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Significance of metabolite extraction method for evaluating sulfamethazine toxicity in adult zebrafish using metabolomics

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ABSTRACT

Recently, environmental metabolomics has been introduced as a next generation environmental toxicity method which helps in evaluating toxicity of bioactive compounds to non-target organisms. In general, efficient metabolite extraction from target cells is one of the keys to success to better understand the effects of toxic substances to organisms. In this regard, the aim of this study is (1) to compare two sample extraction methods in terms of abundance and quality of metabolites and (2) investigate how this could lead to difference in data interpretation using pathway analysis. For this purpose, the antibiotic sulfamethazine and zebrafish (*Danio rerio*) were selected as model toxic substance and target organism, respectively. The zebrafish was exposed to four different sulfamethazine concentrations (0, 10, 30, and 50 mg/L) for 72 h. Metabolites were extracted using two different methods (Bligh and Dyer and solid-phase extraction). A total of 13,538 and 12,469 features were detected using quadrupole time-of-flight liquid chromatography mass spectrometry (QTOF LC–MS). Of these metabolites, 4278 (Bligh and Dyer) and 332 (solid phase extraction) were found to be significant after false discovery rate adjustment at a significance threshold of 0.01. Metlin and KEGG pathway analysis showed comprehensive information from fish samples extracted using Bligh and Dyer compared to solid phase extraction. This study shows that proper selection of sample extraction method is critically important for interpreting and analyzing the toxicity data of organisms when metabolomics is applied.

1. Introduction

Recently, the field of environmental metabolomics have gained popularity in characterizing the interactions of organisms with their environment such as their response to toxic substances. Metabolomic approach has a lot of advantages for understanding organism-toxic substance interactions and in the assessment of the organism's function and health at the molecular level (Bundy et al., 2009). Since the aim of metabolomics is to quantify all metabolites within an organism to provide a valuable insight in the metabolism of cells, extraction of these metabolites from the samples is very important (Dietmair et al., 2010). In fact, sample preparation which includes extraction of metabolites from biological samples is one of the most important considerations in this field (Fan, 2012). In this regard, previous studies have focused on various metabolite extraction procedures for quantitative and qualitative metabolomics. In a study conducted by Lin et al., (2007) different metabolite extraction strategies from fish samples has been evaluated using solvents such as ice cold 6% perchloric acid, acetonitrile/water, methanol/water, and methanol/chloroform/water at different ratios and variations. The group concluded that methanol/chloroform/water (also known as Bligh and Dyer method) has the best reproducibility, ease and speed of procedure, and this method benefits from extracting hydrophilic and hydrophobic metabolites in different fractions (Lin et al., 2007). Meanwhile, Wu et al. (2008) in their study about a high-throughput extraction protocol for NMR and MS-based metabolomics presented different variants of the Bligh and Dyer method coupled with metabolite extraction strategies which they called stepwise, two-step, and all-in-one. The group showed that the two-step method had a high reproducibility for both NMR and MS analytical

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methods which is useful for high-throughput metabolomics studies (Wu et al., 2008). Bligh and Dyer has been used for many applications and has become a standard for lipid determination from biological samples (Iverson et al., 2001). Another extraction method which is used in biomarker identification as well as quantification of specific compounds from biological samples is solid phase extraction (SPE). Solid phase extraction is also suitable for high-throughput analytical approach such as global LC–MS metabolomics (Michopoulos et al., 2009; Rezzi et al., 2008). This method uses sorbents, usually housed in a cartridge format, to extract analytes from a given sample. These analytes are subsequently removed from the sorbent by solvent elution (Vuckovic, 2012). Lee et al. (2007) used solid phase extraction for both targeted and non-targeted metabolomics approach for recognizing metabolic patterns and toxicity evaluation in rats using GC/MS. The group suggested that metabolite analysis by non-targeted metabolomics approach could be performed with solid-phase extraction (Lee et al., 2007). However, these studies only showed the effects of extraction procedures only for the purpose of comparing the metabolite yield in NMR and MS-based metabolomics or for quantifying target compounds from the samples. However, little study has been conducted on how different sample extraction methods affect metabolite generation and potential toxicity analysis after exposure to toxic substances (e.g metabolic pathway analysis). Pathway analysis of metabolites in an organism can help predict the extent of toxicity which may not show in its phenotype/morphological features. This involves a comprehensive understanding on which metabolites as well as specific pathways that might have been affected after exposure to xenobiotic compounds. It is especially important in toxicity studies where the exposure of the test organism did not lead to death and in evaluating low concentration of test compounds (Bugrim et al., 2004; Cui et al., 2007; Kleinstreuer et al., 2011; Schmidt et al., 2009; Wang et al., 2011).

