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New insights into the human metabolism of the *Fusarium* mycotoxins deoxynivalenol and zearalenone☆

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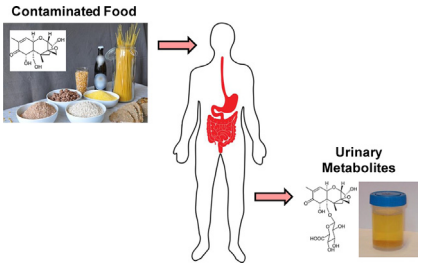
HIGHLIGHTS

- First detailed *in vivo* case study on human deoxynivalenol and zearalenone metabolism.
- Urinary excretion patterns were investigated over a period of eight days.
- A third deoxynivalenol-glucuronide isomer was tentatively identified for the first time in human urine.
- Fate of ingested masked DON forms in humans was preliminary investigated.
- Urinary excretion rate of total zearalenone was estimated.

GRAPHICAL ABSTRACT



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ABSTRACT

This study reports on the detailed investigation of human deoxynivalenol (DON) and zearalenone (ZEN) *in vivo* metabolism through the analysis of urine samples obtained from one volunteer following a naturally contaminated diet containing 138 μg DON and 10 μg ZEN over a period of four days. Based on the mycotoxin intake and the concentrations of mycotoxin conjugates in urine, a mass balance was established. The average rates of DON excretion and glucuronidation were determined to be 68 and 76%, respectively. The investigation of formed glucuronides revealed DON-15-glucuronide as main conjugation product besides DON-3-glucuronide. Furthermore, for the first time in human urine a third DON-glucuronide was detected and the fate of ingested masked DON forms (3-acetyl-DON and DON-3-glucoside) was preliminary assessed. The mean excretion rate of ZEN was determined to be 9.4%. ZEN was mainly present in its glucuronide form and in some samples ZEN-14-glucuronide was directly determined 3–10 h after exposure. For the first time concrete figures have become available for the excretion pattern of DON and ZEN-glucuronides throughout a day, the comparison of total DON in 24 h and first morning urine samples and the urinary excretion rate of total ZEN in humans following exposure through naturally contaminated food. Therefore, valuable preliminary information has been obtained through the chosen experimental approach although the study involved only one single individual and needs to be

Abbreviations: 3ADON, 3-acetyl-deoxynivalenol; α/β-ZEL, α/β-zearalenole; α/β-ZEL-GlcA, α/β-zearalenole-glucuronide; DON, deoxynivalenol; DON-3-Glc, deoxynivalenol-3-glucoside; DON-3-GlcA, deoxynivalenol-3-glucuronide; DON-15-GlcA, deoxynivalenol-15-glucuronide; DON-GlcA, deoxynivalenol-glucuronide; JECFA, Joint FAO/WHO Expert Committee on Food Additives; SCF, Scientific Committee on Food; TDI, tolerable daily intake; ZEN, zearalenone; ZEN-14-GlcA, zearalenone-14-glucuronide; ZEN-GlcA, zearalenone-glucuronide.

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confirmed in larger monitoring studies. The presented experiment contributes to a better understanding of human DON and ZEN *in vivo* metabolism and thereby supports advanced exposure and risk assessment to increase food safety and examine the relationship between these mycotoxins and potentially associated chronic diseases in the future.

1. Introduction

The mycotoxins deoxynivalenol (DON, vomitoxin) and zearalenone (ZEN) are frequent contaminants of grains and cereal products thus representing an important threat to food safety (CAST, 2003). Produced by various *Fusarium* species predominantly pre harvest, they occur worldwide. Hence, dietary exposure through the consumption of contaminated food is frequent in many populations. The trichothecene DON inhibits protein synthesis and modulates immune responses resulting in acute toxicity with symptoms including vomiting, nausea and diarrhea in humans while chronic effects still remain unclear. Toxicological effects and diseases associated with DON exposure were reviewed recently (Pestka, 2010a,b; Turner et al., 2012). However, epidemiological studies are required to critically investigate a potential relationship between the consumption of high DON quantities and the incidence of gastroenteritis and potential chronic diseases (Pestka, 2010a). Zearalenone (ZEN) and its metabolites exhibit potent estrogenic activity, hence it is often referred to as a mycoestrogen. ZEN is implicated in reproductive disorders of farm animals and occasionally in hypoestrogenic syndromes in humans (Zinedine et al., 2007). In addition, it is suspected as a triggering factor for central precocious puberty development in girls (Massart et al., 2008).

