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LC–MS/MS method development for quantitative analysis of acetaminophen uptake by the aquatic fungus *Mucor hiemalis*

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ABSTRACT

Acetaminophen is a pharmaceutical, frequently found in surface water as a contaminant. Bioremediation, in particular, mycoremediation of acetaminophen is a method to remove this compound from waters. Owing to the lack of quantitative analytical method for acetaminophen in aquatic organisms, the present study aimed to develop a method for the determination of acetaminophen using LC–MS/MS in the aquatic fungus *Mucor hiemalis*. The method was then applied to evaluate the uptake of acetaminophen by *M. hiemalis*, cultured in pellet morphology. The method was robust, sensitive and reproducible with a lower limit of quantification of 5 pg acetaminophen on column. It was found that *M. hiemalis* internalize the pharmaceutical, and bioaccumulate it with time. Therefore, *M. hiemalis* was deemed a suitable candidate for further studies to elucidate its pharmaceutical tolerance and the longevity in mycoremediation applications.

1. Introduction

Water is an essential facet to life. However, only 0.3% of the total amount of water on earth is available fresh water, not all of it potable due to anthropogenic pollutants and natural toxins that infer an ecological health risk.

Many reports have been made on the occurrence of anthropogenic water xenobiotics, especially pharmaceuticals in wastewater, surface water, and ground water (Escher et al., 2011; Jones, et al., 2007; Mankes and Silver, 2013; Nunes et al., 2014; Verlicchi et al., 2010; Wu et al., 2012). These pollutants bioaccumulate in the environment, negatively affecting not only man but nature as well.

A pharmaceutical of particular concern is acetaminophen (APAP; *N*-acetyl-*p*-aminophenol or paracetamol), as it is one of the main prescribed analgesics and is freely available for self-medication (Wu et al., 2012; Verlicchi et al., 2010; Escher et al., 2011). In 2000, the amount of paracetamol prescribed in the UK alone was 390×10^3 kg, which is the highest amount of all drugs investigated in a study by Jones (2002). Bedner and MacCrehan (2006) reported a production of 3.6×10^6 kg APAP in 2002 in the USA. Paracetamol is not only present in wastewaters of hospitals and the drug industry but also in natural aquatic environments (Jones, 2002; Nunes et al., 2014; Wu et al., 2012; Verlicchi et al., 2010) due to excretion, inappropriate usage and bedside wasting (Mankes and Silver, 2013).

Numerous publications on environmental pharmaceutical concentrations exist, however, uptake and bioaccumulation studies in aquatic organisms are lacking. The environmental toxicity effects of paracetamol in different aquatic species were evaluated by Nunes et al. (2014) and Escher et al. (2011). Nunes et al. (2014) suggested that the toxic effects of APAP on aquatic organisms are highly variable even among phylogenetically related organisms. Wu et al. (2012) highlighted that even at low concentrations the effects of pharmaceuticals in aquatic environments should not be underestimated since they are substances designed to be biologically active. Also, the combination of pharmaceuticals in water can have a wide range of ecological effects even if the concentration of each pharmaceutical is below the Predicted Ecological Concentration (PEC) (Mankes and Silver, 2013). Paracetamol, ibuprofen and diclofenac are naturally transformed and degraded (Jones et al., 2002), but the consistent release of paracetamol into the environment can outnumber the transformation rate (Petrović et al., 2003).

With the wide spread detection of pharmaceuticals in water sources, necessary treatment regimes need to be put in place, specifically targeting these compounds. There is an indication that even modern wastewater treatment plants cannot completely absorb pharmaceuticals (Verlicchi et al., 2010; Jones et al., 2007) or in other cases, like with paracetamol, toxic breakdown products

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are produced (Bedner and MacCrehan, 2006). Removal efficiencies of approximately 90% from wastewater were reported by Jones et al. (2007), however, the remaining drug concentrations, including paracetamol, were more than several hundred nanograms per litre.

Phytoremediation methods like the Green Liver System® have quite a long research history (Pflugmacher and Sandermann, 1998; Sandermann et al., 1984; Sandermann, 1992) and are already successfully in use (Nimptsch et al., 2008; Pflugmacher et al., 2015). However, due to declining macrophyte growth and even death in winter months, the range of organisms used in the Green Liver System® needs to be expanded. An alternate to using aquatic macrophytes may be aquatic fungi, as they are known for their capacities to adapt to severe environmental constraints and aquatic remediation abilities (Gupta et al., 2011). Remediation using fungi has been demonstrated with heavy metals (Miransari, 2011), polycyclic aromatic hydrocarbons and chlorinated compounds such as pesticides (Kennedy et al., 1990; Fragoeiro, 2005) and recently cyanobacterial toxins (Balsano et al., 2015). Mycoremediation both *in sito* and *ex sito* is primarily achieved by the mycelia, which produce enzymes capable of disrupting lignin and cellulose, structurally similar to many organic xenobiotics. White-rot fungi, a diverse ecophysiological group comprised mostly of basidiomycetous fungi, are known to contain peroxidases and laccases, which aid in degradation. The fungus of interest in the present study, *Mucor hiemalis* (Zygomycota), is used in several industrial processes as it produces lipases and proteases. Furthermore, it is temperature-independent as it sporulates at temperatures as low as 5 °C. *M. hiemalis f. irnsingii* (DSM 14200) demonstrated high glutathione *S*-transferase activity against fluorodifen and a high tolerance against H_2S (Hoque, 2002). The fungus possesses functional groups on its cell wall enabling biosorption of heavy metals, such as chrome VI (Tewari et al., 2005) and nickel (Shroff and Vaidya, 2011) and is also known for its fast and complete remission potential of the herbicide isoproturon (Rønhede et al., 2005).

