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Toxicokinetic profile of fusarenon-X and its metabolite nivalenol in the goat *(Capra hircus)*

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ABSTRACT

The main aim of this research was to evaluate the toxicokinetic characteristics of fusarenon-X (FX) and its metabolite, nivalenol (NIV), in goats. The amounts of FX and NIV in post-mitochondrial (S-9), microsomal and cytosolic fractions of diverse tissues of the goat were also investigated. FX was intravenously (iv) or orally (po) administered to goats at dosages of 0.25 and 1 mg/kg bw, respectively. The concentrations of FX and NIV in plasma, feces and urine were quantified by liquid chromatography tandem-mass spectrometry (LC-ESI-MS/MS). The concentrations of FX in plasma were quantified up to 8 h with both routes of administration. A large amount of NIV (metabolite) was quantifiable in plasma, urine and feces after both administrations. The C_{max} value of FX was 413.39 ± 206.84 ng/ml after po administration. The elimination half-life values were 1.64 ± 0.32 h and 4.69 ± 1.25 h after iv and po administration, respectively. *In vitro* experiments showed that the conversion FX-to-NIV mainly occurs in the liver microsomal fraction. This is the first study that evaluates the fate and metabolism of FX in ruminant species.

1. Introduction

Among the trichothecenes discovered to date, deoxynivalenol (DON), T-2 toxin (T-2), diacetoxyscirpenol (DAS) and Fusarenon-X (FX) are known to contaminate feed globally (Pittet, 1998). Trichothecenes have been classified into A, B, C, and D types. Type A and B trichothecene mycotoxins have a wide range of toxic effects on farm animals and humans (WHO, 1990). In farm animals, they can cause diarrhea, feed refusal, weight loss, decreased production performance, immune suppression and residues in animal food products (Eriksen and Pettersson, 2004).

FX, a type B trichothecene mycotoxin, is mainly produced by the *Fusarium* fungi, which naturally occurs in agricultural commodities such as cereal and cereal products, including wheat, barley, corn, rye, oats, maize and multigrain (Broekaert et al., 2015; Cavalier et al., 2005; Juan et al., 2013; Placinta et al., 1999). FX was first isolated from *Fusarium nivale* strain Fn-2B, its production depends on many factors, including the substrate, temperature and humidity (Cavalier et al., 2005; Juan et al., 2013). Although deoxynivalenol (DON) is the most commonly found globally, the derived products, particularly breakfast cereals and bread, are susceptible to FX contamination, and are very important in the human diet including in baby foods (Placinta et al., 1999; Juan et al., 2013; Yzar and Omurtag, 2008). There are some reports that FX has been frequently detected along with DON and nivalenol (NIV) (IARC, 1993).

FX predominantly occurs in temperate regions of Europe and Asia, because these geographical zones are suitable for *Fusarium* growth and FX production.

FX has been characterized as more potently toxic than the other members of type B trichothecene mycotoxins (IARC, 1993). FX can evoke a ribotoxic stress response, which inhibits protein and DNA synthesis in eukaryotic cells (Aupanan et al., 2017). FX has been reported to induce adverse health effects, particularly apoptosis, in organs containing actively dividing cells such as the small intestine, thymus, spleen, bone marrow, testes, and in cells such as reticulocytes and mitogen-stimulated human lymphocytes; these effects have also been observed after exposure to other trichothecenes (Forsell and Pestka, 1985; Miura et al., 1998; Poapolathep et al., 2001, 2002). Furthermore, FX dose-dependently encourages DNA strand breakage of both dividing cells and differentiated Caco-2 cells (Alassane-Kpembi et al., 2013). FX

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has been reported to induce apoptosis in developing mouse brain, thymus, Peyer's patches, spleen and human jurkat T cells (Aupanan et al., 2015; 2016; Sutjarit et al., 2014).

The European Commission (EC) has regulated acceptable levels of trichothecene mycotoxins in cereal grains, flours, and cereal-based products intended for human and animal consumption (Gareis et al., 2003; EC, 2006) but the limited available information prevents establishing a regulatory limit for FX (Gareis et al., 2003). Nowadays, the European Food Safety Authority Panel on Contaminants in the Food Chain (CONTAM) establishes the tolerable daily intake of NIV as 1.2 mg/kg bw/day based on a lowest-observed-adverse-effect of 0.7 mg/kg bw/day found in long-term dietary studies in mice (EFSA, 2013).

Toxicokinetic profiles of FX and its metabolite NIV have been reported in mice, broiler chickens, ducks and piglets (Poapolathep et al., 2003, 2004; 2008; Saengtienchai et al., 2014) but to the best of the authors' knowledge no data exist on its toxicokinetics in ruminants. Tremendous variations in the species specific toxicokinetic profiles of FX have been observed. Further information on the toxicokinetics of FX is also needed to evaluate possible differences in toxicity between monogastric animals and ruminants. Therefore, this study aimed to evaluate the toxicokinetic characteristics of FX and its metabolite NIV in goats after intravenous (iv) and oral (po) administrations. The amounts of FX and NIV in post-mitochondrial (S-9), microsomal and cytosolic fractions of diverse tissues of the goat were also investigated.

2. Materials and methods

2.1. Animals

Eleven 9-week-old male goats (average weight 7.74 ± 0.44 kg) were purchased from a commercial goat farm (Saraburi Thailand). The experimental animals were housed at the Laboratory Animal Facility, Bureau of Veterinary Biologics, Department of Livestock, Nakhon Ratchasima province, Thailand. Animals were acclimatized to the environment for 1 week prior to the commencement of the study. The animals were fed with dried pangola grass and water *ad libitum*. All experimental procedures on animals were ethically approved by the Animal Ethics Research Committee of the Faculty of Veterinary Medicine, Kasetsart University.

