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Short-term toxicity assessments of an antibiotic metabolite in Wistar rats and its metabonomics analysis by ultra-high performance liquid chromatography coupled to quadrupole time-of-flight mass spectrometry

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ABSTRACT

4-Epi-oxytetracycline (4-EOTC), one of main oxytetracycline (OTC) metabolites, can be commonly detected in food and environment. The toxicity and effects of OTC on animals have been well characterized; however, its metabolites have never been studied systemically. This study aims to investigate 15-day oral dose toxicity and urine metabonomics changes of 4-EOTC after repeated administration in Wistar rats at daily doses of 0.5, 5.0 and 50.0 mg/kg bw (bodyweight). Hematology and clinical chemistry parameters, including white blood cell count, red blood cell count, total protein, globulin and albumin/globulin, were obviously altered in rats of 5.0 and 50.0 mg/kg bw. Histopathology changes of kidney and liver tissues were also observed in high-dose groups. Urinary metabolites from all groups were analyzed using ultra-high performance liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF/MS). Seventeen metabolites contributing to the clusters were identified as potential biomarkers from multivariate analysis, including aminoadipic acid, 6-phosphogluconate, sebacic acid, pipecolic acid, etc. The significant changes of these biomarkers demonstrated metabonomic variations in treated rats, especially lysine and purine metabolism. For the first time in this paper, we combined the results of toxicity and metabonomics induced by 4-EOTC for the serious reconsideration of the safety and potential risks of antibiotics and its degradation metabolites.

1. Introduction

Antibiotics have been widely used to treat infectious diseases of animals or human beings since the late 1950s (Berdy, 2012; Shaw et al., 2011). The application of antibiotics in medical science has been beneficial and has led to healthier subjects, lower disease incidence, and reduced morbidity and mortality. However, in recent decades, the abusive use of antibiotics, particularly the excessive use of veterinary antibiotics, has posed a threat to public health (Gerdes and Ingmer, 2013; Landers et al., 2012; Oliver et al., 2011). Oxytetracycline (OTC) is one of the most commonly used tetracyclines for controlling and preventing bacterial infections, and it can also be used to promote growth (Castanon, 2007; Chopra et al., 1992). Because of its broad-spectrum activity and low cost, the excessive and improper use of OTC leads to the accumulation of OTC and its metabolites in animal tissues and in the environment, which may be toxic and dangerous for public health (Kemper, 2008; Seyfried et al., 2010). Additionally, the residue of OTC and its metabolites can promote the development of antibiotic resistance in bacteria (Halling-Sorensen et al., 2002; Li et al., 2010), even at low-level OTC exposure (20 μg/L) (Knapp, 2008), which is a looming public health crisis.

Recently, there have been many studies on the metabolites of antibiotics. OTC can be degraded into 4-epi-oxytetracycline (4-EOTC), α-apo-oxytetracycline, β-apo-oxytetracycline, and terrinolide (Halling-Sorensen et al., 2002); of these, 4-EOTC is one of the main degradation products (Wang and Yates, 2008; Capolongo et al., 2002; Le et al., 2012). The abiotic degradation pathways of OTC showed the formation of 4-EOTC was present in high concentration which was primarily expected in the mole fraction of 40%–60% with the parent compound in soil water (Halling-Sørensen et al., 2003). It was also reported that as high as 73% to 90% of OTC could be degraded in aqueous (Jiao et al., 2008) and 21–24% OTC could be degraded into 4-EOTC in egg yolk (Zurhelle et al., 2000). The concentration of tetracycline residues in raw milk varied from 47.7 μg/L to 1671 μg/L (Bilandzic et al., 2011; Zheng et al., 2013). OTC and 4-EOTC residues have also been detected in chicken tissues (Le et al., 2012), raw milk (Zheng et al., 2013), manure (Zhao et al., 2010), aqueous and terrestrial environments (Kemper, 2008), indicating that the OTC and 4-EOTC residues are very often detected in agricultural products and environments. The toxicity of OTC

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has previously been studied, and fat metamorphosis and focal cellular change in the livers of male rats were observed after OTC treatment (Dietz et al., 1991; Tropiło et al., 1987). A study of the toxicological effects of OTC to an Indian major carp showed that the hematological and enzymological parameters were altered after exposure to different concentrations of OTC (80, 100, and 120 mg/L) for 25 d (Ambili et al., 2013). However, the toxicity of metabolite 4-EOTC has never been studied. It has been reported that the tetracycline metabolites have the same or even higher toxicity compared with their parent chemical; previous studies have found that 4-EOTC is more toxic (EC_{50}) than the parent compound in a bacterial resistance experiment (Halling-Sorensen et al., 2002; Jiao et al., 2008). In addition, with the accuracy and sensitivity of the developed metabonomic methods, it has been a hot issue on the metabolic effects accompanied with antibiotic usage according to recent researches (Swann et al., 2011; Theriot et al., 2014). The metabolomics analysis of urine, an ideal bio-medium for disease studies, has become a powerful way to detect novel biomarkers in potential biochemical pathways for the explicit understanding of the mechanisms (Zhang et al., 2012). It is necessary to study the toxicity and security of 4-EOTC for the whole understanding of this chemical to guarantee public health.

Therefore, the current sub-chronic study was performed in Wistar rats with a 15-day oral administration of 4-EOTC under different concentrations (0.5, 5.0 and 50 mg/kg bw). Blood and selected tissue samples were collected and analyzed for hematology, serum biochemistry and histopathology. UPLC-Q-TOF/MS method was employed to characterize the metabolomics changes in urine samples in order to provide evidence for the metabolic toxicity of 4-EOTC in vivo for the first time. The results should provide useful information about the safety and potential risks of antibiotics residues, which may be helpful for further studies about antibiotic degradations.

