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THESIS

STUDIES ON TOXICOKINETICS AND TISSUE RESIDUES OF FUSARENON-X AND ITS METABOLITES IN PIGLETS

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Thanit Saengtienchai 2014: Studies on Toxicokinetics and Tissue Residues of Fusarenon-X and Its Metabolites in Piglets. Master of Science (Veterinary Pharmacology and Toxicology), Major Field: Veterinary Pharmacology and Toxicology, Department of Pharmacology.
Thesis Advisor: Associate Professor Amnart Poapolathep, Ph.D. 64 pages.

Fusarenon-X (FX) is one of the type B trichothecene mycotoxin mainly produced by *Fusarium crookwellense*, which naturally occurs in agricultural commodities such as wheat and barley. The toxicokinetic characteristics of mycotoxins especially FX is needed to evaluate because a limited toxicokinetic data are available in food producing animals. To investigate the toxicokinetics of Fusarenon-X (FX) and its metabolites (nivalenol, NIV), FX was administered intravenously (iv) and orally (po) to piglets at a dosage of 1 mg/kg body weight. The concentrations of FX and NIV in plasma, excreta and various tissues were measured simultaneously using LC-MS/MS with electrospray ionization. The plasma concentrations of FX were determined up to 24 h and 48 h in piglets whereas the NIV was detected up to 12 h after iv and po administration, repectively. The plasma concentration at initial time (C_p^0), elimination half life ($t_{1/2\beta}$), oral bioavailability (F_{oral}) of FX were 580.28 ± 140.81 ng/ml, 1.706 ± 0.741 h, and 74.402 ± 18.96% after iv administration, respectively. FX and NIV can be detected in the urine and feces for up to 24 h and 48 h after iv administration and po administration, respectively. FX and NIV were detectable in vital organs up to 24 h after po administration. The peak level of FX in liver, kidney, and spleen were 16.95 ± 9.68 ng/g, 66.29 ± 8.48 ng/g, and 7.35 ± 0.69 ng/g after 3 h following po administration, respectively. *In vitro* incubation of liver postmitochondrial fractions with FX demonstrated that the liver and kidney are capable of the FX-to-NIV metabolism.

Student's signature Thesis Advisor's signature

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LIST OF ABBREVIATIONS

FX	=	Fusarenon-X
NIV	=	Nivalenol
DON	=	Deoxynivalenol
DAS	=	Diacetoxyscirpenol
ZON	=	Zearalenone
3a-DON	=	3-acetyldeoxy-nivalenol
15a-DON	=	15-acetyldeoxy-nivalenol
DMSO	=	Dimethyl sulfoxide
EDTA	=	Ethylenediaminetetraacetic acid
Tris-HCl	=	Tris-Hydrochloride
ACN	=	Acetronitrile
MeOH	=	Methanol
TLC	=	Thin Layer Chromatography
GC	=	Gas Chromatography
HPLC	=	High Performance Liquid Chromatography
LC	=	Liquid Chromatography
LC-MS/MS	=	Liquid Chromatography tandem Mass Spectrometry
ESI	=	Electrospray Ionization
APCI	=	Atmospheric-Pressure Chemical Ionization
ELISA	=	Enzyme Linked Immunosorbent Assay
C_{max}	=	Maximum concentration
T_{max}	=	Time to reach maximum plasma concentration
$t_{1/2\beta}$	=	Elimination half-life
$t_{1/2\alpha}$	=	Distribution half-life
V_{ss}	=	Volume of distribution at the steady state
MRT	=	Mean residence time
Cl	=	Body clearance
F	=	Oral bioavailability

LIST OF ABBREVIATIONS (Continued)

K_{12}	=	Distribution rate constant
K_{21}	=	Redistribution rate constant
ND	=	Not Detected
ATA	=	Alimentary Aleukia
iv	=	Intravenous
ip	=	Intraperitoneal
po	=	Per os / oral
bw	=	Bodyweight
kg	=	Kilogram
g	=	Gram
mg	=	Milligram
µg	=	Microgram
ng	=	Nanogram
ppb	=	Part per billion
ppm	=	Part per million
wk	=	Week
min	=	Minute
h	=	Hour
°C	=	Degree celcius
M	=	Molar
mM	=	Milli molar
SD	=	Standard Deviation
SEM	=	Standard Error of Mean
CV	=	Coefficient of Variation
WHO	=	World Health Organization
WTO	=	World Trade Organization
SPS	=	Sanitary and Phytosanitary
IARC	=	International Agency for Research on Cancer

LIST OF ABBREVIATIONS (Continued)

C	=	Carbon
H	=	Hydrogen
JNK	=	Jun N-terminal kinase
ERK	=	Extracellular signal regulated kinase
MAPKs	=	Mitogen-activated protein kinases
DNA	=	Deoxyriboneucleic acid
RNA	=	Ribonucleic acid
ICR	=	Imprinting control regions
spp	=	Species

STUDIES ON TOXICOKINETICS AND TISSUE RESIDUES OF FUSARENON-X AND ITS METABOLITES IN PIGLETS

INTRODUCTION

Mycotoxins, toxic secondary metabolites, are produced by filamentous fungi. They are growing on agricultural and readily colonies crops in the field or after harvest (Bennett and Klich, 2003; Turner *et al*., 2009). They can be produced in crops and other food commodities both pre- and post-harvest (Bryden, 2007). Mycotoxins are not only to define but they are also challenging to classify due to their diverse chemical structures, biosynthetic origins, the myriad biological effects, and the wide number of different fungal species production (Bennett and Klich, 2003). Mycotoxins are potentially hazardous to humans and animals (Turner *et al*., 2009). When ingested, mycotoxins may cause a mycotoxicosis which can result in an acute or chronic disease episode (Bryden, 2007). Chronic conditions have a much greater impact, numerically, on human health globally (Bryden, 2007). Reduced growth and development, immunosupression and cancer are chronic effects that have a higher incidence following continual exposure to low level mycotoxin ingestion as is experienced in many developing countries (Bryden, 2007). Clinicians often arrange them by the organ they affect (Bennett and Klich, 2003). Thus, mycotoxins can be classified as hepatotoxins, nephrotoxins, neurotoxins, immunotoxins and others (Bennett and Klich, 2003). A reliable risk assessment of mycotoxin contamination for humans and animals relies basically on their unambiguous identification and accurate quantification in food and feedstuff (Peter and Bernhard, 2006). The contamination of foods and feeds gained much global attention in recent years due to its adverse health and economic effects (Luongo *et al*., 2010). Major food commodities affected are cereals, nuts, dried fruit, coffee, cocoa, spices, oil seeds, dried peas, beans and fruit, particularly apples (Turner *et al*., 2009). Mycotoxins might be found in beer and wine resulting from the use of contaminated barley, other cereals and grapes in their production (Turner *et al*., 2009). Various mycotoxins continue to be reported in different commodities in various countries around the world (Ramesh and Vasanthi,

1999). The World Trade Organization (WTO) agreement on the application of Sanitary and Phytosanitary (SPS) measures, signed by various countries to facilitate international trade might introduce newer regulatory policies which could lead to more difficulties for the exporting countries (Ramesh and Vasanthi, 1999). The economic impact of mycotoxins on the food grain and livestock industry has been considerable (Ramesh and Vasanthi, 1999). While rejection of export consignments of groundnut above the regulatory limit would result in economic loss, the distribution of the rejected consignments in the domestic market would increase the consumer risk (Ramesh and Vasanthi, 1999).

FX (3, 7, 15-trihydroxy-4-acetoxy-12, 13 epoxytrichothec-9-e-8-one), a type B trichothecene mycotoxin, is one of the 12, 13-epoxytrichothecenes mainly produced by *Fusarium crookwellense*, which naturally occurs in agricultural commodities such as wheat and barley (International Agency Research for Cancer [IARC], 1993; Poapolathep *et al*., 2008). FX and nivalenol have 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, reticulocytes, and mitogen-stimulated human lymphocytes, as observed in other trichothecenes (Ohta *et al*., 1978; Forsell and Pestka, 1985; Miura *et al.*, 1998; Poapolathep *et al*., 2002). Up to date, limited pharmacokinetic data are available for trichothecene mycotoxins in animals, especially for FX (Poapolathep *et al*, 2008). The toxicokinetic behavior of FX in animals has not been investigated as extensively as DON and T-2. It is well known that species differences affect the disposition of xenobiotics in animals (Walker, 1980). In previous study, Poapolathep *et al*. (2003, 2004, 2008) demonstrated the toxicokinetic characteristics and its metabolites of FX in mice, broilers and ducks.

OBJECTIVES

Recently, pharmacokinetic information and metabolites of mycotoxins especially FX is needed to evaluate because a limited pharmacokinetic data are available in food producing animals. To get insight into the mechanism underlying the toxicity of FX in pig, we studied the fate, residues and the metabolites of FX in piglets based on the pharmacokinetic parameters and toxin depletion in various tissues. Thus, the purposes of this research are as the followings;

1. To get insight into toxicokinetic characteristics of FX and its major metabolite in piglets.

2. To investigate the tissue residues od FX and its metabolites in piglets.

3. To study the metabolism of FX in piglets both *in vitro* and *in vivo*.

4. To standardize the analytical method of FX in biological fluids and animal Tissues using LC-MS/MS

5. To evaluate the risk of assessment of FX in pigs based on the pharmacokinetic parameters.

LITERATURE REVIEW

Health hazards from food can be caused directly by infectious agents and toxic compounds (Berthiller *et al*., 2012). Living microorganisms ingested with food can cause infectious diseases, while toxic substances lead to acute poisoning or have a long-term negative impact on the health of consumers (Berthiller *et al*., 2012). Natural toxins in food are plant secondary metabolites, bacterial toxins, phycotoxins and mycotoxins (Berthiller *et al*., 2012). Human food and animal feed can be contaminated with mycotoxins at various stages in the food chain and the three most important genera of mycotoxigenic fungi are *Aspergillus, Fusarium and Penicillium* (Bryden, 2007). The principal classes of mycotoxins produced by these genera are: aflatoxins (*Aspergillus*), ochratoxins (*Aspergillus* and *Penicillium*), trichothecenes and fumonisins (*Fusarium*) (Bryden, 2007). The disease resulting from mycotoxin exposure is a mycotoxicosis (Bryden, 2007). Mycotoxins, toxic secondary metabolites and low molecular weight natural product, are produced by filamentous fungi. They are growing on agricultural and readily colonise crops in the field or after harvest (Bennett and Klich, 2003; Turner *et al*., 2009). Mycotoxin producing fungi are phytopathogenic organisms that infect living plants in the field and/or greenhouse and saprophytic fungi that colonise plant products at post harvest (Berthiller *et al*., 2012).

Mycotoxins are not only to define but they are also challenging to classify due to their diverse chemical structures, biosynthetic origins, the myriad biological effects, and the wide number of different fungal species production (Bennett and Klich, 2003). Mycotoxins are potentially hazardous to humans and animals (Turner *et al*., 2009). A reliable risk assessment of mycotoxin contamination for humans and animals relies basically on their unambiguous identification and accurate quantification in food and feedstuff (Peter and Bernhard, 2006). They are unavoidable contaminants in foods and a major problem all over the world (Wood, 1982; Woody and Chu, 1992). Many interacting factors in the pathogenesis of a mycotoxicosis make diagnosis difficult as does confirming mycotoxin exposure (Bryden, 2007). Food crops and feed materials can be easily infected by fungal species which may produce mycotoxins during growth, harvest, and storage

(Monbaliu *et al*., 2010). The ability of molds to produce mycotoxins is influenced by environmental factors such as temperature, relative humidity, and drought (Monbaliu *et al*., 2010). Mycotoxins are widely regarded as the natural toxins that can cause the most serious contamination of food and feed grains (Rubert *et al*., 2012). The contamination of foods and feeds gained much global attention in recent years due to its adverse health and economic effects (Luongo *et al*., 2010).

