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Metabolism of clofibric acid in zebrafish embryos (*Danio rerio*) as determined by liquid chromatography–high resolution–mass spectrometry

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ABSTRACT

The zebrafish embryo (ZFE) is increasingly used in ecotoxicology research but detailed knowledge of its metabolic potential is still limited. This study focuses on the xenobiotic metabolism of ZFE at different life-stages using the pharmaceutical compound clofibric acid as study compound. Liquid chromatography with quadrupole-time-of-flight mass spectrometry (LC-QToF-MS) is used to detect and to identify the transformation products (TPs). In screening experiments, a total of 18 TPs was detected and structure proposals elaborated for 17, formed by phase I and phase II metabolism. Biotransformation of clofibric acid by the ZFE involves conjugation with sulfate or glucuronic acid, and, reported here for the first time, with carnitine, taurine, and aminomethanesulfonic acid. Further yet unknown cyclization products were identified using non-target screening that may represent a new detoxification pathway. Sulfate containing TPs occurred already after 3 h of exposure (7 hpf), and from 48 h of exposure (52 hpf) onwards, all TPs were detected. The detection of these TPs indicates the activity of phase I and phase II enzymes already at early life-stages. Additionally, the excretion of one TP into the exposure medium was observed. The results of this study outline the high metabolic potential of the ZFE with respect to the transformation of xenobiotics. Similarities but also differences to other test systems were observed. Biotransformation of test chemicals in toxicity testing with ZFE may therefore need further consideration.

1. Introduction

Biological effects of a large number of compounds have been investigated using the zebrafish embryo (ZFE) as an alternative test system in toxicological research to reduce or replace classical *in vivo* studies (Lieschke and Currie, 2007; Embry et al., 2010; Sipes et al., 2011). High-throughput screening represents an adequate experimental setup for assessing the toxicity and studying e.g. lethal concentrations (Ali et al., 2012; Padilla et al., 2012), and it has been shown that the results are comparable to toxicological studies using adult zebrafish (Lammer et al., 2009; Strahle et al., 2012; Sanz-Landaluze et al., 2015). As long as effects on the test organisms are compared to the external exposure concentration the underlying toxicokinetic (TK) and toxicodynamic (TD) processes remain unknown. Toxicokinetic includes absorption, distribution, metabolism, and excretion of the test compound, which all influence the internal concentration in the ZFE (Escher et al., 2011). Analytical approaches using liquid or gas chromatography with mass spectrometric detection are available for the quantification of internal concentrations and various uptake rates for different chemical classes have been reported (El-Amrani et al., 2012; Kühnert et al., 2013; Brox et al., 2014a). For compounds such as benzocaine or benz[a]anthracene, it has been suggested that biotransformation explains decreasing internal concentrations in early life-stages of the ZFE (Kühnert et al., 2013; Brox et al., 2014a). Gene expression of phase I and phase II enzymes in ZFE such as cytochrome P450 (CYP), glutathione S-transferase (GSH), UDP-glucuronosyltransferase (UGT), or sulfotransferase (SULT) support this assumption (Goldstone et al., 2010; Kurogi et al., 2013; Christen and Fent, 2014; Hansen and Harris, 2015).

Phase I and phase II biotransformation of a toxic compound may reduce or increase toxic effects depending on whether the transformation products (TPs) are less or more toxic than the parent compound (Carlsson et al., 2011; Weigt et al., 2011; Padilla et al., 2012; Zhu et al., 2015). However, the knowledge on the potential of ZFE to transform xenobiotics after uptake is limited (Sipes et al., 2011). Activation and detoxification of test compounds requires enzyme activity and this may be independently proven by the determination of TPs, preferably by liquid chromatography coupled to mass spectrometry (LC–MS). Previous studies on the metabolism of xenobiotics in ZFE predominantly used tandem-mass spectrometry (tandem-MS) and older life-stages of the ZFE starting at 72 h post-fertilization (hpf) (Alderton et al., 2010; Hu et al., 2012; Jones et al., 2012).

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These life-stages are supposed to be fully capable to form TPs. In these studies, phase I TPs such as hydroxy-ibuprofen after the exposure to ibuprofen (Jones et al., 2012) or paracetamol after the exposure to phenacetin have been identified (Alderton et al., 2010). Furthermore, phase II metabolism such as sulfatation, glucuronidation, or glucosylation occurs and conjugates with polyphenols or testosterone have been shown to be formed by ZFE (Alderton et al., 2010; Hu et al., 2012). For the detection by tandem-MS techniques, however, potential TPs must either be known *a priori* or need to share a high degree of structural similarity with the parent compound to allow their determination by neutral loss or precursor ion scan. These conditions are not necessarily fulfilled in biodegradation studies.

Liquid chromatography coupled to high-resolution mass spectrometry (LC-HRMS) with suitable data processing strategies offers a more generic approach to detect and to identify yet unknown TPs. In this study, an analytical approach is presented for the identification of TPs as well as the determination of internal concentration-time profile in very early life-stage (<72 hpf) of the ZFE. Time-resolved investigations starting at 4 hpf would provide more information on the onset of metabolic activity.