A number of different type of pharmaceutical compounds are found in aquatic environment (Bendz et al., 2005). Among them, antibiotics have been given much concern because they are naturally bioactive compounds (Kümmerer, 2009). The presence of these antibiotics in the environment could lead to the exposure of non-target species to these biologically active compounds which have been proven to have adverse effects on them (Isidori et al., 2005; Park and Choi, 2008; Robinson et al., 2005). For this reason, studies have been focused on evaluating the effects of antibiotics especially on their toxicity to non-target organisms (Christensen et al., 2006; Ebert et al., 2011). Although these studies tell us the toxic effects of antibiotics measured by death, growth inhibition, reproduction, and other toxicological endpoints of the test organisms, effects on the biological function specifically the behavior of their metabolites have not been fully understood under sublethal concentrations of antibiotics. In this study, the antibiotic sulfamethazine was used for the acute exposure while the model organism is zebrafish, a freshwater fish. Sulfamethazine (SMZ) is a sulfonamide antibacterial which is used to treat bacterial infections causing bronchitis, prostatitis and urinary tract infections. This antibiotic was found in environmental matrices such as soil and groundwater (Aust et al., 2008; Cabeza et al., 2012) and has been shown to have moderate toxicity on the crustacean *D. magna*, the marine-bacteria *V. fischeri,* and, the fish *O. latipes* (De Liguoro et al., 2009; Kim et al., 2007). On the other hand, zebrafish has become a favorite model organism for biologists who are trying to understand developmental processes in vertebrates (Glass and Dahm, 2004). This fish has also been used in different scientific studies involving fields such as toxicology, integrative physiology, behavioral genetics, etc. (Hill et al., 2005; Norton and Bally-Cuif, 2010). Therefore, the aim of this study is to compare the metabolite yields of both Bligh and Dyer and solid-phase extractions which will be used to determine the effects of varying concentrations of sulfamethazine in zebrafish through pathway analysis. To the author's knowledge, there has not been a published study discussing extraction procedures and its relevance to metabolic pathway analysis of zebrafish (*Danio rerio)* exposed to xenobiotic compounds.

2. Materials and methods

2.1. Reagents and chemicals

Sulfamethazine (SMZ) with purity of $>99\%$ was from Sigma Life Science, Sigma-Aldrich Co. and all solvents used in the experiments were HPLC grade. Sulfamethazine stock solution was dissolved using 0.1 M sodium hydroxide and kept in a glass container protected from light and stored at 4 °C.

2.2. Zebrafish exposure

D. rerio AB strain (zebrafish) was purchased from Seoul Aquarium, Daejeon, South Korea. Adult zebrafish (14mm average body length) were approximately 90 days old (3 months) at the time of purchase. Fish were placed in a 20 L tank with two bubblers for proper oxygenation. The water temperature was maintained at 28.5 °C and the fish were fed daily with commercial fish feed (Jaqno Color Charasin) (Lawrence, 2007). The exposure set-up to different concentrations of sulfamethazine was done by placing ten fish to nine 3-L aquaria containing 2 L of water dosed with 10, 30, and 50 mg/L of the antibiotic (Fig. 1). These sulfamethazine concentrations were selected as sub-lethal concentrations from a preliminary 72 h exposure testing. Meanwhile, the fish used as control were placed in aquaria containing water mixed with the solvent for sulfamethazine (0.1 M NaOH) The aquaria were then mounted in a frame (100 cm × 30 cm × 150 cm) with a controlled temperature at 28.5 °C via a recirculating water system. The exposure was done for 72 h and the fish were processed thereafter.

2.3. Extraction of metabolites

Extraction of metabolites using Bligh and Dyer and SPE methods were done following previously described procedures with slight modifications (Bligh and Dyer, 1959; Shao et al., 2005). Both extraction methods are elaborated in the Supplemental information.

2.4. ^{1}H-NMR spectroscopy

Nuclear magnetic resonance spectroscopy samples were prepared according to Park et al. (2009) with minimal modifications. Briefly, samples were thawed and 100 μL aliquots were transferred to Eppendorf tubes. 500 μL of deuterium oxide (D2O) and 66 μL of a mixture of deuterium oxide and 4, 4-dimethyl-4-silapentane-1-sulfonic acid (DMS) (1:6). The solution was vortexed and transferred to the NMR tubes.^{1}H NMR spectra were measured at 600 MHz on a Bruker Biospin AVANCE 600 MHz spectrometer with water pre-saturation at 25 °C. Spectra were measured with 64 scans in 16,384 data points over a spectral width of 6600.7 Hz. The acquisition time was 2.55 seconds (d1 = 0, pulse = 5 μs, presaturation = 1 s, and acquisition = 1.5 s) with line broadening at 0.3 Hz. The chemical signals obtained from the analysis were compared for both the metabolite extraction samples.

2.5. Q-TOF LC/MS

Before analysis, fifty microliter aliquot of the extracts were