Major food commodities affected are cereals, nuts, dried fruit, coffee, cocoa, spices, oil seeds, dried peas, beans and fruits, particularly apples (Turner *et al*., 2009). Mycotoxins might be found in beer and wine resulting from the use of contaminated barley, other cereals and grapes in their production (Turner *et al*., 2009). Various mycotoxins continue to be reported in different commodities in various countries around the world (Ramesh and Vasanthi, 1999). The term mycotoxin was coined in 1962 in London, England, during which approximately 100,000 turkeys died (Blout, 1961; Forgacs**,** 1962; Bennett and Klich, 2003). When this mysterious turkey X disease has been linked to peanut (groundnut) contaminated with secondary metabolites of the fungi, *Aspergillus flavus* (aflatoxins), it is possible that scientists sensitive material molds mystery which may be life-threatening. (Bennett and Klich, 2003). Mycotoxin production of fungi is a complex process currently not fully understood (Kokkonen *et al*., 2010). It is known that both the fungal growth and their toxigenic potential are affected by environmental factors, such as temperature, humidity, water activity, pH and both nutrient composition and availability (Kokkonen *et al*., 2010). Under field conditions, there are many factors which influence the mycotoxin production: climate, agricultural practices, host plant and the presence of other microorganisms (Kokkonen *et al*., 2010).

Table 1 Some diseases in humans have been involved with mycotoxins.

Disease	Mycotoxin source	Fungus
Akakabio-byo	Wheat, barley, oats, rice	*Fusarium spp.*
Alimentary toxic aleukia	Cereal grains (toxic bread)	*Fusarium spp.*
Balkan nephropathy	Cereal grains	*Penicillium spp.*
Cardiac beriberi	Rice	*Aspergillus spp.*, *Penicillium spp.*
Celery harvester's disease	Celery (Pink rot)	*Sclerontinia*
Ergotism	Rye, cereal grains	*Claviceps purpurea*
Hepatocarcinoma	Cereal grains, peanuts	*Aspergillus flavus*, *A. parasiticus*
Kwashiorkor	Cereal grains	*Aspergillus flavus.* *A. parasiticus*
Neural tube defects	Maize	*Fusarium verticillioides*, *F.proliferatum*
Oesophageal tumors	Corn	*Fusarium verticilloides*, *F.proliferatum*
Onyalai	Millet	*Phoma sorghina*
Reye's syndrome	Cereal grains (grain dust)	*Aspergillus*
Stachybotryotoxicosis	Cereal grains, (grain dust)	*Stachybotrys atra*

Source: Bryden (2007)

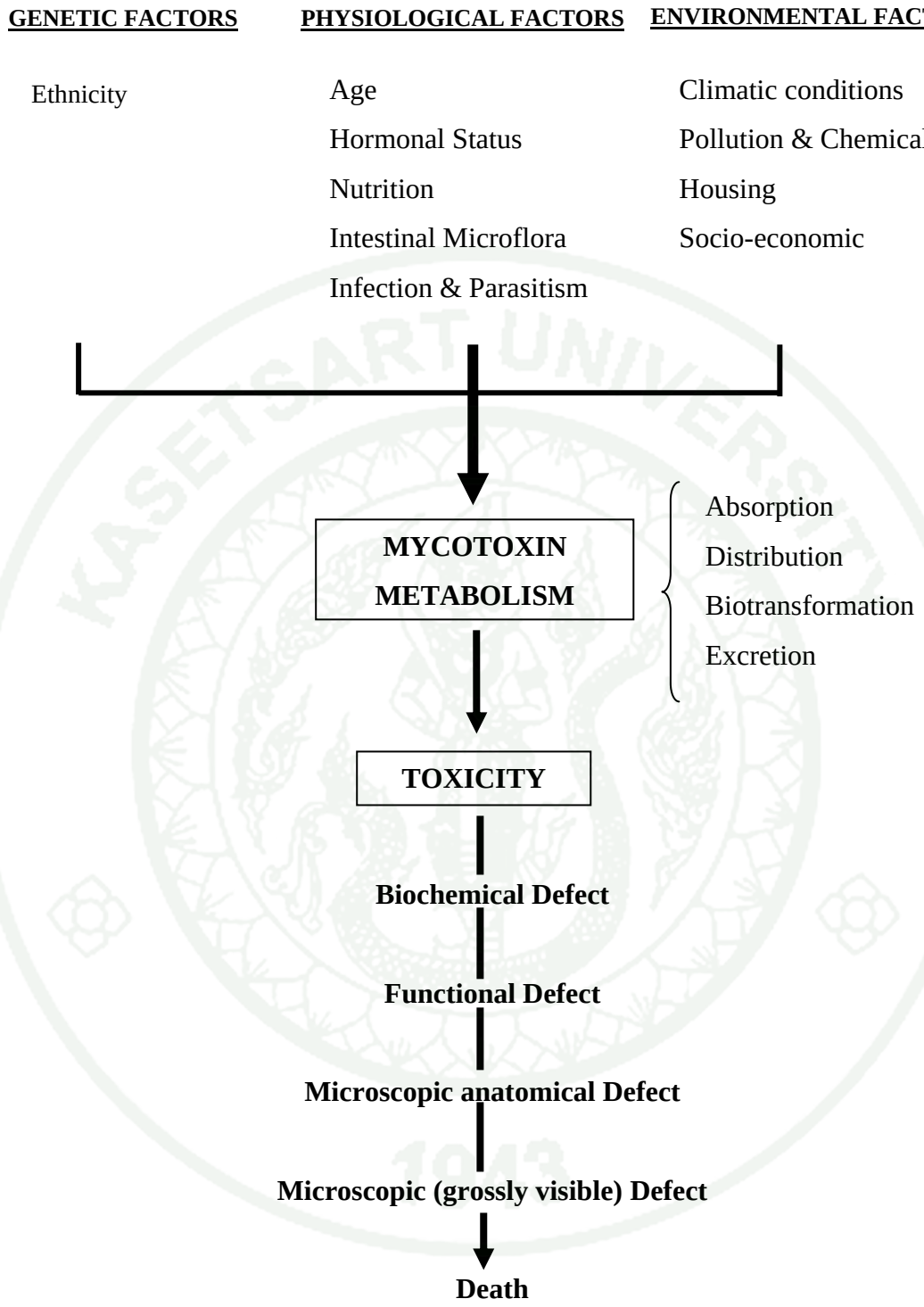


Figure 1 A simplified representation of some general relationships in a mycotoxicosis.

Source: Bryden (2007)

In environmental monitoring, mycotoxins were measured in food, air, or other samples. In biological monitoring, the presence of residues adducts and metabolites are assayed directly in tissues, fluids, and their excretion (Hsieh, 1988). Various mycotoxins continue to be reported in different commodities in various countries around the world (Ramesh and Vasanthi, 1999). The WTO Agreement on the application of SPS measures, signed by various countries to facilitate international trade might introduce newer regulatory policies which could lead to more difficulties for the exporting countries (Ramesh and Vasanthi, 1999). The economic impact of mycotoxins on the food grain and livestock industry has been considerable (Ramesh and Vasanthi, 1999). While rejection of export consignments of groundnut above the regulatory limit would result in economic loss, the distribution of the rejected consignments in the domestic market would increase the consumer risk (Ramesh and Vasanthi, 1999).

Clinicians often arrange them by the organ they affect (Bennett and Klich, 2003). Thus, mycotoxins can be classified as hepatotoxins, nephrotoxins, neurotoxins, immunotoxins and other (Bennett and Klich, 2003). There are many attempted to classify them. Organic chemists have attempted to classify them by their chemical structures (e.g., lactones, coumarins) (Bennett and Klich, 2003). Physicians have attempted to classify by the illnesses they cause (e.g., St. Anthony's fire, stachybotryotoxicosis). Mycologists attempted to classify by the fungi that produce them (e.g., Aspergillus toxins, Penicillium toxins) (Bennett and Klich, 2003). Toxicologists tend to concentrate their efforts on hazardous chemicals such as polyaromatic hydrocarbons, heavy metals, and organic pesticides (Bennett and Klich, 2003). Because they have devoted less effort to natural products, agriculturalists, chemists, microbiologists, and veterinarians who are often unfamiliar with the basic principles of toxicology have conducted most of the mycotoxin research (Bennett and Klich, 2003). Mostly, the main human and veterinary health burden of mycotoxin exposure is related to chronic exposure (e.g., cancer induction, kidney toxicity, immune suppression). However, the best-known mycotoxin episodes are manifestations of acute effects (e.g., turkey X syndrome, human ergotism, stachybotryotoxicosis) (Bennett and Klich, 2003). Human exposure to mycotoxins is further determined by environmental or biological monitoring (Bennett and Klich,

2003). The final toxic effects, appearing in consumers exposed to a mixture of mycotoxins, are related to the toxicokinetic behavior, the metabolism, and the toxicodynamic effects of mycotoxins (Monbaliu *et al*., 2010).

The requirement to apply these regulatory limits has prompted the development of a number of analytical methods for the identification and quantification of mycotoxins in various samples, such as food, feed, and other biological matrices (Krska *et al*., 2008). The chemical diversity of mycotoxins and their varying concentration ranges in a wide range of agricultural commodities, foods and biological samples poses a great challenge to analytical chemists (Krska *et al*., 2008). The different chemical and physicochemical properties of the mycotoxins require specific extraction, cleanup, separation and detection methods (Krska *et al*., 2008). Therefore, most methods target only individual mycotoxins or at best a group of closely related mycotoxins (Krska *et al*., 2008). The analytical methods of mycotoxins that are developed and reported in many literature are not usually directly applied to biological fluids and organs, but the methods that have been developed achieve good performance at low concentration levels and are also sufficient for biological fluids and organ analysis (Rubert *et al*., 2012; Berthiller *et al*., 2005; Kadota *et al*., 2011). A large variety of analytical methods is used for mycotoxin determination, including chromatographic methods such as TLC, GC or LC and immunochemical methods such as ELISA (Berthiller *et al*., 2012). Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) is the technique of choice for mycotoxin analysis because of highly reliable, versatility, specificity and selectivity (Monbaliu *et al*., 2010; Rubert *et al*., 2012).

Trichothecenes are a large group of structurally related mycotoxins mainly produced by the fungi of Fusarium genus. Other species, such as *Stachybotrys, Myrothecium, Cephalosporium, Verticimonsporium, and Trichotheciu* are the minor species producing trichothecenes. The trichothecenes are most commonly found in cereals, and particularly in barley, maize, oats, and wheat (Conkova *et al*., 2003; D'Mello *et al*., 1999; Eriksen and Petterson, 2004). These mycotoxins are chemically stable to heating and survive food processing (Montes *et al*., 2012). Consequently, a regular contamination can be expected for cereal-based foods, posing a potential risk to human health (Montes *et al*., 2012). Trichothecene levels are depending on climatic, as well as on seasonal and geographic conditions, because the influence of several factors can lead to a change in the Fusarium profile (Ibanes-Vea *et al*., 2012). Trichothecenes occur worldwide in grains and other commodities grown in cooler climates (Haschek and Beasley, 2009). Mild temperatures tend to encourage fungal growth and cool temperatures increase toxin production (0–15°C) (Haschek and Beasley, 2009).

Trichothecenes tend to be produced in toxic concentrations in years of wet weather when harvests are delayed and prolonged (Haschek and Beasley, 2009). Trichothecenes in the class of sesquiterpenoids contain an olefinic group, an epoxide, and variable numbers of hydroxyl and acetoxy groups. They have a tetracyclic 12, 13-epoxytrichothecene skeleton in common, and can be divided into four categories depending on their functional groups (Ueno, 1977; World Health Organization [WHO], 1990) (Figure 2). Members of group A are not contain carbonyl on C-8. The examples are represented by T-2 toxin, HT-2 toxin, and diacetoxyscirpenol. Hydrolysis of ester groups leads to the formation of a basic trichothecene moiety with one to five hydroxyl groups. Group B differs from group A by the presence of a carbonyl group on C-8. Group C members, such as crotocine, have another epoxy group between the C-7 and C-8 or C-8 and C-9 positions, respectively. Compounds in group D, such as satratoxin G, include a macrocyclic ring between C-4 and C-15 (Qinghua *et al*, 2010). In European agricultural commodities, type-A trichothecenes occur less frequently and at lower concentrations than DON and type-A trichothecenes have a higher toxicity than those of type-B (Ibanes-Vea *et al*., 2012).

