracts was characterized based on comparison of the relative ion abundance ratio and retention features. The compounds that were characterized and quantified in grocery store fish species. The values of detected compounds from grocery store samples are approximately 1 to 3 orders of magnitude lower than the fish that were collected from the environmental sites [39-42,64].

### Biological Transformation and Effects of PPCPs

Biotransformation of any chemicals is a critical consideration because the compounds get metabolized in biological system forming different species or breakdown products that may induce numerous issues over the period of time. The effects of PPCPs differ from those of conventional pollutants because drugs are intentionally designed to interact with cellular receptors at low concentrations and to cause specific biological effects. Unintended adverse effects can also occur from interaction with non-target receptors. Environmental toxicoLOGY focuses on acute effects of exposure rather than chronic effects. Effects on aquatic life are a major concern because aquatic organisms receive much lower exposure risks than do human, and are exposed with continual and multi-generational basis with higher concentrations of PPCPs in untreated water possible low dose effects. The risks posed to aquatic organisms by trace level concentration of the PPCPs are largely unknown. Some of the known potential impacts on organisms include delayed development in fish, delayed metamorphosis in frogs, and a variety of reactions including altered behavior and reproduction [1,2]. Overall, the behavior and fate of pharmaceuticals and their metabolites in the aquatic environment and organism are not well established. The low volatility and increase polarity of pharmaceuticals indicates that distribution in the environment occurs primarily through aqueous transport, but also via food chain dispersal. Recent studies have indicated that many pharmaceuticals and metabolites are environmentally persistent, bioactive, and have potential for bioaccumulation [49,50].

Acute aquatic toxicities of drugs and metabolites were examined on marine bacterium (Vibrio fischeri), a freshwater invertebrate (Daphnia magna), and the Japanese medaka fish (Oryzias latipes) by Kim et al. [69]. They demonstrated that Daphnia was the most susceptible among the tested organisms. Correa and Hoffmann [70] studied the variation of magnitude of response effect of drugs d-amphetamine, sodium pentobarbital, diazepam, β-carboline, and saline before and after inducing into of knife-fish (Gymnatus carapo). They concluded a reduction of the degree of alertness by the barbiturate and a decrease in emotionality and/or stress by the benzodiazepine with the novelty response. Brandao et al. [71] evaluated the biochemical and behavioral effects of neuro-active anticonvulsant drugs (diazepam, carbamazepine, and phenytoin) on pumpkin-seed sunfish (Lepomis gibbosus) and showed behavioral changes of sunfish through oxidative stress parameters such as glutathione reductase, glutathione S-transferases, catalase and lipid peroxidation.

Mottaleb et al. [47,72,73] investigated the biotransformation and toxicokinetics of PPCPs known as nitro musks fragrance ingredients, musk xylene (MX), and musk ketone (MK) using trout fish as model. The fish were exposed to nitro musks compounds. Details regarding fish exposure, extraction, and analysis of breakdown product or metabolites have been reported [47,72,73]. Previously we demonstrated the formation of amine (cysteine - hemoglobin) adducts through enzymatic biotransformation reaction between MX/MK and...
trout [47,74]. The reduction of a nitro-group in MX and MK led to the formation of amine adducts of hemoglobin that could be suitable as a biochemical endpoint useful for exposure monitoring and assessment of potential hazards resulting from MX and MK compounds [47,72-74].

Recently our group [48] also reported that the bound metabolites obtained from liver proteins may be used as indicators of internal exposure to chemical carcinogens. Table 3 illustrates the relationship between exposure time and the uptake of different dosages of MX and MK compounds over a period of 7 days. The metabolites of nitro musks or other related nitroarenes, bound to the cysteine sulfhydryl group (–SH) of proteins in liver as biomarkers of exposure, could potentially be used to assess continuous exposure over a longer time range, and thus, may be better suited for risk assessment than quantitation of urinary metabolites [75]. The biological transformation processes of MX and MK to their corresponding amine metabolites, with cysteine containing proteins in the liver results in adducts formation are shown in Figure 6. Nitroarenes are enzymatically reduced to nitroso reactive intermediates, nitrosoarenes, capable of covalently binding with the –SH group of cysteine amino acids in proteins to form an acid/base labile sulfonamide adducts that hydrolyzes to aromatic amines in the presence of aqueous base [76]. The aromatic amines were considered to be good dosimeters for the target tissue [77].

Spike recovery studies and limit of detection (LOD) measurements of 2-amino-MX, 4-amino-MX and 2-amino MK metabolites were accomplished as 95-114% with relative standard deviation <10% and 0.91-3.8 ng/g, respectively. Table 4 illustrated the concentration of metabolites observed in the trout liver [48]. The half-lives of 2-AMX, 2-AMK and 4-AMX metabolites were estimated to be 2 - 9 days in the trout liver based on the assumption of first-order kinetics. The individual values of the elimination rate constants and the half-lives of the 2-AMX, 2-AMK and 4-AMX metabolites in the fish liver suggested that the toxicokinetics are more complex than a simple first-order reaction because additional internal biological processes transpire in the living organisms. Nitromusks (MX and or MK) were identified as inducers of hepatic cytochrome P450 2B enzymes and P450 1A1 and 1A2 isoenzymes [78,79] and are non-genotoxic [80,81]. Although the non-genotoxic carcinogenesis is not fully understood, it is believed that a non-genotoxic mechanism, such as increased cell proliferation, might be responsible for the increase in the liver tumors [82]. In our investigation [48], the MX and or MK-cysteine-protein adducts in fish liver were used to monitor nitro musks hazards as biomarkers of exposure.