For this issue, liquid chromatography with quadrupole-time-of-flight mass spectrometric detection (LC-QToF-MS) in combination with all ion fragmentation is used. The data analysis involves suspect and non-target screening approaches (Zhu et al., 2011). These two strategies cover well-known and putative metabolites but also unexpected metabolic products and can be applied to any test compounds regardless its molecular structure. The pharmaceutical compound clofibric acids has been chosen as study compound for the following reasons: (a) adverse effects on aquatic organisms (Emblidge and Delorenzo, 2006; Runnalls et al., 2007) including adult zebrafish (Coimbra et al., 2015) have been described; (b) it is widely present in the environment (Reemtsma et al., 2006); (c) the metabolism of clofibric acid and of other xenobiotic carboxylic acids (XCA) in humans and other vertebrates has been described (Cayen, 1985; Darnell and Weidolf, 2013). Therefore, clofibric acid appeared to be a good candidate to study the metabolism in ZFE and to compare it with the metabolism in other, primarily higher organisms.

It has been shown before that clofibric acid is taken up comparatively slowly and this has been explained by the predominance of its anionic form (Brox et al., 2014a). In the case that metabolism is faster than uptake, it leads to decreasing internal concentrations of the parent compound but biotransformation may also occur when no such decrease is discernible. The liver morphogenesis, that is supposed to play a key role for biotransformation, is starting already at approximately 24 hpf while liver-specific markers occur even earlier (16 hpf) (Tao and Peng, 2009). The analytical approach presented here should be of use for ecotoxicological research to obtain a detailed insight into biotransformation processes and evidence on enzyme activity in very early life-stages of the ZFE.

2. Material and methods

2.1. Chemicals, reagents and standards

Clofibric acid (CAS RN 882–09-7) was obtained from ICN Biomedicals (Eschwege, Germany). Calcium chloride dehydrate (10035-04-8), magnesium sulfate heptahydrate (10034-99-8), sodium hydrogencarbonate (144-55-8), and potassium chloride (7447-40-7) for the preparation of the standard water (ISO-water (ISO, 1996)) were purchased from Sigma–Aldrich (Munich, Germany). Methanol and ammonium acetate (both ULC/MS grade) for UPLC-QToF analysis were obtained from Biosolve (Valkenswaard, The Netherland). The aqueous exposure solution of clofibric acid was prepared in ISO-water (ISO, 1996) and the final concentration was well below the water solubility.

2.2. Exposure experiments

Fertilized eggs were identified according to Kimmel et al. (Kimmel et al., 1995) and were collected under a light microscope. 25 ZFE were placed into a 50 mL glass flask and were exposed to clofibric acid (exposed ZFE). To obtain control samples, 25 ZFE were incubated in ISO-water in parallel (control ZFE). For both groups of samples, three replicates were prepared for each sampling. Incubation took place in a climatic chamber (26 ± 1 °C) with a light–dark cycle (12 h/12 h). For quality assurance, an aliquot of the exposure medium was taken at the start of the experiment. Additionally the stability was checked by incubation in parallel without ZFE (stability control). Aliquots from the exposure medium as well as of the stability control were taken at every sampling point. The external concentration of clofibric acid was 50 mg/L in all experiments. The exposure concentration (50 mg/L) was below the reported LC_{50} value (>600 mg/L) (Kehrer, 2008), and no lethal effects on the ZFE were observed (OECD, 2013). After 96 h of exposure, all individuals had hatched.

Two experimental setups were performed: a) a screening experiment for the identification of TPs and b) the investigation of the time-resolved formation of TPs. For the screening experiment, ZFE were pre-incubated until 72 hpf in ISO-water and were then exposed to the study compound for 24 h. For internal concentration-time profiles, the experiment was started approximately 4 hpf and samples were taken after 3, 6, 24, 48, 72, and 96 h of exposure (approx. 7, 10, 28, 52, 76, and 100 hpf). For the identification of TPs in ZFE (screening experiment), one experiment was performed. The time-resolved results of the internal concentration of the identified TPs are based on four individual exposure experiments.

2.3. Sample preparation

The sample preparation is described by Brox et al. (Brox et al., 2014a). Briefly, alive embryos were transferred from the exposure medium onto a metal mesh and washed with double distilled water. The mesh was first dried using tissue paper and the washed ZFE were then transferred into a Eppendorf tube and were shock-frozen with liquid nitrogen. 500 μL methanol were used for extraction and samples were sonicated for 15 min. Contrary to the previous approach (Brox et al., 2014a), ZFE were not dechorionated in these experiments. As the chorion and perivitellin space affect the quantification of the study compounds (Brox et al., 2014b) and since this study focuses on TPs that are formed in the ZFE dechorionation was not required. The methanolic extract was concentrated by a factor of approximately 2.88 by evaporating an aliquot of 360 μL of the methanolic extract to dryness using argon gas and dissolving the solid in 125 μL water/methanol (80/20, v/v). Samples of the exposure medium were analyzed without dilution. External concentration of clofibric acid was checked at every sampling point of the exposure and quantification was performed according to Brox et al. (Brox et al., 2014a).

2.4. UPLC-QToF-MS

For the analysis, a UPLC Acquity I-Class System (Waters, Milford, USA) and a Synapt G2S QToF mass spectrometer (Waters, Milford, USA) equipped with an electrospray interface was used. Separation was carried out on a Waters Acquity UPLC BEH C18 column (2.1 × 100 mm, 1.7 μm; Waters) using water and methanol as eluents, both with 10 mM ammonium acetate and 0.3% acetic acid (pH 5). Column temperature was set to 45 °C and 10 μL of the sample were injected. The gradient with a flow rate of 450 μL/min was as follows: 0.0 min, 2% B; 0.25 min, 2% B; 12.5 min, 99% B; 13.0 min 99% B; 13.01 min, 2% B; 17.0 min, 2% B.

Electrospray ionization was performed in positive and negative mode using a capillary voltage of 0.7 and −2 kV, respectively. The source temperature was set to 140 °C and the desolvation temperature

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