Due to their toxic potential, regulatory limits were introduced for both mycotoxins in many countries, including the European Union enforcing the most rigorous policy (European Commission, 2006). In addition, detailed risk assessments for DON as well as for ZEN were carried out by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) resulting in a provisional maximum tolerable daily intake (PMTDI) of 1.0 μg DON and 0.5 μg ZEN per kg bodyweight (FAO/WHO, 2000, 2001). In 2010, the JECFA updated its evaluation for DON and concluded to include its acetylated forms 3-acetyl-deoxynivalenol (3ADON) and 15-acetyl-deoxynivalenol (15ADON) to define the proposed value as a group PMTDI (FAO/WHO, 2010). Due to lack of data DON-3-glucoside (DON-3-Glc) was not included in this calculation so far, although recent studies in rats and *in vitro* indicated the possibility that these plant metabolites might be hydrolysed to the precursor toxin in humans, contributing to overall toxicity (Berthiller et al., 2011; Nagl et al., 2012). The European Scientific Committee on Food (SCF) performed a risk assessment on ZEN and concluded a temporary TDI of 0.2 μg/kg bodyweight (SCF, 2000). These TDI values have been an important basis for the current mycotoxin legislation established in the European Union which are designed to protect consumers to exceed the TDI.

Human DON and ZEN metabolism was rarely investigated in the past, mainly due to very low concentrations that occur in biological fluids following exposure *via* contaminated food. Extensive studies on the excretion profiles of DON in different animal species were conducted in the 1980's. They revealed the ubiquitous formation of DON-glucuronides (DON-GlcA) by indirect methods and a significant difference in urinary excretion and glucuronidation between species (Côté et al., 1986; Lake et al., 1987; Prelusky et al., 1986). This species dependent variation was recently confirmed by an *in vitro* study investigating the hepatic metabolism of human and six animal liver microsome mixtures (Maul et al., 2012). However, the first investigation of the human DON excretion pattern was performed in 2003, when total DON was proposed as a biomarker of exposure in urine after enzymatic hydrolysis using β-glucuronidase (Meky et al., 2003). The developed indirect method was applied in various DON exposure studies (reviewed by Turner, 2010 and Turner et al., 2012) and additionally used to examine urinary metabolite profiles in 34 UK adults (Turner et al., 2011). Urine samples previously analyzed for total DON after enzymatic hydrolysis were re-measured without this treatment to indirectly determine the amount of DON-glucuronide to be approximately 91% (range 85–98%) of total DON. Furthermore, total urinary DON (sum of free DON + DON-GlcA) was validated as a biomarker of exposure with an average urinary excretion rate of 72% (Turner et al., 2010). Recently, our group established an LC–MS/MS based method to directly quantify DON-GlcA in human urine using a chemically synthesized, NMR confirmed DON-3-glucuronide (DON-3-GlcA) reference standard (Warth et al., 2011). Within the course of a pilot study to investigate DON exposure toward Austrian adults, we detected a second DON-glucuronide, which was tentatively identified as DON-15-GlcA. These results were opposed to a previous work, which only could detect one DON-glucuronide in human urine by MS/MS experiments, which were based on theoretical masses (Lattanzio et al., 2011). In the Austrian study, the newly identified metabolite DON-15-GlcA was shown to be the predominant conjugate, accounting for approximately 75% of total DON-glucuronide. The average glucuronidation rate was determined to be 86% (range 79–95%) (Warth et al., 2012a). Fecal excretion of DON, mainly as its detoxified metabolite deepoxy-DON, was reported in cow, sheep, pig and rat (Côté et al., 1986; Prelusky et al., 1986; Eriksen et al., 2003; Nagl et al., 2012) in varying importance but not investigated in humans so far to the best of our knowledge. However, due to an average urinary excretion rate of 72% (Turner et al., 2010) it can be derived that excretion *via* feces is not the main route in humans. Two recent *in vitro* studies examined the metabolism of DON and its plant metabolite DON-3-Glc by the human fecal microbiota and found that DON can be released from its glycosylated form (Dall'Erta et al., 2013; Gratz et al., 2013).

Zearalenone metabolism was studied in various animals, especially in pigs as they are particularly sensitive to associated adverse effects such as decreased fertility. Biotransformation takes place in two major pathways: Hydroxylation forms the phase-I-metabolites α-zearalenol (α-ZEL) and β-zearalenol (β-ZEL), while conjugation of ZEN and its reduced forms with glucuronic acid and sulfate leads to the formation of typical phase-II-conjugation products. This was recently also confirmed in Caco-2 cells, which represent a widely accepted *in vitro* system for human intestinal absorption and metabolism (Pfeiffer et al., 2011). Comprehensive reviews were published by the JECFA committee (FAO/WHO, 2001) and by Metzler et al. (Metzler et al., 2010). In the latter, the authors point at the lack of pharmacokinetic data of ZEN in humans. Knowledge on zearalenone *in vivo* metabolism is based on a single experiment from 1981, where the metabolite pattern in 24 h urine was analyzed following ingestion of 100 mg ZEN at once by a male volunteer (Mirocha et al., 1981). Zearalenone-glucuronide (ZEN-GlcA) and α-ZEL-GlcA were the main metabolites, besides a minor amount of β-ZEL-GlcA was excreted. All analytes were determined after enzymatic hydrolysis and neither free nor sulfated metabolites were detected. Using the concentrations of the urinary metabolites, it can be estimated that about 10–20% of the ZEN dose, was recovered in