2.2. Chemicals and reagents

Standard FX and NIV were purchased from Wako Pure Chemical Industries Ltd. (Kyoto, Japan). Other analytical grade reagents and chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Purified water was produced using the Milli-Q water purification system from Millipore, Inc (Bedford, MA, USA). The solutions for iv and po administrations were prepared in one batch at a concentration of 2 mg/ml by dissolving standard FX in 0.01 M phosphate buffer saline pH 7.4 containing 10% (v/v) dimethyl sulfoxide (DMSO).

2.3. Toxicokinetic study

Ten goats were randomly divided into two groups (n = 5). After overnight fasting, each group was administered FX iv (into the right jugular vein) or po at a dosage of 0.25 or 1.0 mg/kg bw, respectively. Blood samples (2.5–3.0 ml) were collected from the left jugular vein of each goat with heparinized syringes at 0, 5, 15 and 30 min and 1, 2, 4, 6, 8, 12, 24, 48, and 72 h, whereas the urine and feces were collected at 0–2 h, 2–4 h, 4–8 h, 8–12 h, 12–24 h and 24–48 h after FX administration. The urine and faecal samples were immediately collected from the individual animals. The plasma was separated by centrifugation at 1968 g for 15 min and immediately frozen at −20 °C until analyzed. The urine and faecal samples were also stored at −20 °C until analyzed.

2.4. Extraction and clean up

The extraction method of FX and NIV in plasma, urine and feces was based on a previously published method (Poapolathep et al., 2008). Briefly, 1 ml of goat plasma or urine, or 5 g of feces were extracted with 3 ml of acetonitrile (ACN)-water (3/1, v/v). Two g of ammonium sulfate were added, and shaken for 30 s by a vortex mixer. The ACN fraction was separated by centrifugation at 1968 g for 15 min. The supernatant was collected in the glass tube. These extraction steps were then repeated for 2 additional cycles. The supernatant fractions were combined and purified using the solid phase extraction cartridge (C18 Sep-pak silica cartridge) (Waters Corp., Milford, MS, USA) as described previously (Poapolathep et al., 2008). The eluate was completely evaporated under a nitrogen stream at 40 °C on a heating block. The residue was reconstituted with 500 µl of methanol-water (1:4) v/v with 5 mM ammonium acetate, and injected onto a 0.22 µm syringed filter (Sartorius AG, Goettingen, Germany) before being analyzed by liquid chromatography tandem-mass spectrometry (LC-MS/MS).

2.5. Preparation of postmitochondrial (S-9), microsomal and cytosolic fractions

In order to study the metabolites of FX, one 9-week-old male goat was sacrificed with thiopentone sodium at a dosage of 20 mg/kg of bw by iv administration. Various tissues including the liver, kidney, heart, lung, muscle, rumen, reticulum, omasum, abomasum, duodenum, jejunum, ilium, colon and rectum were immediately collected and frozen in liquid nitrogen and stored at −80 °C until assayed. The post-mitochondrial (S-9), microsomal and cytosolic fractions were prepared as described (Esaki and Kumagai, 2002; Poapolathep et al., 2008). Briefly, a portion of the thawed tissue sample was cut into small pieces using scissors and homogenized in a motor driven glass homogenizer. The homogenates were centrifuged at 10,000 rpm for 10 min to separate the supernatant designated as the S-9 fraction. S-9 fraction was then centrifuged at 105,000 × *g* for 1 h to collect the cytosolic fraction. The pellet was resuspended and recentrifuged at 105,000 × *g*. Supernatant was discarded and the microsomal fraction was resuspended in 20 mM Tris buffer pH 7.4 containing 1 mM ethylene diamine tetraacetic acid (EDTA) and 0.25 M sucrose. All preparation procedures were performed on ice and all sample fractions were kept at −80 °C until analyzed.

2.6. Protein assay

Protein concentrations in S-9, microsomal, and cytosolic fractions were determined as described by Esaki and Kumagais (Esaki and Kumagai, 2002). In brief, 2 ml of S-9, microsomal, or cytosolic fractions from each tissue were added to 5 ml of the dye reagent: water, 1:4 v/v, mixture. The samples were then incubated at room temperature for 5 min and measured spectrophotometrically at 595 nm using a GENESYS TM 20 spectrophotometer (Thermo Fisher Scientific, Washington, USA). Bovine serum albumin (Bio-Rad Protein Assay, Bio-Rad Lab, CA) was used as the standard for the protein assay.

2.7. Determination of S-9, microsomal and cytosolic activity to form NIV

Conversion of FX by S-9, microsomal and cytosolic activities was performed according to a published method (Esaki and Kumagai, 2002). In brief, 1 mg protein of S-9, microsomal and cytosolic fractions was pre-incubated at 37 °C in an incubation buffer consisting of potassium phosphate buffer (100 mM, pH 7.4), G-6-P (5.6 mM), NADPH (2 mM) and G-6-P dehydrogenase (0.5 unit). After pre-incubation for 5 min, FX (5 mg/10 ml of methanol) 5 µg of FX in 10 µl of methanol was added and the mixtures were continuously incubated whilst being shaken for 0, 15, 30, 45 and 60 min. The reaction was terminated by adding an equal volume of ice-cold chloroform, followed by vigorous