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Aerobic activated sludge transformation of methotrexate: Identification of biotransformation products

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ABSTRACT

This study describes the biotransformation of cytostatic and immunosuppressive pharmaceutical methotrexate. Its susceptibility to microbiological breakdown was studied in a batch biotransformation system, in presence or absence of carbon source and at two activated sludge concentrations. The primary focus of the present study are methotrexate biotransformation products, which were tentatively identified by the ultra-high performance liquid chromatography–quadrupole – Orbitrap-MS. Data-dependent experiments, combining full-scan MS data with product ion spectra were acquired, in order to identify the molecular ions of methotrexate transformation products, to propose the molecular formulae and to elucidate their chemical structures. Among the identified transformation products 2,4-diamino-N^{10}-methyl-pteroic acid is most abundant and persistent. Other biotransformation reactions involve demethylation, oxidative cleavage of amine, cleavage of C–N bond, aldehyde to carboxylate transformation and hydroxylation. Finally, a breakdown pathway is proposed, which shows that most of methotrexate breakdown products retain the diaminopteridine structural segment. In total we propose nine transformation products, among them eight are described as methotrexate transformation products for the first time.

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1. Introduction

Among various classes of pharmaceuticals, cytostatic chemotherapy drugs, also called anticancer or antineoplastic agents, are of particular environmental concern because they are potentially carcinogenic, mutagenic and genotoxic (Zounková et al., 2007). The fate of these compounds in the environment is largely unknown, but they can contaminate wastewater treatment effluents and consequently aquatic ecosystems (Zounková et al., 2007).

Methotrexate (MET) is an antimetabolite and antifolate drug used in treatment of cancer and autoimmune diseases (Nagulu et al., 2009), such as psoriasis, rheumatoid arthritis, Crohn's disease, and intrinsic asthma. Chemically MET is *N*-[4-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-L-glutamic acid. A major part of the molecule includes diamino-pteridine ring linked by a methylene bridge to *para*-methylaminobenzoic acid, which is joined by an amide linkage to glutamic acid (Fig. 1).

MET acts by inhibiting *dihydrofolic* acid *reductase*, which reduces dihydrofolates to tetrahydrofolates, before they can be utilised as carriers of one-carbon groups in the synthesis of purine nucleotides and thymidylate. In this way MET interferes with DNA synthesis and cellular replication. Actively proliferating tissues such as malignant cells, bone marrow, fetal cells, buccal and intestinal mucosa, and cells of the urinary bladder are in general more sensitive to this effect of MET (FDA, 2013).

After absorption, MET undergoes hepatic and intracellular metabolism to polyglutamated forms, which can be converted back to MET by *hydrolase* enzymes. Overall, MET is not significantly metabolised; with intravenous administration, 80–90% of the administered dose is excreted unchanged in the urine within 24 h (FDA, 2013; Foye and Sengupta, 1995). Though, a small amount of metabolism to 7-hydroxymethotrexate (7-OH-MET) may occur at doses commonly prescribed (FDA, 2013), whereas 2,4-diamino-N^{10}-methyl-pteroic acid (DAMPA) is formed after high-dose MET treatment (Donehower et al., 1979). Renal excretion is the primary route of elimination and is dependent upon dosage and route of administration.

The mixture of parent compound and metabolites is excreted from the body and typically enters the sewerage system eventually reaching surface waters (Kosjek et al., 2013). MET was determined in hospital effluents at concentrations of 1 μg L^{-1} (Aherne et al., 1985) and 17 ng L^{-1} (Yin et al., 2010), and in sewage treatment

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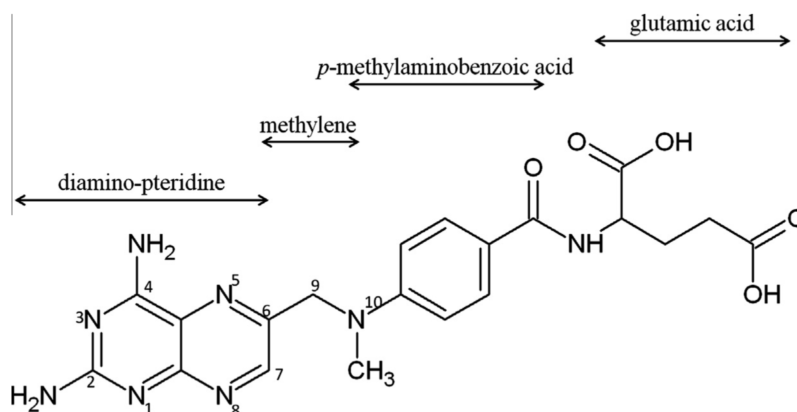

Fig. 1. Chemical structure of MET.

plant effluents at levels of 12.6 ng L^{-1} (Castiglioni et al., 2005) and 2.1–20 ng L^{-1} (Negreira et al., 2013a), whereas it was below the detection limit in surface, ground and potable waters (Aherne et al., 1985; Negreira et al., 2013a).