The primary fungal species producing the trichothecene mycotoxins are listed in table 2. Trichothecene mycotoxins including Fusarenon-X (FX), non-protein toxins, are highly cytotoxic to eukaryotic cells and act by inhibiting protein and DNA (Miura *et al*., 1998). Thus, they cause specific damage to the tissues containing many rapidly proliferating cells such as lymphoid tissues (Miura *et al*., 1998). However, the identification of trichothecenes are over 150 members, data about their natural occurrence in foods are mostly limited to T-2 toxin, diacetoxyscirpenol (DAS), deoxynivalenol (DON), fusarenon-X (FX), and nivalenol (NIV) due to their high toxicity and occurrence (Pittet, 1998; Voyksner *et al*., 1987; Yoshizawa *et al*., 1980; Poapolathep *et al*., 2002). DON may be the most commonly occurring trichothecene in nature (Qinghua *et al*, 2010). T-2 toxin does not occur as much as DON, but its toxicity is higher than that of DON (Qinghua *et al*, 2010). In addition, the acetyl derivatives of trichothecenes, 3-acetyldeoxy-nivalenol (3-aDON), 15-acetyldeoxynivalenol (15-aDON), and fusarenon-X (FX), co-occur regularly all over the world (Qinghua *et al*, 2010).

Toxicokinetics

The toxicokinetics of trichothecenes has been subjected of various reviews (Swanson and Corley, 1989; Yagen and Bialer, 1993; IARC, 1993). Trichothecenes undergo all four basic reactions in xenobiotic metabolism (Ramesh, 2007). Phase I hydrolysis and oxidation and phase II glucuronide conjugation occur in the body tissues, while reduction of the 12,13-epoxide is thought to occur through microbial action in the gastrointestinal tract; although T-2 toxin is the only trichothecene for which all four basic reactions or pathways occur simultaneously in the same animal (Swanson and Corley, 1989). The ability to remove the epoxide oxygen (deepoxidation) is an important step in the detoxification of trichothecenes (Ramesh, 2007). The toxicokinetics of trichothecenes is important for the understanding potential impact on animals (Ramesh, 2007). The kinetics of all trichothecenes has not been investigated completely, but the existing data shows that the toxins are rapidly absorbed (Erikson and Pettersons, 2004). Trichothecenes do not accumulate to a significant extent in the body and are rapidly excreted within a few days in urine and feces after po administration (Swanson and Corley, 1989). Trichothecenes are rapidly

excreted without any accumulation in any tissue, and only traces of the toxins are found 24 h after po or iv exposure (Erikson and Pettersons, 2004). Trichothecenes do not accumulate in animals due to the rapid excretion and only traces (<50 ng/g) can be found in animal-derived food products (Erikson and Pettersons, 2004). The oral bioavailability of trichothecenes is generally low as a result of physiological instability and first-pass metabolism (Yagen and Bialer, 1993). Intestinal microorganisms present an additional pathway for the detoxification of ingested trichothecenes in some animal species (Sudakin, 2003). Reduction of the trichothecene C-12,13 epoxide has been demonstrated to occur within the gastrointestinal tract of rats, cattle, and swine (Swanson *et al*., 1988). In pigs, T-2 toxin, DON and NIV can be detected in blood less than half an hour after oral exposure to the toxins (Erikson and Pettersons, 2004). The main route of excretion after iv injection is the urine, but after oral exposure a significant proportion is also excreted in the faeces (Swanson and Corley, 1989; Prelusky *et al*., 1988).

Trichothecenes undergo a variety of different metabolic reactions in animals (Erikson and Pettersons, 2004). The major reactions are hydrolysis to split off side groups, hydroxylations and de-epoxidation. Acetylated toxins are rapidly metabolised to the de-acetylated form of the toxin (e.g. T-2 toxin to HT-2 toxin, fusarenon-X (FX) to NIV, 3-acetyl DON to DON) (Erikson and Pettersons, 2004). The deacetylation reactions are fast and catalyse by specific esterases (Swanson and Corley, 1989). A 12,13 de-epoxide metabolite of trichothecenes has been detected after oral exposure to NIV or DON in rats, mice, cattle and sheep (Erikson and Pettersons, 2004). A de-epoxide metabolite was found after iv injection of T-2 toxin in pigs (Erikson and Pettersons, 2004). De-epoxidation of trichothecenes has also been reported from incubations of trichothecenes with the microflora from the intestines of a range of species or with micro-organisms from the rumen of cows (Swanson and Corley, 1989; Swanson *et al*., 1988; Kollarczik *et al*., 1994). These incubation studies have also showed that the microflora is able to deacetylate trichothecenes (Erikson and Pettersons, 2004). De-epoxide metabolites of trichothecenes have also been detected in plasma and urine in sheep and cows, but only in low amounts could be determined (Erikson and Pettersons, 2004). This reduction of the epoxide ring is probably carried out by anaerobic gastrointestinal micro-organisms (Swanson and Corley, 1989). Some

trichothecenes, such as DAS, T-2 toxin and HT-2 toxin and metabolites there of, are extensive glucuronide conjugated, while little or no such conjugation has been found in monogastric animals given DON or NIV (Swanson and Corley, 1989). The absorption of trichothecenes is rapid also in ruminants, but only low proportions of the administered toxin are accounted for in studies with cattle and sheep, even when the de-epoxide metabolite is included (Erikson and Pettersons, 2004). This indicates that other unknown metabolites may be formed in ruminants (Erikson and Pettersons, 2004).

From a large number of mycotoxins, only a few states that will affect the pigs performances (Viljoen, 2008). The risk of mycotoxins in pig contaminated-feed, depending on the age and health of the pigs and the level of toxins in the extreme is death (Viljoen, 2008). However, low levels of mycotoxins can reduce pig performance and general well-being (Viljoen, 2008). When pigs fed with mycotoxin harmful, toxic chemicals can affect the central nervous system, pork, liver, kidneys, immune system and reproductive processes. (Viljoen, 2008). Aflatoxin, zearalenone, and tricothecene (vomitoxin and T-2 toxin) are the most often reported mycotoxins in swine feed. Each toxin is produced by a different mould (Viljoen, 2008). The conditions that promote the growth of fungi are varies. However, high humidity and warm temperatures are responsible for the growth of most fungi feed (Cranshaw, 2008). The pig is most sensitive to trichothecenes (Erikson, 2003). The kinetic parameters of DON have been related to intravenous and acute and chronic oral DON exposures in pigs (Coppock *et al*., 1985; Prelusky *et al*., 1988, 1990; Prelusky and Trenholm, 1991; Goyarts and Dänicke, 2006). In pigs fed graded levels of T-2 toxin in standard pig ration for 8 weeks, no significant differences in body weight gain and feed consumption were observed between the test and control pigs (Agag, 2005). Young pigs refused a ration containing 16 ppm T-2 toxin, but not a diet containing 10-12 ppm (Agag, 2005). The no observed effect level in ration was estimated to be less than 1 ppm based on differences in body weight gain (Weaver *et al*., 1978a). Dietary levels of T-2 toxin as low as 0.5 ppm were found to cause a reduction in feed intake in pigs (Rafai *et al*., 1995a). T-2 toxicosis is due to elevation of tryptophan in the brain. Tryptophan, a mediator of appetite, is a precursor of serotonin (Smith and seddon, 1998). Infertility with some lesions in the uteri and ovaries result from

consumption of feed contaminated with 1 to 2 ppm of T-2 toxin (Jacobsen *et al*., 1993). The intravenous administration of T-2 toxin to pigs at doses of 4 or 8 mg/ kg bw, resulted in increased plasma concentrations of epinephrine, norepinephrine, thromboxane B_2 and 6- keto- prostaglandin F (Lorenzana *et al*., 1985; WHO, 1990).

Mechanism of action

Health risk associated with human exposure to trichothecenes is widely recognized (Montes *et al*., 2012). There are depending on the degree they are consumed in a diversified diet (Montes *et al*., 2012). In order to protect health consumers, the European Commission has legislated maximum levels for trichothecenes in cereal grains, flours, and cereal-based products intended for human and animal consumption (Montes *et al*., 2012; European Commission, 2006). Trichothecenes initiate a wide range of toxic effects on farm animals and humans. (Haschek and Beasley, 2009) They causes of apoptosis and/or necrosis in the lymphoid, hematopoietic, and gastrointestinal systems resulting in leukopenia, vomiting, and diarrhea that can be lethal (Haschek and Beasley, 2009) Alimentary toxic aleukia (ATA), a typical disease for human, was found to be associated primarily with the ingestion of moldy cereal infected with T-2 toxin (Joffe, 1974 and 1978). In addition, trichothecenes are toxic to the skin and testes (Qinghua *et.al*, 2010). Immune suppression and increased susceptibility to infection may occur, especially in the late phase of the disease (Qinghua *et.al*, 2010). The toxic effects from trichothecenes largely resemble those following radiation exposure (radiomimetic) due to effects on rapidly dividing cells in the intestine, bone marrow, and testis (Haschek and Beasley, 2009). Trichothecenes have multiple effects on eukaryotic cells, including inhibition of protein, RNA and DNA synthesis, alteration of membrane structure and mitochondrial function, stimulation of lipid peroxidation, induction of programmed cell death or apoptosis, and activation of cytokines and chemokines. The effects of these toxin on eukaryotic cell function was shown in table 3. Trichothecenes including deoxynivalenol (DON) are the most common group causing animal disease, effects range from feed refusal, vomiting to immunosuppression and loss of productivity (Viljoen, 2008). Although DON can be

acutely lethal when ingested in large quantities, moderate- to low-level ingestion of the toxin can cause poor performance and altered immune function (Pier *et al*., 1980a, 1980b). Monogastric animals, particularly swine, exhibit the greatest sensitivity to DON, while chickens and turkeys, followed by ruminants, appear to have higher tolerance (Prelusky *et al*., 1994). The adverse effects of the trichothecenes mycotoxin in animals are show in table 4.

Trichothecenes are potent inhibitors of protein synthesis due to binding to the 60S ribosomal unit and this is believed to be the main mechanism of toxicity. (Haschek and Beasley, 2009) Inhibition of protein synthesis occurs through interference with peptidyl transferase activity, with an intact C-9, 10 double bonds and the C-12, 13 epoxide are required for this inhibition. (Haschek and Beasley, 2009) They can also activate MAPKs and induce apoptosis in a process known as the ‘‘ribotoxic stress response’’ (Haschek and Beasley, 2009). In addition, trichothecenes can activate p38, Jun N-terminal kinase (JNK) and extracellular signal regulated kinase (ERK) MAPKs *in vitro* and *in vivo*. (Haschek and Beasley, 2009) They effect on rapidly dividing cells in the lymphoid, hematopoietic, and gastrointestinal systems (Haschek and Beasley, 2009).

