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Products of methotrexate during chlorination

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ABSTRACT

Methotrexate (MTX) is a cytotoxic drug widely used in the treatment of tumors, autoimmune diseases and severe asthma. jen00883This drug has been frequently detected in the aquatic environment with concentrations up to μg/L levels. The MTX present in environmental water might be transformed and removed during chlorination disinfection treatment. In this work, the fate of MTX during aqueous chlorination was investigated in laboratory batch experiments, and the transformation products of MTX were identified. Aqueous solutions of MTX (1 mg/L) were chlorinated by sodium hypochlorite solution at room temperature under neutral pH conditions. Chlorinated products were pre-concentrated with solid-phase extraction (SPE) cartridges and determined by liquid chromatography electrospray ionization tandem mass spectrometry (LC–ESI–MS/MS). The reaction of MTX chlorination exhibited pseudo-first-order kinetics and the half-life time of MTX degradation was calculated to be 1.65 min, when the initial chlorine concentration was 2 mg/L. Two chlorinated MTX congeners, 4-amino-3-chlorinated-N10-methylpteroylglutamic (monochloro-MTX) and 4-amino-3,5-dichloro-N10-methylpteroylglutamic (dichloro-MTX) were found in the chlorinated solution. Monochloro-MTX was successfully fractionated by high performance liquid chromatography (HPLC) and its structure was further identified using ^{1}H nuclear magnetic resonance (NMR) analysis. The presence of the two products in real hospital wastewater was then examined and both compounds were detected. Finally, the effects of MTX and monochloro-MTX on the cell cycle progression *in vitro* were evaluated using zebrafish liver cell line. It was found that both compounds could inhibit the proliferation of zebrafish liver cells through S phase arrest and their effects on the cell cycle profile had no significant difference.

Introduction

The presence of pharmaceuticals in the aquatic environment was first reported in 1970s (Hignite and Azarnoff, 1977). Since then, these compounds have been frequently reported to occur in influents and effluents of wastewater treatment plants (WWTPs), as well as in surface water, ground water and even in drinking water (de Jongh et al., 2012; Kasprzyk-Hordern et al., 2008; Kim et al., 2007; Lopez-Serna et al., 2012; Roberts and Thomas, 2006; Wang et al., 2010; Watkinson et al., 2009; Yu and Chu, 2009). In view of their potential risk to aquatic organisms and human health, their occurrence in environmental water has raised growing attentions, with the improvements of analytical techniques allowing to detect traces of chemicals in any type of water (Ferrer and Thurman, 2012; Gros et al., 2012).

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Among various classes of pharmaceuticals, cytotoxic drugs are of special environmental concern in recent years (Besse et al., 2012), because this group of drugs was demonstrated to be carcinogenic, mutagenic and/or teratogenic in animal experiments and epidemiological studies (DeMeo et al., 1995; Falck et al., 1979; IARC, 2014; Jackson et al., 1996; Santos and Pacheco, 1995). Methotrexate (MTX) is one of the most common cytotoxic drugs, which has been widely used in the therapy of solid tumors and leukemias since the 1940s (Farber and Diamond, 1948; Panetta et al., 2008), and later as an immunosuppressive agent in organ transplantation, in the treatment of autoimmune diseases and in the therapy of severe asthma (Genestier et al., 1998; Groff et al., 1983; Hanno et al., 1980). This pharmaceutical is designed to kill rapidly growing cells by interrupting folate metabolism and consequently inhibiting DNA synthesis (Rajagopalan et al., 2002). Due to its mode of action, MTX can exert severe and even fatal toxic effects on both animals and humans at relatively low doses. The oral LD50 of MTX in rats is about 180 mg/kg bw while the intraperitonea (i.p.) LD50 is 6–25 mg/kg bw in rats and 94 mg/kg body weight in mice (Ferguson et al., 1950). Symptoms of bone-marrow depression, hepatotoxicity, neurotoxicity and renal damage, such as leucopenia, liver necrosis, aphasia and hematuria, can occur in patients treated with MTX, depending on the dosage and duration of therapy (IARC, 1981). Furthermore, MTX has been considered as a potential carcinogen, teratogen and mutagen to animals and humans (IARC, 1981, 1987).