Currently, the Green Liver System®, developed by Prof. Dr. Pflugmacher Lima and his team, has focused on using aquatic macrophytes to assimilate and biotransform xenobiotics (Pflugmacher, 2015). The present study aimed to investigate the possibility of using the aquatic fungus, *M. hiemalis*, to take up the pharmaceutical APAP in order to expand the application possibilities of the Green Liver System®. It was therefore necessary to develop and implement a reliably sensitive, reproducible in-house APAP quantification method together with a suitable extraction method from the fungus in question. Previous studies have focused on APAP method development and validation in plasma and urine samples (Van Eeckhaut et al., 2009; Hewavitharana et al., 2008; An et al., 2012; Gicquel et al., 2013). Nonetheless, to the best of the authors' knowledge, this is the first report on the development and testing of an APAP quantitative analysis method in an aquatic organism.

2. Methods and materials

2.1. Chemicals and reagents

All chemicals used during the present study were analytical grade unless specified otherwise and purchased from Sigma-Aldrich. APAP powder was used to prepare a stock solution (1 μg/mL) from which the necessary dilutions were made; all of which in MS grade water.

2.2. Instrumentation and method development

All quantitative analyses were performed on an Agilent 1200 infinity series liquid chromatography system coupled to an Agilent 6460 triple quadrupole mass spectrometer with Agilent Jet Stream electron spray ionization (AJS-ESI) technology. The APAP LC–MS/MS method was configured and optimized using the above-mentioned stock solution. Firstly, the optimum tandem mass spectrometer parameters were established by direct infusion of 1 μL of 100 ng/mL APAP stock. A full mass spectrum was executed to determine the most abundant precursor ions and adducts followed by a product ion scan.

Optimal parameters for MS/MS operation, established using the MassHunter Source and iFunnel Optimizer software, were as follows: The drying gas temperature was set at 280 °C at a flow of 8 L/min and the sheath gas temperature was set at 300 °C at a flow of 12 L/min. Nitrogen was used as drying gas and argon as collision gas. The capillary voltage and nozzle voltage was set at 5000 and 2000 V, respectively and the nebulizer pressure used was 40 psi. The MRM mode (positive mode) was used with a mass transfer of the precursor ion 152 (Q1) to the product ions 110 and 93 (Q3). A fragmentor voltage of 100 V was applied, and collision energies of 16 and 26 V for each product ion, respectively. The accelerator voltage used was 1 V.

Liquid chromatographic separation of APAP was optimized and established on a reverse phase Kinetex® C18 (100 Å, 2.6 μm, 50×2.1 mm^2) column (Phenomenex) equipped with a SecurityGuard Ultra C18 (2.1 mm) pre-column (Phenomenex). Optimal separation and adequate retention was achieved using water (MS grade) (A) and methanol (MeOH) (MS grade) (B), both containing 0.2% formic acid, as mobile phases. Linear gradient elution was applied starting with 5% B and increased to 80% over 6 min. The MeOH percentage was further increased to 95% and held at this percentage for 2 min, resulting in a retention time of 4.4 min for APAP. A post time of 4 min ensued before the next injection. A constant flow rate of 0.3 mL/min was used with a sample injection volume of 10 μL. The column oven was kept at a constant temperature of 35 °C for the duration of the analysis.

System control was managed by using the Agilent MassHunter data acquisition for triple quadrupole mass spectrometer, Version 06.00 software. Agilent MassHunter qualitative software, Version 06.00 was used for the quantitative analysis of APAP in samples.

2.3. Method performance evaluation

The developed method performance was evaluated according to Huber (1999). A seven-point standard curve (dilution pattern: 1:10:2:5:2:5:2) was constructed by plotting the peak areas of the APAP molecular ion (mass transfer of 416 (Q1) and 176 and 194 (Q3)) as a function of concentration. All analyses were conducted in triplicate unless stated otherwise. The calibration curve was statistically evaluated by linear regression to determine linearity as a validation parameter (IBM SPSS Statistics Standard package for Mac). Additionally, accuracy and precision was calculated for each of the calibration points. The precision was calculated as the percentage relative standard deviation (%RSD) of APAP obtained from replicates at each calibration point (%RSV = 100 × (standard deviation/mean of concentrations)). Accuracy was calculated for each calibration point by the MassHunter software, as a percentage deviation relative to the nominal concentration of each point. Intermediate precision was measured by repeating the calibration analysis on three consecutive days, followed by evaluation with an analysis of variance (ANOVA) on SPSS. The sensitivity and robustness of quantification was established in a matrix of APAP in a crude fungal extract, prepared by cell disruption and spiking with 100 μg/L APAP. The limit of detection (LOD) and limit of