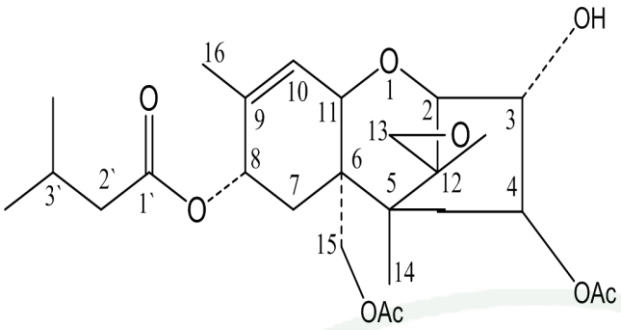
Fusarenon-X (FX; 3, 7, 15-trihydroxy-4-acetoxy-12, 13 epoxytrichothec-9-e-8-one), a type B trichothecene mycotoxin, is one of the 12, 13-epoxytrichothecenes mainly produced by *Fusarium crookwellense*, which naturally occurs in agricultural commodities such as wheat and barley (IARC, 1993; Poapolathep *et al*., 2008). Montes *et al*., 2012 found the contamination of FX in breakfast cereal. This is a first report of FX detection in food, especially in Spain (Montes *et al*., 2012). There have been reported that FX induced adverse health effects, particularly apoptosis, in organs containing actively dividing cells such as the small intestine, thymus, spleen, bone marrow, testes, reticulocytes, and mitogen-stimulated human lymphocytes, as observed in other trichothecenes (Ohta *et al*., 1978; Forsell and Pestka, 1985; Miura *et al*., 1998; Poapolathep *et al*., 2002). FX has been classified as the class 3 by IARC classification. For the definition of class 3 carcinoginicity refers to is inadequate or limited in humans and animals. (IARC, 2011). Recently, the limited pharmacokinetic data are available for trichothecene mycotoxins in animals, especially for FX

(Poapolathep *et al*, 2008). Poapolathep *et al*. (2008) reported the FX disposition fit an open 2-compartment pharmacokinetics model in broilers and ducks. FX can be metabolized to NIV via deacetylation in mice, excreted mainly in urine (Poapolathep *et al*, 2003). The liver and kidney are the organs responsible for the FX-to-NIV conversion (Poapolathep *et al*, 2003). The metabolic pathways of FX and NIV were shown on figure 4.

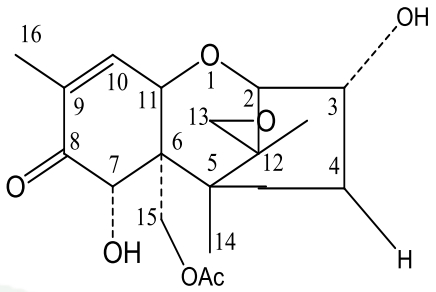
In general, FX is well known to be cytotoxic to mammalian cells (Miura *et al*., 2002). Although, FX has been observed to occur frequently with deoxynivalenol (DON; 3, 7, 15-trihydroxy-12, 13-epoxytrichothec-9-e-8-on) in agricultural products (Yoshizawa, 1983; Miller *et al*., 1991). The fate and disposition of FX in animals has not been extensively studied as DON (Popolathep *et al*, 2003). It is well known that species differences affect the fate of drugs and chemicals in animals (Walker, 1980). In our previous investigation, we demonstrated that FX is rapidly converted to NIV in mice after po administration (Poapolathep *et al*., 2003, 2004). However, the previous findings in mice cannot be directly estimated to other animal species. (Poapolathep *et al*., 2002, 2003) Not only FX is absorbed from the gastrointestinal tract rapidly and metabolized to NIV after being absorbed but also shown that the liver and kidney are the organs responsible for the FX- to- NIV conversion in mice (Poapolathep *et al*., 2003). In addition, FX can transfer to fetal mice via placenta (Poapolathep *et al*., 2004). Both *in vivo* and *in vitro* metabolisms of NIV and FX in female ICR mice were investigated by utilizing ^{3}H-NIV and ^{3}H-FX (Poapolathep *et al*., 2003). During *in vivo* study, a large proportion of ^{3}H-FX was found to be excreted as ^{3}H-NIV in urine and feces (Poapolathep *et al*., 2003). ^{3}H-NIV was mostly excreted in the unchanged form, except for an unknown metabolite (Poapolathep *et al*., 2003). FX can be metabolized to NIV via deacetylation in mice poultry and ducks, excreted mainly in urine (Poapolathep *et al*., 2003, 2008).

In swine, there are few publications on the toxicokinetics of FX and NIV (Poapolathep *et al* , 2002, 2003, 2008). After NIV was administered with 0.05 mg / kg, bw in swine, the metabolites were found in plasma, urine and feces as glucuronic acid or sulfate conjugates or deepoxy-NIV (Hedman and Petterson, 1997). When NIV were prolonged feed for 3 weeks to investigate the effects of time-dependent

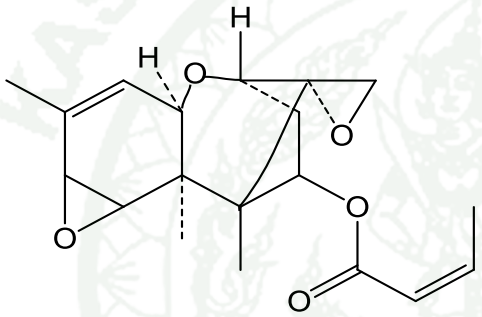
exposure to NIV with the ability to create deepoxy-NIV (Hedman *et al*., 1997). When NIV was incubated at 37°C with the feces collected from the pigs exposed to NIV for 48 h, 99.1 ± 1.3% of NIV was transformed to deepoxy-NIV (Qinghua *et al*, 2010). However, no metabolite was detected in incubations with feces collected prior to the exposure period (Qinghua *et al*, 2010). Deepoxy-NIV was also found in the anaerobic incubation with pig feces collected at different pig farms (Eriksen *et al*., 2002). No deepoxidation ability was found in samples of feces or ileum content from pigs at the start of the feeding trial or during the first 2 weeks when the pigs were fed uncontaminated feed (Qinghua *et al*, 2010). However, after the pigs were exposed to feces from pigs known to have the deepoxidation ability for 1 week, the deepoxidation ability was found in fecal and ileal incubates from 4 of the 5 pigs (Qinghua *et al*, 2010). A conclusion that the deepoxidation ability was able to be transferred between pigs in a stock was proposed. In summary, NIV is able to be transformed to deepoxy-NIV in pigs exposed to NIV for a long period (Qinghua *et al*, 2010). The deepoxidation ability may be transferred between pigs in a stock (Qinghua *et al*, 2010).



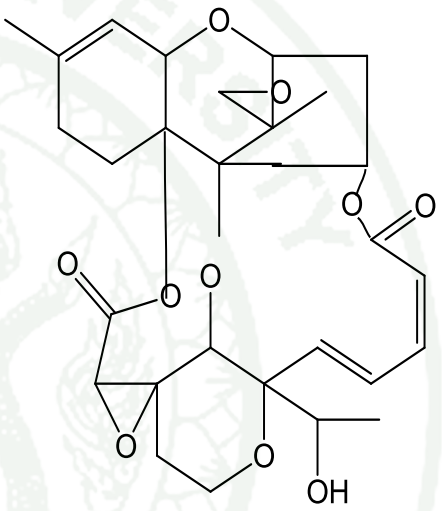
Group A: T-2 toxin



Group B: Deoxynivalenol



Group C: Crotocin



Group D: Satratoxin G

Figure 2 Chemical structures of Trichothecenes classification

Source: Qinghua *et al* (2010)

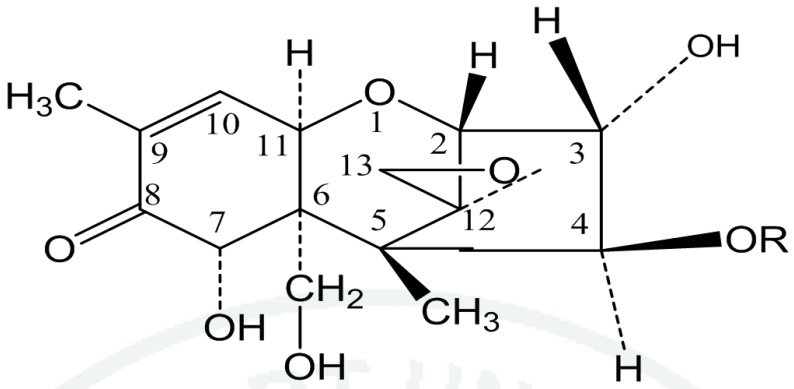


Figure 3 Chemical structures of Fusarenon-X (R= Ac)

Source: Poapolathep *et al* (2003)

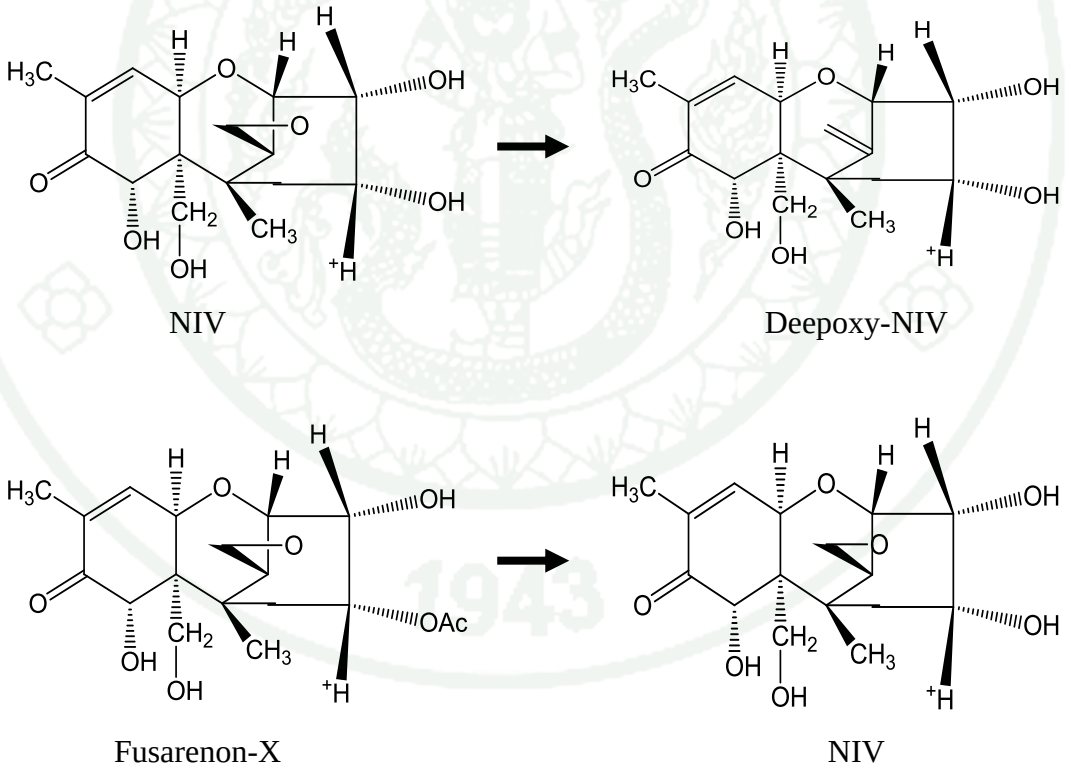


Figure 4 The metabolic pathways of FX and NIV in animals

Source: Qinghua *et al* (2010)

Table 2 The LD_{50} and lethal dose of trichothecene toxins and their comparative toxicity

Group	Fungi	Trichothecene	Acute LD_{50} Values (mg/kg)				Lethal dose (mg/kg)
			Mouse			Pig	
			iv or ip	po	Inhalation	iv	inhalation
A	Fusarium spp.	T-2 toxin	3.0 – 5.3	3.8 – 10.5	0.16	1.21	1.5 – 3.0
		HT-2 toxin	6.5 – 9.0				
		Diacetoxyscirpenol (DAS)	9.6 – 23.0	15.5 – 46.0		0.37	
		Monoacetoxyscirpenol					
B	Fusarium spp.	Deoxynivalenol (DON, vomitoxin)	70.0 – 76.7	46.0			
		Nivalenol	4.0 - 6.3				
		Fusarenon-X	3.4	4.5			
C	Cephalosporium spp.	Crotocin	700 – 810	1,000			
D	Myrothecium spp.	Verrucarins					
		Verrucarin A and B	0.5(A)-7.0(B)				
		Roridins					
		Roridin A	1.0 (A)				
	Stachybotrys spp.	Satratoxins					

Source: Haschek and Beasley (2009)