Precise data on the consumption of MTX worldwide cannot be obtained, but the amount was expected to be numerous with regard to its comprehensive use. After being administered, MTX is not completely metabolized in the human body, and approximately 60% to 90% of the parent compound is excreted unchanged in the urine (Turci et al., 2003). Therefore, this pharmaceutical can enter the hospital and municipal wastewater, reach WWTPs, and consequently be released into aquatic environment due to inefficient elimination. In one of our previous studies, this drug was observed in effluent samples taken from 14 hospitals with concentrations ranging from 4 to 4689 ng/L (Yin et al., 2010a). In the influent samples of municipal WWTPs, it was measured at concentrations of <5.9 and 23.0 ng/L (Ferrando-Climent et al., 2013). It has been detected even in WWTP effluents at concentrations of 12.6 and 30 ng/L by Castiglioni et al. (2005) and Catastini et al. (2008), respectively. Though the presence of MTX in environmental water was at these very low concentrations, it should be further monitored and assessed for environmental risk considering its distinctive mechanism of action and high biological activity.

Owing to its low cost, chlorination is generally applied in the disinfection of wastewater and drinking water in many countries to ensure elimination of potentially dangerous microbes (Gibs et al., 2007; Glassmeyer and Shoemaker, 2005; Lee and von Gunten, 2010). During the disinfection process, the pharmaceuticals present in the water can react with free chlorine to form chlorinated byproducts. Identification of possible chlorination products of pharmaceuticals is of particular importance as the products formed during the disinfection might be more harmful than the parent compounds (Bedner and MacCrehan, 2006; Kamoshita et al., 2010; Legay et al., 2010; Xu et al., 1997). Therefore, the effect of chlorination has been investigated for a number of pharmaceuticals in aqueous media in the previous literature (Chamberlain and Adams, 2006; Dodd et al., 2005; Li et al., 2011). However, the fate of MTX after chlorination has received very low attention despite its potential health risk and occasional detection in wastewater. To the best of our knowledge, only Roig et al. (2014) have reported the transformation of MTX during chlorination treatment in a recent paper. One monochlorinated product was observed for MTX in their study and the toxicity of the proposed product was predicted by *in silico* analysis. Nevertheless, the structure and toxicity of the observed transformation product were not clearly confirmed in this research. Further experiments and analysis are needed to better characterize the behavior of MTX in chlorinated environmental water.

In our present study, the fate of MTX during chlorination was studied in laboratory kinetic experiments. The products of MTX chlorination were identified based on liquid chromatography electrospray ionization tandem mass spectrometry (LC–ESI–MS/MS) and nuclear magnetic resonance (NMR) methods, and their occurrence in real hospital wastewater was also examined. Finally, the effect of one chlorinated product on cell cycle progression was evaluated *in vitro* with zebrafish liver cell line to preliminarily investigate its toxicity.

1. Materials and methods

1.1. Chemicals and materials

MTX ($C_{20}H_{22}N_8O_5$, molecular weight: 454.44) with purity >99% was purchased from Sigma Chemical Co. (St, Louis, MO, USA). The MTX stock solution was made by dissolving 1 mg of MTX in 1 mL of water/methanol (1:1, V/V) containing 0.01 mol/L hydrochloric acid and stored at −18°C. Stock solutions of chlorine were prepared by diluting a commercial solution of sodium hypochlorite (NaOCl, 13% active chlorine, Acros Organics, Morris Plains, NJ, USA), stored at 4°C in the dark and periodically standardized by N,N-diethyl-pphenylenediamine (DPD) methods (APHA et al., 2005). Working dilutions were prepared freshly on the day of use.

Formic acid of HPLC grade was purchased from Acros Organics. Ultra-pure water was obtained by using an in-house Milli-Q® Ultra-pure water system (Millipore, Bedford, MA, USA). Methanol and acetonitrile of high performance liquid chromatography (HPLC) grade were from Fisher Scientific (Fair Lawn, NJ, USA). Sodium dihydrogen phosphate, sodium hydroxide solution and L-ascorbic acid were of analytical grade and purchased from Sinopharm Chemical Reagent Corp. (Beijing, China). Dimethyl sulfoxide-d6 (DMSO-d6) and tetramethylsilane (TMS) with purity higher than 99.9% were purchased from Sigma Aldrich (St. Louis, MO, USA). Oasis HLB solid-phase extraction (SPE) cartridges (500 mg, 6 mL) were obtained from Waters (Milford, MA, USA).

1.2. Kinetic experiments

Chlorination experiments were performed in amber laboratory bottles equipped with a dispenser. The initial concentrations of MTX and chlorine were 1 and 2 mg/L, respectively. Chlorine concentrations were analyzed with the DPD method. The reaction solutions were buffered using sodium dihydrogen

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