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Uptake, biotransformation and elimination of selected pharmaceuticals in a freshwater invertebrate measured using liquid chromatography tandem mass spectrometry

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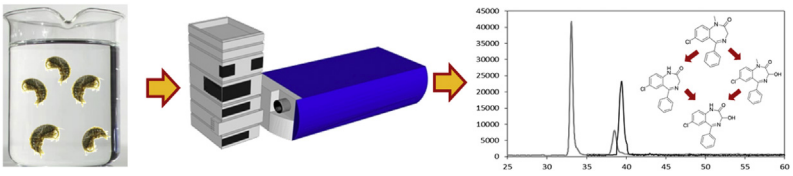
HIGHLIGHTS

- Bioconcentration factors of 9 pharmaceuticals in *G. pulex* ranged from 0 to 73 L kg^{-1}
- *G. pulex* are capable of pharmaceutical biotransformation.
- Five pharmaceutical metabolites were determined up to 94.5 ng g^{-1}
- Biotransformation can affect BCF_{total} estimates if metabolites are not accounted for.

GRAPHICAL ABSTRACT



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ABSTRACT

Methods were developed to assess uptake and elimination kinetics in *Gammarus pulex* of nine pharmaceuticals (sulfamethazine, carbamazepine, diazepam, temazepam, trimethoprim, warfarin, metoprolol, nifedipine and propranolol) using targeted LC-MS/MS to determine bioconcentration factors (BCFs) using a 96 h toxicokinetic exposure and depuration period. The derived BCFs for these pharmaceuticals did not trigger any regulatory thresholds and ranged from 0 to 73 L kg^{-1} (sulfamethazine showed no bioconcentration). Metabolism of chemicals can affect accurate BCF determination through parameterisation of the kinetic models. The added selectivity of LC-MS/MS allowed us to develop confirmatory methods to monitor the biotransformation of propranolol, carbamazepine and diazepam in *G. pulex*. Varying concentrations of the biotransformed products; 4-hydroxypropranolol sulphate, carbamazepine-10,11-epoxide, nordiazepam, oxazepam and temazepam were measured following exposure of the precursor compounds. For diazepam, the biotransformation product nordiazepam was present at higher concentrations than the parent compound at 94 ng g^{-1} dw. Overall, the results indicate that pharmaceutical accumulation is low in these freshwater amphipods, which can potentially be explained by the rapid biotransformation and excretion.

1. Introduction

Extensive research into organic environmental micropollutants has enabled the elucidation of the mechanisms for the uptake and

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accumulation in biota (Barber et al., 1988, 1991; Mackay and Fraser, 2000). Uptake was mainly considered to occur by passive diffusion across cellular membranes and traditional models relied heavily on physico-chemical properties such as octanol-water partition coefficients (log*P*) to describe and predict xenobiotic concentrations in biota (Kanazawa, 1981; Neely et al., 1974; Veith et al., 1979). Such earlier works often focussed on neutral compounds (Fu et al., 2009; Klosterhaus et al., 2013; Wu et al., 2013), but more recently identified micropollutant classes, such as pharmaceuticals, are somewhat different in that they are often ionisable and have a wider range of molecular polarity. Additional mechanisms such as ion trapping, carrier mediated transport and partitioning to non-lipid components (protein binding) could also influence the accumulation of pharmaceutical residues in the environment (Fu et al., 2009; Klosterhaus et al., 2013; Stott et al., 2015). As most of the reported work has focussed on vertebrates such as fish (Gobas et al., 1986; Kanazawa, 1981; Spacie and Hamelink, 1982; Veith et al., 1979), the bioaccumulation of compounds in invertebrates is not well understood.