Table 3 Trichothecene effects on eukaryotic cell functions

Compound	References
Inhibit protein synthesis	Ehrlich and Daigle (1987), McLaughlin *et al*. (1977)
Inhibit RNA and DNA synthesis	Rosenstein and Lafarge-Frayssinet (1983), Thompson and Wannemacher (1986)
Stimulate lipid peroxidation	Rizzo *et al*. (1994), Vila *et al*. (2002)
Alter cellular membrane function	Bunner and Morris (1988)
Inhibit mitochondrial and electron transport chain function	Pace *et al*. (1988)
Induce apoptosis	Pestka *et al*. (1994), Shinozuka *et al*. (1998), Islam *et al*. (1998)
Activate MAPKs	Zhou *et al*. (2005)
Modulate immune responses	Corrier (1991), Bondy and Pestka (2000)
Alter neurotransmitters	Prelusky *et al*. (1992), Swamy *et al*. (2004)
Induce gene expression of numerous chemokines and cytokines	Azcona-Olivera *et al*. (1995), Zhou *et al*. (1997), Moon and Pestka (2002), Ji *et al*. (1998), Pestka *et al*. (2005)

Source: Ramesh (2007)

Table 4 The adverse effects of the trichothecenes mycotoxins in animals

Compound	Effects
T-2 toxin	Feed refusal; weight loss; decreases red blood cell count; reduces leucocyte count; reproductive disorders; increases mortality of piglets after birth, reduces plasma glucose in piglets, pathological changes in liver and stomach, increases infection rate; alimentary toxic aleukia (ATA); induces apoptosis in the thymus and spleen; inhibits the synthesis of DNA and RNA
DON	Food refusal; vomiting; digestive disorders; weight loss; decreases levels of serum protein; oxidative stress and blood phagocytic activity in broilers; cytotoxic effect on human primary hepatocytes
NIV, FX	Gastrointestinal erosions; nephropathy; reduction of feed intake; cytotoxicity
DAS	Reduces feed intake and weight gain; oral lesions; gastrointestinal lesions; diarrhea

Source: Qinghua *et al.* (2010)

Table 5 Deoxynivalenol (DON) and T-2 toxicity in various animal species.

Animal species	Dose	Symptoms of toxicity	Reference
Pigs	10 ppm	Neutrophils affected	Takayama *et al*., 2005
	5.7 mg/kg	Elevated liver RNA concentration and increased protein Synthesis	Danicke *et al*., 2006
	0.21 ppm DON + 0.004 ppm ZON or 9.75 ppm DON + 0.358 ppm ZON in diet (sow)	Lesions in spleens and liver of sows No pathological lesions in piglet	Tiemann *et al*., 2008
Poultry	4 or 16 mg/kg diet (7 day oldchicks)	Reduced body weight and feed intake, plaque formation and buccal ulceration	Hoerr *et al*., 1982
Ruminants	15.6 mg/kg DON (sheep)	No effects for 28 days Weight loss (extended feeding)	Harvey *et al*., 1986
	10 - 20 mg/kg T-2 toxin in feed (calves)	Sloughing of papilla and omasum ulceration	Cheeke *et al*., 1998a
	3-5 ppm (dairy cow)	Reduced IgA , serum albumin and globulin	Korosteleva *et al*., 2007

Source: Upadhaya *et al*. (2010)

MATERIALS AND METHODS

Materials

1. Standard Fusarenon-X (Wako® company)
2. Standard Nivalenol (Wako® company)
3. 25% Dimethyl sulfoxide (DMSO)
4. Acetronitrile
5. Milli Q water
6. Ammonium sulfate
7. Distilled water
8. Sterile water
9. Methanol
10. Ammonium acetate
11. Sucrose
12. Dithiothreitol hydrochloride
13. EDTA
14. Tris-HCL
15. 70% Alcohol
16. Heparin
17. Thiopental sodium
18. Liquid nitrogen
19. Povidone iodine
20. Sterile cotton ball
21. Syringe 1 ml.
22. Syringe 3 ml.
23. Syringe 5 ml.
24. Needle No. 21 size 1 ½ inch
25. Glass test tube
26. Surgical knife No.4
27. Surgical blade No. 22
28. Knife

29. Stainless steel tray
30. Eppendorf tube
31. Glass test tube 10 ml.
32. Heparin tube
33. EDTA tube
34. Zip lock
35. Liquid chromatography tandem mass spectrometry with electrospray ionization (LC-ESI-MS, Shimadzu LC8030)
36. Centrifuge
37. Ultracentrifuge
38. Obital shaker
39. Nitrogen evaporator
40. Homoginizer
41. Vortex mixer
42. Sonicator
43. Blender
44. Water® C18 cartridge Sep-pak
45. Pasteur pipette
46. Dropper
47. Phenomenax® analytical column
48. Centrifuge tube with screw cap
49. Stainless test tube rack
50. Surgical glove
51. Cooler box
52. Dosing sheet

Methods

1. Animals

A Forty-three healthy 4-wks-old female piglets (average weight 7.44 ± 0.76 kg) were obtained from commercial pig farm in Chonburi Province, THAILAND. The experimental animals were housed in individual stainless-steel cages at the Laboratory Animal Unit, Faculty of Veterinary Medicine, Kasetsart University, and acclimatized to the environment for 1 wk. The piglets were fed with a nursery commercial diet and water *ad libitum* throughout the experiment. All experimental procedures carried out on the animals were approved by the Animal Ethics Research Committee of Faculty of Veterinary Medicine, Kasetsart University.

2. Toxin preparation

Standard FX was purchased from Wako Chemical Co, (Tokyo, Japan). For oral group, standard FX 40 mg were weighing with digital scales in 25 ml beaker and then dissolved with 25% DMSO solution 10 ml. The final standard stock solution for oral group was 4 mg/ml. On the other hand, standard FX 40 mg were weighing with digital scales in 25 ml beaker and then dissolved with 25% DMSO solution 5 ml. This standard final solution, 8 mg/ml, was for intravenous administration group.

3. Standard solution preparation

a. For spiked sample

Five milligram of standard FX were dissolved with 1.25 ml of 25 % DMSO solution and 3.75 ml of PBS , pH 7.4. The stock solution was 1 mg/ml. The concentration for spiked sample need were 500 ppb, 250 ppb, 125 ppb, 50 ppb, 10 ppb, and 5 ppb, respectively.

b. For carilbration curve

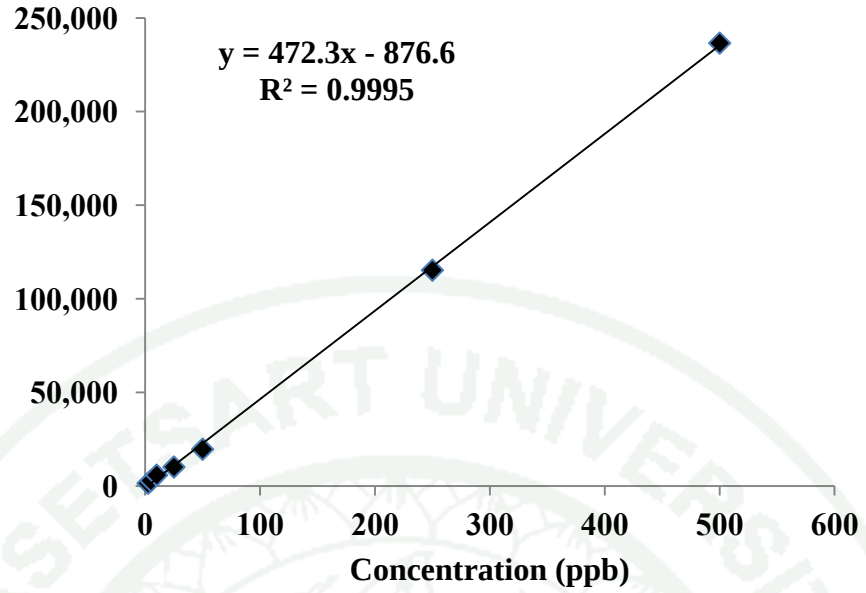
The standard solution of FX and NIV were purchased from Wako® company, JAPAN. This solution was dissolved in acetronitrile. The concentration was 100 µg/ml. The standard concentration of FX and NIV were prepared for standard curve following; 500 ppb, 250 ppb, 100 ppb, 50 ppb, 25 ppb, 10 ppb, 5 ppb, 2.5 ppb, and 1 ppb, respectively. The carilbration curve of FX and NIV were show in figure 5.

4. Experimental design for toxicokinetic study

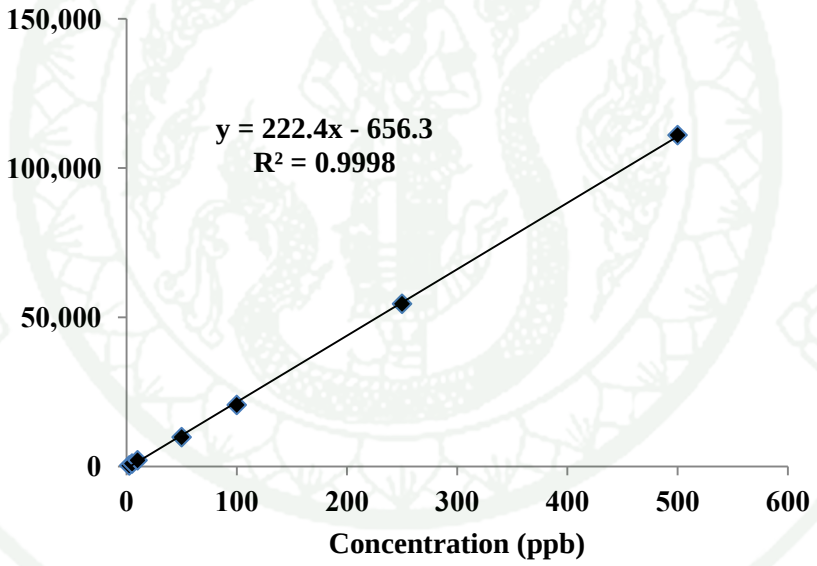
To obtain the toxicokinetic data, ten piglets were divided into 2 groups. (n=5) Each group was administered intravenously (iv) or orally (po) with FX at a dosage of 1 mg/kg bw. Blood samples were collected from jugular veins with heparinized syringes at 0, 5, 10, 30, 45 min and 1, 2, 4, 8, 12, 24, and 48 h, respectively. Plasma were separated by centrifugation (1,912 x g) for 15 min. Urine and feces were also collected at 0, 1, 4, 8, 12, 24, and 48 hrs after administration. All the plasma, urine and feces were frozen at -20°C until analysis.