2. Materials and methods

2.1. Chemicals and reagents

4-EOTC was purchased from Acros Organics (NJ, USA), which can be used as a standard with a purity of 97%. All other chemicals were analytical grade purity. Acetonitrile (HPLC-grade) and methanol (HPLC-grade) were acquired from Fisher Scientific Ltd. (Rathburn, Walkerburn, UK), formic acid (solution 96%, HPLC-grade) was acquired from Tedia company, Inc. (USA). Water was purified by a Millipore (Bedford, MA) Milli-Q water system.

2.2. Animals and housing

Healthy male and female Wistar rats, aged 5 weeks and weighing 140–150 g, were purchased from Shanghai Animal Center. The animals were housed in plastic cages with stainless steel wire cover under standard conditions. The living temperature is 22 ± 2 °C with a relative humidity of 55 ± 10%, and a light/dark period of 12/12 h. Standard rat food and sterile water were available ad libitum. All animals were allowed to acclimatize for 5 d before the treatment.

All procedures in this study were performed according to the guidelines of the Council of Animal Care of Zhejiang University.

2.3. Dosing and experimental design

The 4-EOTC was prepared in normal saline (9 g NaCl dissolved in 1000 mL Milli-Q water) 12 h before each administration. The 15-day repeated dose oral subchronic toxicity study was performed in male and female rats by randomly dividing them into the following four groups (12 rats each: 6 males and 6 females): control, low-dose, medium-dose and high-dose, which were administered normal saline, 0.5 mg/kg bw, 5.0 mg/kg bw and 50.0 mg/kg bw, respectively. The dose depended on the usual concentration of reported antibiotic residues in milk products or food stuffs and on the limited concentration of antibiotic residues in milk in guidelines (Heitzman, 1992; Hou et al., 2015; Zheng et al., 2013). All animals were weighed early in the morning and were intragastrically administered antibiotics by lavage needle according to their body weight once daily for 15 consecutive days. The animals were sacrificed 24 h after the last administration.

Clinical observations were performed daily throughout the study, including abnormal posture, mental state and daily activity. Body weight was recorded twice a week during the study. Food and water consumption was measured twice a week per cage. The amount of food was calculated before it was added to each cage, and the remaining food was measured on the next day to calculate the food and water consumption.

The animals were fasted overnight prior to necropsy and blood collection. Hematologic analyses were performed just after blood collection, which was placed in centrifuge tubes containing EDTA-Na_2. The red blood cell count (RBC), white blood cell count (WBC), platelet count (PLT), hemoglobin concentration (HGB), hematocrit (HCT) level, and lymphocyte count (LYM) were analyzed using an auto hematologic analyzer (ADUIA2120 of Siemens, GER).

Serum samples were obtained after the centrifugation of whole blood at 3000 rpm for 10 min. An automatic biochemistry analyzer (ACCUTE TBA-40FR of Toshiba, JPN) was used to examine the serum for alanine aminotransferase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), total protein (TP), albumin (ALB), blood urea nitrogen (BUN), creatinine (CREA) and total cholesterol (TC).

Using the metabolic cages, urea samples were collected from half of the rats in each group after an overnight fast and before sacrifice, and were centrifuged at 8000 rpm for 10 min at 4 °C, then stored at −80 °C until preprocessing for UPLC-TOF/MS. The protein, pH, specific gravity, glucose, occult blood, ketone body, and bilirubin were measured using a urea analyzer (Uritest-500B of Urit, CHN).

At the end of the treatment (day 16), the rats were euthanized by anesthesia and sacrificed. The brain, liver, kidneys, lungs, spleen, pancreas, and stomach were collected and examined for gross pathology. The kidneys and liver were individually isolated and weighed to calculate the ratios of the organ weight to body weight. For histopathology evaluation, the kidney and liver tissues were fixed in 10% neutral buffered formalin, processed according to the standard procedure and stained with hematoxylin–eosin (HE). Subsequently, a pathologist who was unaware of the study being performed examined the pathology changes using a light microscope.

2.4. Metabolite profiling

UPLC analysis was performed on Waters Acquity UPLC system (Waters, Milford, MA, USA), equipped with an autosampler and binary solvent delivery system. Prior to the analysis, urine samples were thawed and centrifuged at 12,000 rpm for 10 min at 4 °C. 200 μl of the supernatant from each urine sample was collected and 400 μl methanol was added to the supernatant and vortex-mixed for 1 min. The mixtures were filtered through 0.22 μm membrane filter twice before transferred into the autosampler. The chromatography was performed on a Waters Acquity BEH C18 column (100 × 2.1 mm i.d., 1.7 μm, Waters, Milford, MA, USA). The mobile phase consisted of (A) 0.1% formic acid in water and (B) acetonitrile containing 0.1% formic acid. Reversed phase separation parameters were initially 10% B (2 min) followed by a linear gradient from 10 to 50% B over 8 min and subsequently a ramp from 50 to 90% B over 4 min. After a hold at 90% B for 0.1 min, and a ramping step down to 10% B over 1 min, the system was re-equilibrated at 5% B for 1 min. A blank of pure acetonitrile was analyzed every five samples to wash the column.

MS analysis was performed using positive electrospray ionization mode using a Waters Q-TOF Premier (Micromass MS Technologies, Manchester, UK) equipped with an electrospray ionization (ESI) source operating in positive ion mode. The nebulization gas was set to 600 L/h