The absence of MET in the aquatic environment and its low-level occurrence in treated wastewater samples may be justified by its significant biodegradation (Kiffmeyer et al., 1998). Kiffmeyer et al. (1998) reported its nearly complete removal from an activated sludge system within 14 d. The same research group revealed that MET was biotransformed into OH-MET during their biotransformation experiments (Kiffmeyer et al., 1998). In contrast with the parent compound, OH-MET was not found biodegradable (Kiffmeyer et al., 1998) and might therefore occur at detectable concentrations in the environment, though its presence is yet to be determined (Kosjek and Heath, 2011).

To the best of our knowledge, except for the study performed by Kiffmeyer et al. (1998), there is no information about MET biodegradation, which is the key process preventing MET to enter the aqueous environment. Besides OH-MET, the parent compound may transform into other transformation products (TPs): a group of structurally related compounds with an unknown identity, fate, effects and threat they pose to the aquatic organisms. Their identification is essential, not only to provide a comprehensive risk assessment on drug residues in the environment, but also to design improved treatment technologies for persistent trace contaminants. Several pharmaceutical TPs were identified during recent years; some examples include TPs formed from acetaminophen (De Laurentiis et al., 2014), diclofenac and ketoprofen (Salgado et al., 2013; Kosjek et al., 2008, 2011).

The present study aims to contribute to the existing knowledge on fate of MET, when exposed to microorganisms, and in particular, to identify the TPs formed during the biological breakdown process. In this view, we studied the susceptibility of MET to biodegradation under specific conditions, including presence or absence of carbon source and different activated sludge concentration. Our main focus was formation, detection and identification of TPs that were formed during these biotransformation experiments. The detection was performed with the aid of knowledge-based biotransformation prediction system and by spectral and chromatographic search software, whereas identification was achieved with an ultra-high performance liquid chromatography coupled to quadrupole – Orbitrap mass spectrometry.

2. Experimental

Caution: MET is cytotoxic, genotoxic, teratogenic and possibly mutagenic (Padmanabhan et al., 2009; Pfizer Inc, 2014), *and should be handled with care.*

2.1. Standards and chemicals

MET × $3H_2O$ (*N*-[4-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-L-glutamic acid; CAS: 59-05-2) was of ⩾99% purity and was obtained from TOCRIS Bioscience (Minneapolis, MN, USA). Deuterated MET (methotrexate-methyl-d3, MET-d3) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). All solvents and chemicals used for chromatographic separation (methanol, water, formic acid) and chemicals used for biomass inhibition (formaldehyde, FDH) or standard dissolution (dimethyl sulfoxide, DMSO) and nutrient-mineral medium (yeast and meat extract, casein peptone, CH_3COONH_4, NH_4Cl, K_2HPO_4, KH_2PO_4, $CaCO_3$, $MgCO_3$, NaCl, $FeSO_4 \times 7H_2O$) were of analytical reagent grade purity.

2.2. Setup of the biotransformation experiments

Biodegradability of MET was studied by conducting batch biotransformation experiments, which were set up in 0.5 L glass bottles with a total wetted volume of 0.4 L and were aerated with an aquarium pump. The experiments were performed at room temperature and protected from light. Activated sludge (AS) used in the biotransformation experiments was obtained on the day of their set-up from a nitrification basin at a wastewater treatment plant treating 22–30 million of m^3 of wastewater per year (360,000 population units), where wastewater originates mostly from municipalities, storm water, hospitals and health centres, pharmaceutical and food industry, and waste recycling. After being brought to our laboratory, the AS was allowed to settle, and 50 mL or 10 mL inoculums of the AS were added into the test bottles. FDH was added where applicable (Table 1), and the solution was left to shake for more than 4 h in order to allow the complete biomass inhibition (Süzer, 2013). Deionised water (DIW) was added as the alternative to FDH to account for the identical total volume in each test bottle.

The experiments were performed in nutrient-mineral or in salt-only medium and, to account for possible abiotic degradation, control experiments were run in DIW with (E) or without (E2) the addition of FDH (Table 1). 400 μL of 1 mg mL^{-1} MET solution in DMSO was added to the 0.4 L test solutions to give a final concentration of 1 mg L^{-1} MET, whereas the concentration of DMSO did not exceed 0.1% of the total volume of the test solution. The detailed description of the experimental set-up is given in Table 1.

The nutrient-mineral medium mimicked the composition of a municipal wastewater and contained yeast and meat extract, casein peptone, ammonium acetate and salts NH_4Cl, K_2HPO_4, KH_2PO_4, $CaCO_3$, $MgCO_3$, NaCl, $FeSO_4 \times 7H_2O$. The detailed description of the nutrient-mineral medium is given elsewhere (Kosjek et al.,

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