5. Experimental design for residue study

Twenty-five piglets were orally with FX at a dosage of 1 mg/kg of bw. The remaining five piglets served as controls and were orally administered DMSO in 0.9% physiologic saline. Animals were sacrificed with thiopentone sodium at a dosage of 20 mg/kg of bw by iv administration. Tissues samples including liver, kidney, spleen, muscle, intestine and bile were collected at 0, 1, 3, 6, 12 and 24 h after po administration, respectively. Urine and feces are also collected at 0, 1, 4, 8, 12, 24 and 48 h after administration. All samples were frozen at -20°C until analysis.



a.) Fusarenon-X

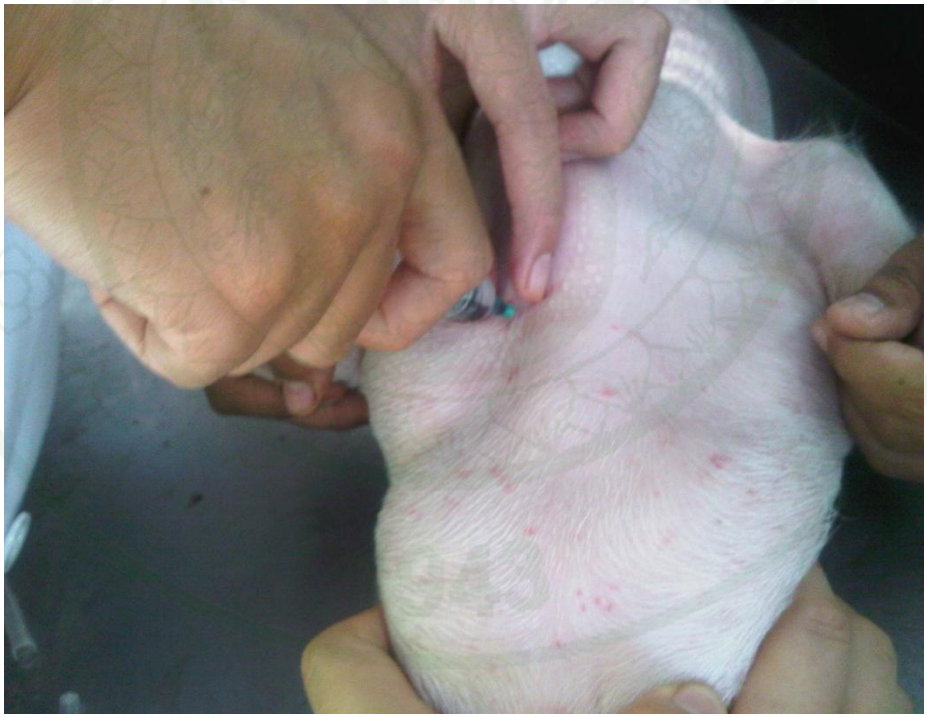


b.) Nivalenol

Figure 5 The linearity standard curves of FX (a) and NIV (b)

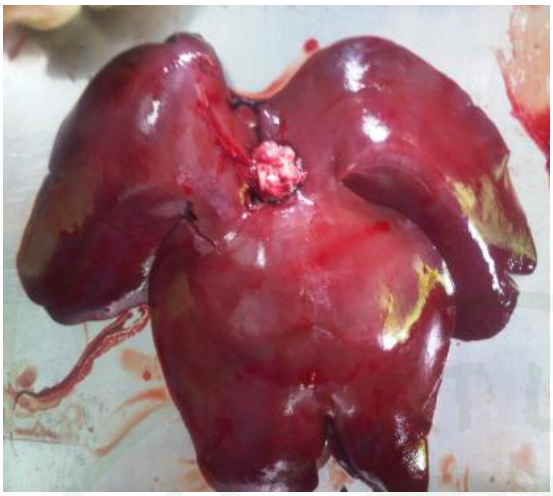
Figure 6 A healthy 4-wks-old female piglets (average weight 7.44 ± 0.76 kg) were housed in stainless-steel cages at the Laboratory Animal Unit, Faculty of Veterinary Medicine, Kasetsart University.

a) Restraint in experimental animals

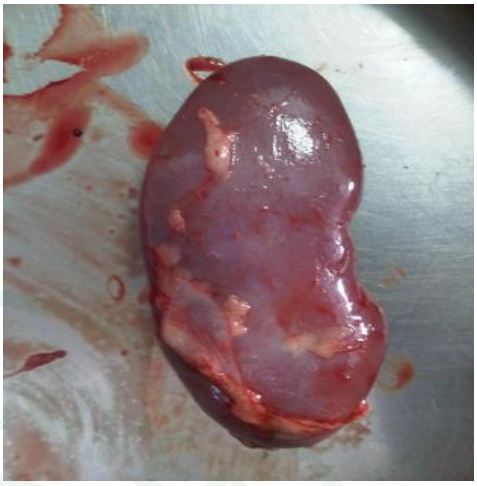


b) Blood collection in experimental animals

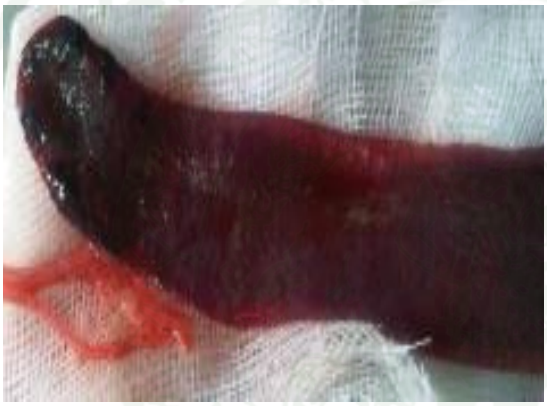
Figure 7 Procedure of restraint (a) and blood collection (b) in experimaental animals.



a. Liver



b. Kidney



c. Spleen

d. Small Intestine

e. Muscle

Figure 8 The organs were collected for residues study

6. Metabolism of FX to NIV *in vitro* study

Three 5-wk-old female piglets were killed with thiopentone sodium at a dosage of 20 mg/kg of BW by iv administration. The livers and kidneys were immediately removed and frozen in liquid nitrogen and stored at -80°C until used. Postmitochondrial fractions were prepared by the previous method (Esaki and Kumagai, 2002; Poapolathep *et al*, 2008). The postmitochondrial fractions of liver and kidney were incubated and sheaken (60 cycles/min) with FX at 37°C for 15, 30, 45, and 60 min, respectively.

7. Tissue homogenization for residue study

Five gram of various tissues; liver, kidney, spleen, muscle and small intestine were homogenized with homogenizing buffer. The buffer consist of 0.25M sucrose, 0.2 mM dithiothreiol hydrochloride, 1 mM EDTA and 10 mM tris HCl pH 7.4, respectively. After homogenized, all of tissues were kept at - 20°C until extracted and clean-up step.

8. Extraction and Clean-up

Plasma, urine, feces, various homogenized tissues and postmitochondrial fractions of the liver and kidney were extracted in the 3 mL of acetonitrile (ACN)-water (3:1). Ammonium sulfate was added to the mixture (Poapolathep *et al*., 2008), and then the ACN fraction was separated by centrifugation at (1,912 x g) for 15 min. Extraction was repeated 2 additional times. The parent and metabolites in the ACN fraction were purified with a C_{18} Sep-pak silica cartridge (Waters Corp., Milford, MS) as described previously (Poapolathep *et al*., 2008). The elute was evaporated to dryness under a nitrogen stream at 40°C in a heating block. The residue was redissolved with 500 µl of methanol-water (1:4) v/v with 5 mM of ammonium acetate (Berthiller *et al*., 2005, Kadota *et al*., 2011) and then analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS).

9. LC parameter

The LC-MS/MS has been described in previous publications. (Berthiller *et al*., 2005 and Kodata *et al*., 2011). In briefly, the system was composed of LC-ESI-MS (LCMS 8030, Shimadzu, JAPAN). The residue was separated on a Synergi 4 μm Polar-RP 80A analytical column with guard column (150 mm length × 4.6mm i.d., 4 μm, Phenomenex, Cheshire, England). The column was maintained at a temperature of 45°C. The flow rate was 0.4 ml/min. The injection volume was 5 μl. Mobile phase A was 10 mM ammonium acetate in distilled water and mobile phase B was methanol. A linear binary gradient was applied changing from 5 to 95% mobile phase B within 15 min for FX and 10 min for NIV, respectively. The gradient elution was shown in table 6 .

10. MS parameter

A Shimadzu (LCMS 8030) triple quadrupole mass spectrometer equipped with an electrospray source analyzed the multiple reaction monitoring (MRM) negative ion mode with the following setting: Nebulizing gas flow 3.0 l/min, Drying gas flow 15.0 l/min, DL temperature 250°C, Heat Block Temperature 400°C and CID gas 230 kPa. The molecular ions and fragments employed for FX and NIV were shown on table 7 .

11. Fortification procedure

To evaluate recovery, FX and NIV were added to 1 ml of plasma, urine, and bile, and 5 g of feces and various tissues, and 2.5 g of spleen, respectively, to yield final concentrations of 5, 10, 50, 125, 250, 500 ng/ml, repectively. The spiked samples were then analyzed in triplicate as described in the extraction and clean-up procedure. The average (±SD) recoveries of FX and NIV in plasma and various organ were shown in table 8.

Table 6 Gradient elution method used by LCMS 8030

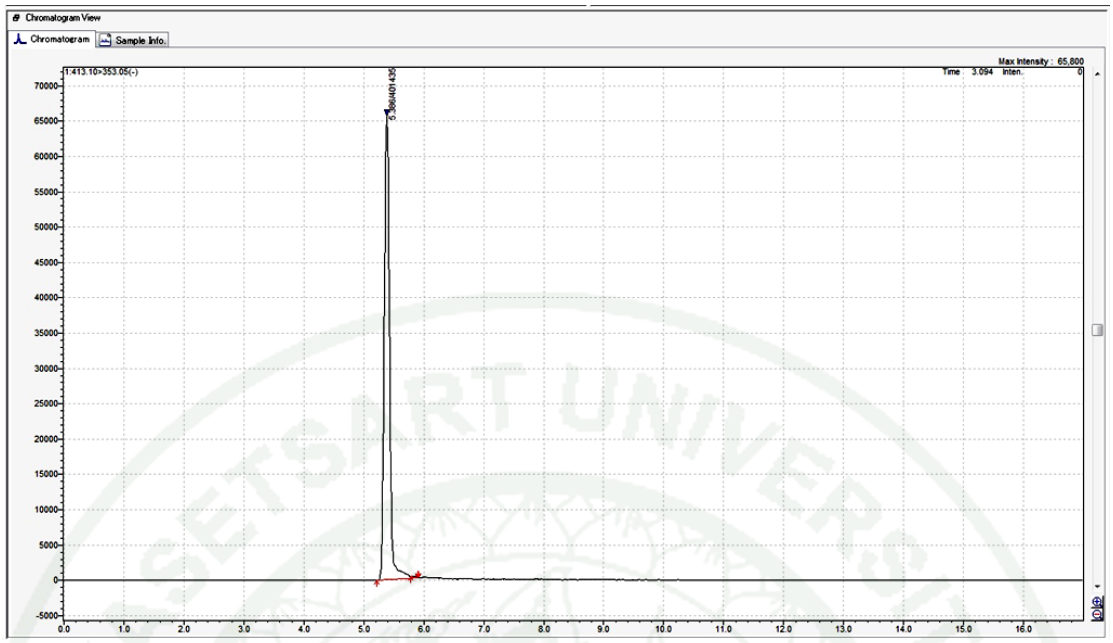
Times (mins)	% A	% B
0.00	100	0
0.30	95	5
2.00	5	95
15.00	5	95
15.01	95	5

Table 7 The molecular ions and fragments employed of FX and NIV

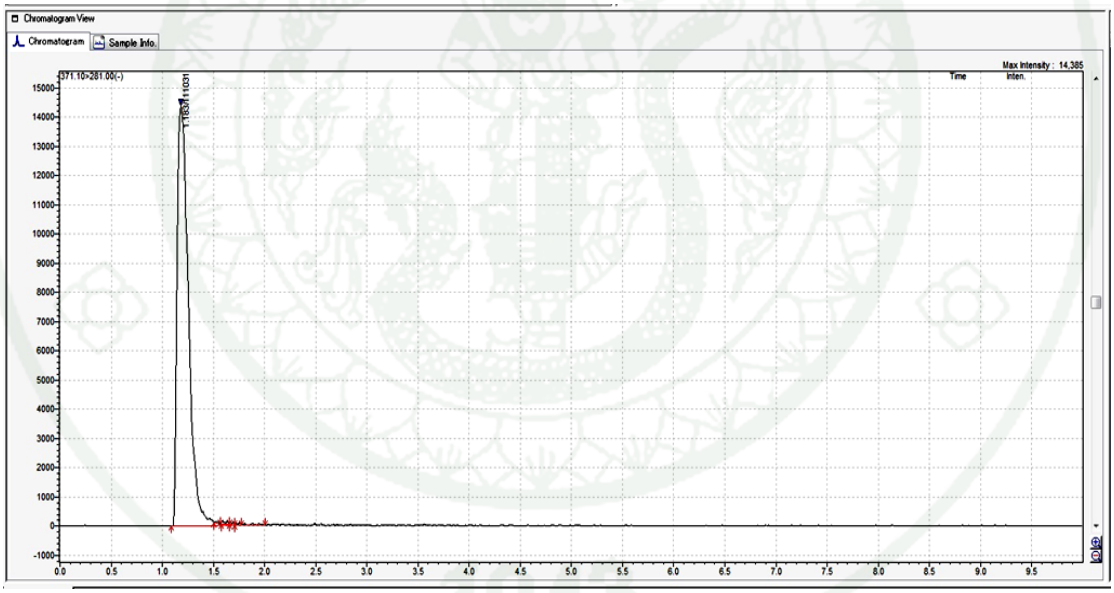
Compounds	Molecular weight (g/mol)	m/z	Collision Energy (CE) (eV)
FX	354.35		
Q1		413.1 to 355.05	10
Q3 (1)		413.1 to 59.15	25
Q3 (2)		413.1 to 262.95	20
NIV	312.32		
Q1		371.10 to 281.1	15
Q3		371.10 to 59.10	45

Table 8 The average recoveries of FX and NIV in plasma and various organs of piglets (±SD).