**Conclusions**

PPCPs are increasingly being used in human and animal applications for numerous purposes. The ultimate fate of these chemicals are in aquatic systems, where organisms get exposed over an extended period of time through wastewater treatment plants and other sources. Continuous loading of the parent compounds and metabolites of PPCPs will reach harmful concentrations that adversely affect the freedom of aquatic creatures. Thus, periodic measurements of exposure level of those compounds are very important. Modern analytical techniques such as LC-MS and GC-MS have made it possible to detect extremely low levels of those chemicals. To address the challenge of PPCPs as emerging contaminants, regulatory authorities or agencies or health care professionals need to work collectively. An important and effective way aspect to reduce the load of PPCPs and their metabolites in wastewaters and surface...
Table 3: In vivo trout exposure dosing schedule with nitro musk compounds and salmon oil vehicle.

| Exposure period (Day) | Exposure level MX or MK (mg/g) | Nitro musk metabolites (ng/g) |
|-----------------------|--------------------------------|-------------------------------|
|                       | MX conc. (mg/mL) | Fish wet weight, (g) | Liver wet weigh, (g) | MX dose / trout, (mg) | Average dosing level, (mg/g) | MK conc. (mg/mL) | Fish wet weigh, (g) | Liver wet weigh, (g) | MK dose / trout, (mg) | Average dosing level, (mg/g) |
| 1-Day                 |                  |                           |                     |                       |                             |                  |                         |                         |                        |                             |
| 10                    | 0.01             | 202                       | 0.75               | 2.0                    | 257                       | 0.88             | 2.6                    | 0.01                     |
| 30                    | 0.03             | 180                       | 0.75               | 5.4                    | 199                       | 0.70             | 6.0                    | 0.03                     |
| 100                   | 0.10             | 236                       | 0.76               | 24.0                   | 272                       | 1.26             | 27.0                   | 0.10                     |
| 300                   | 0.30             | 250                       | 0.88               | 75.0                   | 190                       | 0.70             | 57.0                   | 0.30                     |
| Control               |                  | 206                       | 0.76               |                       |                            | 0.20 mL, exposed with salmon oil only |
| 3-Days                | 0.03             | 208                       | 0.79               | 6.3                    | 278                       | 1.22             | 8.4                    | 0.03                     |
| Control               |                  | 253                       | 0.89               |                       |                            | 0.25 mL, exposed with salmon oil only |
| 7-Days                | 0.03             | 212‡                      | 0.71               | 6.3                    | 121                       | 0.30             | 3.6                    | 0.03                     |
| Control               |                  | 273                       | 1.12               |                       |                            | 0.27 mL, exposed with salmon oil only |

†Trout was found dead and the liver sample was not collected (NC). ‡Trout was found sick (not equilibrium condition) and the collected liver specimen was not used in this study for composite preparation. All control liver samples were mixed together to make one control composite specimen. The collected MX and or MK exposed liver samples were composited mixing three liver for each dosing level with exception of sick or dead fish liver. (Source: Reproduced with permission from (48), © Elsevier).

Table 4: Concentration of nitro musk metabolites in trout liver samples using hydrolyzed extraction.

| Exposure period (Day) | Exposure level MX or MK (mg/g) | Nitro musk metabolites (ng/g) |
|-----------------------|--------------------------------|-------------------------------|
|                       | MX conc. (mg/mL) | Fish wet weight, (g) | Liver wet weigh, (g) | MX dose / trout, (mg) | Average dosing level, (mg/g) | MK conc. (mg/mL) | Fish wet weigh, (g) | Liver wet weigh, (g) | MK dose / trout, (mg) | Average dosing level, (mg/g) |
| 1-Day                 |                  |                           |                     |                       |                             |                  |                         |                         |                        |                             |
| 0.01                  |                    | 94.0                     | 2404.4              | 115.4                 |
| 0.03                  |                    | 492.0                    | 12588.5             | 505.5                 |
| 0.10                  |                    | 444.1                    | 10325.9             | 426.6                 |
| 0.30                  |                    | 259.1                    | 5147.3              | 396.1                 |
| 3-Days                | 0.03               | 213.6                    | 6097.6              | 357.8                 |
| 0.03                  |                    | 113.5                    | 2988.3              | 298.0                 |
| 7-Days                | 0.03               |                            |                       |                        |                             |                  |                         |                         |                        |                             |
| Controls              | None               |                            |                       |                        |                             |                  |                         |                         |                        |                             |

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waters is to develop new sewage treatment processes. This requires understanding the fate of PPCPs during sewage treatment plants for implementation of better removal techniques. Consumers need to be aware of the consequences of PPCPs to aquatic organisms and ecosystems, and should follow the regulatory agencies disposal guidelines to make our environment friendly for all living organisms. At the same time, scientists and toxicologists should continue to investigate the transport, fate, toxicity and their potential physiological and psychological effects on humans and wildlife as well as relationship between bioaccumulation and diseases.

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