The OECD 305 guidelines are widely used for estimating the bioconcentration factor (BCF) or bioaccumulation factor (BAF) in fish and have also been applied to invertebrates, such as bivalves and amphipods (Ashauer et al., 2006, 2010; Meredith-Williams et al., 2012; OECD; Sordet et al., 2016). Estimations using these guidelines can be determined using steady-state or kinetic measurements. Kinetic measurement estimates are based on non-linear regression to generate uptake (k_1) and elimination rate constants (k_2) and used together to estimate BCF/BAF. Models assume that rate constants do not change. However, recent investigations in our laboratory showed that the OECD model led to significant lack-of-fits for measured data in invertebrates (Miller et al., 2016). The lack-of-fits were shown to arise from a potentially decreasing k_1 trend over time for a proportion of compounds tested. This finding was significant as such models could lead to under/over estimation of BCF/BAFs during risk assessment of chemicals in invertebrates. Possible causes for the decreases in k_1 could be related to several factors such as growth, metabolism and sorption processes. Metabolism in particular, is generally not considered in bioconcentration studies. Many analytical methods rely on measurement of total radioactivity of a labelled compound using liquid scintillation counting (LSC) (Arnot and Gobas, 2006) as an alternative to confirmatory analytical tools based on liquid chromatography (LC) or gas chromatography (GC) coupled to mass spectrometry (MS). However for small freshwater invertebrates, only a few published LC or GC-MS-based methods exist for parent compound determination (Grabicova et al., 2015; Inostroza et al., 2016; Miller et al., 2015; Sordet et al., 2016). A reason for the small number of published methods is that sensitivity at environmentally relevant concentrations is often challenging due to their small size. Similarly, simply increasing the sample mass to be extracted (via pooling of individuals) is often undesirable due to extra MS signal suppression or enhancement effects caused by the matrix. Therefore, a delicate balance is required to ensure sufficient MS sensitivity and that reliable quantifications can be performed. In the absence of confirmatory analytical methods, biotransformation during the exposure period could have a significant effect on invertebrate BCF/BAF estimation using scintillation counting methods (de Wolf et al., 1992; Oliver and Niimi, 1985; Opperhuizen and Sijm, 1990). Xenobiotics can also induce or inhibit their own metabolism or the metabolism of other compounds that will affect the clearance rate and hence the BCF/BAF (Golan et al., 2011). Moreover, as standardised methods to measure metabolic products and the kinetics of biotransformation currently do not exist, it is difficult to assess the influence of biotransformation in accumulation (Cowan-Ellsberry et al., 2008). Several authors have applied *in vitro* intrinsic clearance rates to extrapolate to whole body biotransformation rates for predictive BCF modelling (Arnot et al., 2008; Nichols et al., 2013). However, these extrapolations measure only the loss of parent compound to predict whole body biotransformation. Thus, *in vitro* clearance rates may not reliably reflect whole body metabolic rates (Nichols et al., 2006).

To date only three studies have measured biotransformation products and their associated toxicokinetics in invertebrates (Ashauer et al., 2012; Jeon et al., 2013b; Rösch et al., 2016). Two of these works used LC coupled to high resolution MS (HRMS) or LC with a radioactivity detector to model the uptake and elimination profiles of organic micropollutants and their biotransformation products in *G. pulex* (Ashauer et al., 2012; Rösch et al., 2016). Ashauer et al. showed that the measurement of biotransformation improved the accuracy of BCF estimates when compared to estimates using total radioactivity counts (Ashauer et al., 2012). A further constraint to the study of xenometabolism is that *a priori* knowledge of biotransformation products in aquatic organisms is lacking leading to difficulty when developing targeted analytical methods (Celiz et al., 2009). Current methods have focussed on the determination of organic pollutants in fish, with little attention given to invertebrates or pharmaceutical biotransformation. Therefore, it is essential that methods are developed that can determine pharmaceutical biotransformation products to more reliably assess the affect metabolism has on bioconcentration models.

The aim of this work was to assess the bioconcentration of a selection of nine pharmaceuticals in *G. pulex* using targeted LC-MS/MS methods described in (Miller et al., 2015). In this regard, the method developed for pharmaceutical occurrence was used for the determination of selected known pharmaceutical biotransformation products of carbamazepine, diazepam and propranolol. The method showed good performance in terms of linearity, recovery, precision and robustness. In particular, BCFs were estimated using the OECD 305 guidelines and kinetic parameters were checked for constancy over time. Finally, the optimised LC-MS/MS method was used for the identification and determination of biotransformation products of propranolol, carbamazepine and diazepam. As few published works have studied pharmaceutical bioconcentration and biotransformation, the work presented herein addresses the knowledge gaps concerning their bioaccumulation and biotransformation in invertebrates at environmentally relevant concentrations using a minimised test design.

2. Materials and methods

2.1. Reagents, chemicals and consumables

HPLC grade methanol, acetonitrile, acetone, ethyl acetate, dichloromethane and dimethyldichlorosiloxane were purchased from Fischer Scientific (Loughborough, UK). Analytical grade ammonium acetate was sourced from Sigma-Aldrich (Dorset, UK). Propranolol hydrochloride, warfarin, sulfamethazine, carbamazepine, nimesulide, (±)-metoprolol (+)-tartrate salt, temazepam, diazepam, nifedipine, oxazepam, nordiazepam, carbamazepine-10,11-epoxide, and sulfamethazine were all obtained from Sigma-Aldrich (Steinheim, Germany). Trimethoprim, was ordered from Fluka (Buchs, Switzerland). Stable isotope-labelled standards including carbamazepine-d_{10}, propranolol-d_7, temazepam-d_5 and diazepam-d_5 were ordered from Sigma-Aldrich. Sulfamethazine-d_4, nifedipine-d_4, metoprolol-d_7, trimethoprim-d_3 and warfarin-d_5 were ordered from QMX Laboratories (Essex, UK). The propranolol biotransformation products; 4-hydroxypropranolol, 4-hydroxypropranolol sulphate and 4-hydroxypropranolol glucuronide were sourced from Santa Cruz Biotechnology (Heidelberg,