	FX (%)	**NIV (%)**
Plasma	99.41 ± 0.91	95.65 ± 2.99
Liver	90.16 ± 4.95	86.51 ± 1.71
Kidney	89.36 ± 3.82	86.21 ± 1.31
Spleen	85.99 ± 9.42	81.24 ± 2.10
Bile	85.44 ± 5.84	81.56 ± 3.03
Muscle	75.17 ± 5.22	73.95 ± 2.76
Intestine	75.34 ± 4.57	74.25 ± 1.80
Urine	97.93 ± 2.15	93.14 ± 0.35
Feces	66.46 ± 3.84	65.69 ± 1.01



a.) Fusarenon-X



b.) Nivalenol

Figure 9 Chromatogram of FX and NIV standard at 500 mg/ml.

12. Toxicokinetic parameter

The toxicokinetic characteristics of FX in piglets were described by two compartmental pharmacokinetic models using the Phoenix Win Nonlin® version 6.3 (Pharsight Corporation, St.Louis, MO, USA), where AUC was the area under the curve, K_{el} the elimination rate constant, $t_{1/2\beta}$ the elimination half-life, $t_{1/2\alpha}$ the distribution half-life, and Cl the body clearance, and K_{12}; K_{21} the micro-rate constants. The oral bioavailability (F) was calculated using the equation as follow**:**

$$(\%)\ F\ (po) = (AUC\ po) / (AUC\ iv) \times 100$$

13. Statistical Analysis

Plasma concentration curves of FX and NIV were shown as mean (±SD) of piglets. Pharmacokinetic parameters were shown as mean (±SD). The liver and kidney conversion of FX to NIV in mitochondrial fractions values of 3 piglets were shown as standard error of means (±SEM).

Place of works

The experiment were examined at Faculty of Veterinary Medicine, Kasetsart University, THAILAND and Laboratory of Toxicology, Department of Environmental Science, Graduate School of Veterinary Medicine, Hokkaido Univetsity, JAPAN

RESULTS AND DISCUSSIONS

Results

Toxicokinetic parameters

The determination of FX and NIV concentrations showed that FX and NIV were measurable in plasma of piglets following a single iv or po administration of FX at a dosage of 1 mg/kg bw. Figure 10a) showed the plasma concentration-time plot of FX and NIV in piglets following iv administration of FX. FX was detected up to 24 h whereas NIV was measurable up to 12 h after iv administration of FX. Fig 10b) showed the plasma concentration-time plot of FX and NIV in piglets following po administration of FX. FX was detected from 5 min to 48 h whereas NIV was measurable from 5 min to 12 h following po administration of FX. Peak plasma concentrations (C_{max}) of FX and NIV were 580.28 ± 140.81 ng/ml and 518.35 ± 60.83 ng/ml at 5 min, respectively, after po administration of FX. The limit of quantification (LOQ) of FX was 1.11 ng/ml. LC-MS/MS profile in plasma showed that a large proportion of FX was changed as NIV form. The FX disposition fit an open 2-compartment pharmacokinetic model. The values of elimination half life ($t_{1/2\beta}$), volume of distribution at steady state (V_{ss}), clearance (Cl) and oral bioavailability (F) were 1.706 ± 0.741 h, 0.009 ± 0.002 ml, 0.01 ± 0.01 ml/h and 74.402 ± 18.956%, following iv administration, respectively. Toxicokinetic parameters of FX in plasma after single iv administration were shown in table 9.

Tissue residues

LC-MS/MS profile in various tissues including liver, kidney, spleen, muscle and intestine showed that FX was also changed to its active metabolites, NIV. The maximum level of FX in liver, kidney, and spleen were 165.95 ± 9.68 ng/g, 66.29 ± 8.48 ng/g, and 7.35 ± 0.69 ng/g at 3 h after po administration of FX, while the maximum levels of NIV were 136.70 ± 27.11 ng/g, 62.00 ± 7.93 ng/g and 4.60 ± 1.31 ng/g at 6 h after po administration, respectively. The levels of FX and NIV in various tissues including liver, kidney, spleen, bile, muscle and intestine after FX administration were shown in table 10. Consistent with this, FX and NIV can be detected in the urine and feces up to 24 h and 48 h after iv and po administration of FX, respectively. The large proportions of NIV were also determined in urine after FX administration (Table 11).

Metabolism of FX to NIV *in vitro* study

To study the tissue capable of the conversion of FX to NIV in piglets, FX was incubated with liver and kidney S-9 fractions at 15, 30, 45 and 60 min, respectively. Then, the amount of NIV formed was determined. The result showed that FX to NIV conversion was noted clearly in the liver and kidney, the activities were shown 90.91% and 89.72% in liver and kidney at 60 min, respectively, after incubation. The data of the conversion of FX to NIV in piglets were shown in table 12.

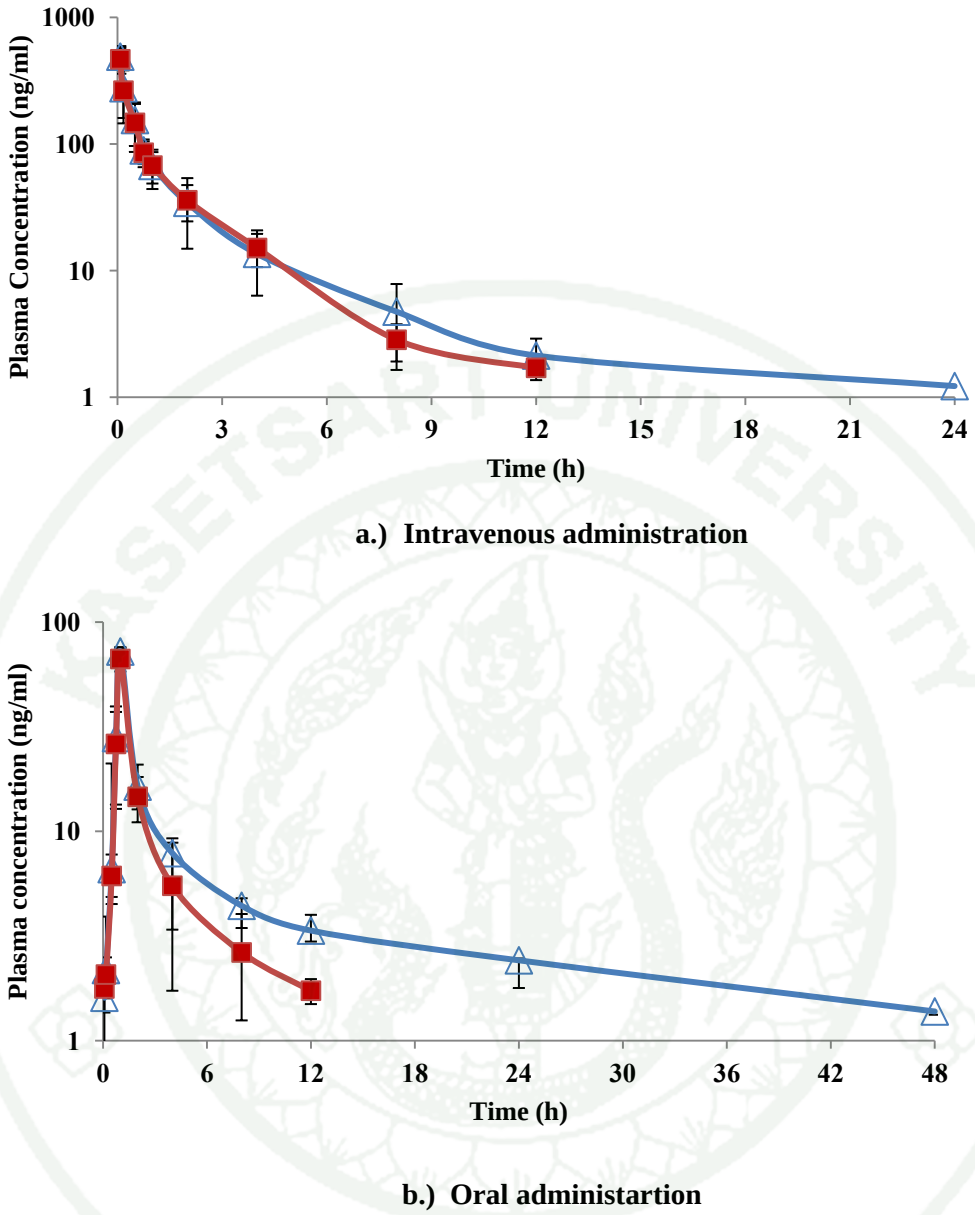


Figure 10 Mean values (±SD) of fusarenon-X (FX) (△) and nivalenol (NIV) (■) concentrations in plasma piglets at a dosage of 1 mg/kg bw of FX; a.) intravenous administration, b.) oral administration (n=5).

Table 9 Mean ± SD values of toxicokinetic parameters of FX following single intravenous administration at a dosage of 1 mg/kg bw of FX in piglets (n=5)

Toxicokinetic Parameters (units)	**FX**	**CV (%)**
K_{el} (h)	0.39 ± 0.10	14.74
K_{12} (1/h)	1.55 ± 0.94	36.32
K_{21} (1/h)	0.99 ± 0.52	42.71
AUC (h.ng/ml)	343.85 ± 35.75	10.39
$t_{1/2\alpha}$ (h)	0.19 ± 0.06	24.69
$t_{1/2\beta}$ (h)	1.71 ± 0.74	33.36
Cl (ml/h)	0.01 ± 0.01	10.41
Vss (ml)	0.01 ± 0.01	22.07
C_{max} (ng/ml)	580.28 ± 140.81	11.43
T_{max} (h)	0.98 ± 0.26	29.10
MRT (h)	1.71 ± 0.49	28.72
F_{oral} (%)	74.40 ± 18.96	

Table 10 The residue concentrations of FX and NIV in various organs following single oral administration at 1 mg/kg bw of FX in piglets

Time (h)	Liver		Kidney		Spleen		Muscle		Intestine	
	FX	NIV	FX	NIV	FX	NIV	FX	NIV	FX	NIV
1	23.26 ± 8.74	22.03 ± 8.27	3.68 ± 3.01	3.45 ± 2.82	2.95 ± 0.24	2.85 ± 0.10	3.22 ± 4.95	2.31 ± 1.33	3.22 ± 1.50	2.12 ± 1.33
3	165.95 ± 9.68	94.44 ± 3.73	66.29 ± 8.48	12.61 ± 5.22	7.35 ± 0.69	3.83 ± 0.31	5.82 ± 4.78	4.42 ± 1.35	12.61 ± 2.77	11.22 ± 2.46
6	103.10 ± 11.21	136.70 ± 27.11	32.50 ± 1.60	62.00 ± 7.93	5.66 ± 0.60	4.60 ± 1.31	11.56 ± 1.50	7.34 ± 1.79	19.86 ± 3.84	17.67 ± 3.42
12	33.03 ± 13.47	12.40 ± 1.77	7.93 ± 0.66	4.19 ± 2.52	3.98 ± 0.24	3.61 ± 0.42	20.91 ± 5.70	13.30 ± 2.51	7.94 ± 0.93	7.07 ± 0.83
24	3.52 ± 0.51	2.52 ± 0.47	1.49 ± 0.60	ND	2.01 ± 0.40	1.40 ± 0.24	1.61 ± 1.26	1.53 ± 0.21	1.61 ± 1.26	1.27 ± 0.42

ND = not detected

Table 11 The residue concentrations of FX and NIV in urine and feces after single oral administration at a dosage of 1 mg/kg bw of FX in piglets

Time (h)	Concentration (Mean±SD)							
	iv administration				po administration			
	Urine (ng/ml)		Feces (ng/g)		Urine (ng/ml)		Feces (ng/g)	
	FX	NIV	FX	NIV	FX	NIV	FX	NIV
1	84.87 ± 3.57	75.45 ± 8.88	11.48 ± 0.32	9.85 ± 0.08	9.34 ± 0.19	2.70 ± 0.30	8.81 ± 0.15	7.66 ± 0.14
4	21.56 ± 3.42	48.73 ± 3.50	20.72 ± 0.23	13.98 ± 0.83	84.71 ± 15.28	9.17 ± 2.63	11.69 ± 0.27	9.73 ± 0.87
8	6.72 ± 0.38	28.82 ± 0.12	33.13 ± 1.06	28.82 ± 0.93	16.95 ± 9.24	66.13 ± 3.78	30.56 ± 0.31	26.63 ± 0.20
12	3.29 ± 0.20	10.53 ± 2.02	9.37 ± 0.62	7.71 ± 0.10	9.26 ± 0.24	12.61 ± 0.37	8.05 ± 0.12	7.05 ± 0.04
24	1.81 ± 0.11	1.66 ± 0.04	2.81 ± 0.35	1.82 ± 0.08	4.33 ± 0.88	4.75 ± 0.43	3.71 ± 0.03	1.66 ± 0.08
48	ND	ND	ND	ND	1.25±0.03	1.57±0.03	1.79±0.28	1.34±0.06

ND = not detected

Table 12 The conversion rate of FX to NIV ratio in liver and kidney (n=3)

Time (min)	Concentration (ng/g)			
	Liver		Kidney	
	FX	NIV	FX	NIV
15	182.68 ± 7.63	48.65 ± 1.22	171.29 ± 1.01	48.55 ± 8.29
30	139.51 ± 26.06	78.49 ± 6.68	134.38 ± 0.78	85.28 ± 2.46
45	123.98 ± 17.80	132.99 ± 24.85	124.46 ± 1.89	123.82 ± 1.02
60	100.38 ± 1.80	174.14 ± 7.27	94.17 ± 0.40	157.83 ± 1.31
Conversion rate (%)	**90.91**		**89.73**	

Discussion

The present study used LC-MS/MS with electrospray ionization to determined FX and its active metabolite NIV in plasma, urine, feces and various tissues following iv or po administration in piglets. The toxicokinetic characteristics of FX in piglets were described by two compartmental pharmacokinetic models. The FX plasma level in piglets was detectable up to 24 and 48 h after iv and po administration, respectively. A large proportion of NIV was determined in plasma of piglets. The NIV was detected in plasma from 5 min to 12 h in orally given with FX, indicating FX was absorbed and metabolized rapidly. These results corresponded well with our previous research, showing FX was rapidly converted to NIV in plasma of mice, broilers and ducks (Poapolathep *et al*., 2003, 2008). The values of oral bioavailability, elimination half-life and body clearance of FX were 74.40 ± 18.96%, 1.71 ± 0.74 h and 0.01 ± 0.01 ml/h, respectively. The oral bioavailability of FX was higher in piglets than mice, broilers and ducks (Poapolathep *et al*., 2003, 2008). The elimination half-life of FX was also longer in piglets than in broilers but shorter than that in ducks (Poapolathep *et al*., 2008). The LC-MS/MS profile of urine and feces showed a large proportion of NIV after administration of FX in piglets. These finding clearly that FX is excreted almost in the NIV form in urine and feces of piglets. The results also corresponded well to our previous investigation of FX in mice, broilers and ducks showing that a large propotion of FX was changed as NIV following FX administration (Poapolathep *et al*., 2003, 2008). In addition, the FX and NIV in various tissue including liver, kidney, spleen, intestine and muscle were detectable from 1 h to 24 h after po administration of FX. It indicated that FX has the ability to distribute in the various tissues as well but in different levels.

The *in vitro* study of FX metabolism indicates that the liver and kidney are capable for the FX to NIV conversion in piglets. Consistent with this, the liver and kidney have also been noticed to be major organs for FX and NIV conversion *in vitro* in mice, rat rabbit, broiler and ducks (Ohta *et al*., 1978; Poapolathep *et al*., 2003, 2008).

CONCLUSION AND RECOMMENDATION

Conclusion

In conclusion, the study described here is the pharmacokinetic profiles and residue depletions of FX. FX and its metabolite to NIV were detectable in plasma, urine, feces and edible tissues of piglets after a single iv or po administration of FX at a dosage of 1 mg/kg bw. Based on our present study, indicating FX is efficiently absorbed from gastrointestinal tract and extensively penetrates to various tissues. The liver and kidney are the major organ responsible for the FX-to-NIV conversion. FX can be excreted mainly in urine as NIV form of piglets.

Recommendation

Due to limited information of toxicokinetics and residues of trichothecene mycotoxins in food producing animals, adequate information on disposition and depletion is also needed to establish the regulatory limit for human consumption and the ecological impact of mycotoxins in the environment. Therefore, data derived from this study is also useful for assuring food safety especially FX in swine. However, we recommended that toxicokinetics and residues of the other trichothecenes should be examined for assessing consumer health risk.

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APPENDIX

Appendix Table 1 Dosing sheet for piglets given with FX after oral administration.

Species	Piglets	**Breed**		**Sex**	Female
Sample size	5	**Route**	PO		

	R1	**R2**	**R3**	**R4**	**R5**
Weight	7.00	7.20	6.40	6.60	7.90
Dose (mg/kg)	1.00	1.00	1.00	1.00	1.00
Dosing	7.00	7.20	6.40	6.60	7.90
FX 4 mg/ml	1.75 cc.	1.80 cc.	1.60 cc.	1.65 cc.	1.97 cc.
	Time of blood collection				
0 min	11.14	11.17	11.30	11.33	12.07
5 min	11.19	11.22	11.35	11.38	12.12
10 min	11.24	11.27	11.40	11.43	12.17
30 min	11.44	11.47	12.00	12.03	12.37
45 min	11.59	12.02	12.15	12.18	12.52
1 h	12.14	12.17	12.30	12.33	13.07
2 h	13.14	13.17	13.30	13.33	14.07
4 h	15.14	15.17	15.30	15.33	16.07
8 h	19.14	19.17	19.30	19.33	20.07
12 h	23.14	23.17	23.30	23.33	24.07
24 h	11.14	11.17	11.30	11.33	12.07
48 h	11.14	11.17	11.30	11.33	12.07

Appendix Table 2 Dosing sheet for piglets given with FX after intravenous administration.

Species	Piglets	**Breed**		**Sex**	Female
Sample size	5	**Route**	IV		

	V1	**V2**	**V3**	**V4**	**V5**
Weight	9.00	7.50	6.70	8.30	7.50
Dose (mg/kg)	1.00	1.00	1.00	1.00	1.00
Dosing	9.00	7.50	6.70	8.30	7.50
FX 8 mg/ml	1.12 cc.	0.94 cc.	0.83 cc.	1.03 cc.	0.94 cc.
	Time of blood collection				
0 min	10.14	10.17	10.30	10.33	11.07
5 min	10.19	10.22	10.35	10.38	11.12
10 min	10.24	10.27	10.40	10.43	11.17
30 min	10.44	10.47	11.00	11.03	11.37
45 min	10.59	11.02	11.15	11.18	11.52
1 h	11.14	11.17	11.30	11.33	12.07
2 h	12.14	12.17	12.30	12.33	13.07
4 h	14.14	14.17	14.30	14.33	15.07
8 h	18.14	18.17	18.30	18.33	20.07
12 h	22.14	22.17	22.30	22.33	23.07

Extraction and Clean-up process

1. Thaw the frozen plasma , urine, feces and homogenized various tissues at 4°C.
2. 1 ml of plasma and urine, 5 g of feces and tissues were mixed carefully with 1 ml of a mixture of ACN:water (3:1).
3. Add 2 spoon of NH_3SO_4 then vortex 10 second.
4. Centrifuge the mixture at 4,301 X g for 15 min.
5. The upper fraction was collected.
6. For the lower fraction were repeated step 2-5 again without step 3 for 2 times.
7. C_{18} water cartridge was conditioned with 3 ml of ACN.
8. Passthrough the collected sample with glass syringe without vacuum.
9. Add 1 ml of ACN for wash the cartridge and the final step.
10. The elute were evaporate to dryness with nitrogen stream at 40°C.
11. The dry residue were kept in -20°C until analysis with LC-MS/MS.

Protocol for tissues homogenization

1. 5 g of tissues; liver, kidney, spleen, muscle and small intestine, were put in 50 ml centrifuge tube with sacrew cab.
2. Add 10 ml of a mixture of ACN:water (3:1).
3. Homogenized for fine minze.
4. Add 2 spoon of NH_3SO_4 then vortex 5 min.
5. Centrifuge the mixture at 4,301 X g for 15 min.
6. The upper fraction was collected.
7. For the lower fraction were repeated step 2-5 again without step 4 for 2 times.
8. C_{18} Water® cartridge was conditioned with 2 ml of ACN.
9. Passthrough the collected sample with glass syringe without vacuum.
10. Add 1 ml of ACN for wash the cartridge and the final step.
11. The elute were evaporate to dryness with nitrogen stream at 40°C.
12. The dry residue were kept in -20°C until analysis with LC-MS/MS.

Protocol for S-9 fraction collection

1. After collected from control group, the liver and kidney were washed with normal saline immediately.
2. The liver were flushed with 40 ml of normal saline via hepatic portal vein.
3. Put the liver and kidney in to liquid nitrogen immediately.
4. All liver and kidney were kept at -80°C.
5. 1 g of freezed organ were add with 1.5 ml of homogenizing buffer.
6. Homogenized with homogenizer for tender and keep the tempuratue under 4°C.
7. Centrifuge at 1,912 X g in 4°C for 15 min.
8. 500 µl of supernatant were collcted in to microtube and then kept in -80°C immediately until analysis.

Homogenizing buffer for S-9 fraction and kidney analysis

1. 0.25 M Sucrose (MW 342.3 g/mol)
2. 0.2 mM DTT hydrochloride (MW 154.25 g/mol)
3. 1 mM EDTA (MW 416.2 g/mol)
4. 10 mM Tris-HCl (MW 157.64 g/mol)

The preparation of standard stock solution for fortification procedure.

Stock solution = 1 mg/ml
= 1000 µg/1000 µl
= 1 µg/µl

1. Standard preparation for 1,000 ng and 500 ng (No.1)
    - 400 µl of master stock solution + 3,600 µl + PBS = 100 µg/ml

2. Standard preparation for 250 ng, 125 ng, and 50 ng (No.2)
    - 200 µl of stock solution from 1 + 1,800 µl of PBS = 10 µg/ml

3. Standard preparation for 10 ng and 5 ng (No.3)
    - 100 µl of stock solution from 2 + 900 µl of PBS = 1 µg/ml

Appendix Table A3 The volume of the standard solution for fortification procedure in sample**.**

Concentration	Sample		
	1 ml of plasma, urine, and bile	**5 g of various organs**	**2.5 g of spleen**
500 ng	5 µl (1)	25 µl (1)	12.5 µl (1)
250 ng	2.5 µl (2)	125 µl (2)	62.5 µl (2)
125 ng	12.5 µl (2)	62.5 µl (2)	31.25 µl (2)
50 ng	5 µl (2)	25 µl (2)	12.5 µl (2)
10 ng	10 µl (3)	50 µl (3)	25 µl (3)
5 ng	5 µl (3)	25 µl (3)	12.5 µl (3)

(1) = from stock solution no.1
(2) = from stock solution no.2
(3) = from stock solution no.3

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