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Metabolism of the masked mycotoxin deoxynivalenol-3-glucoside in rats

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HIGHLIGHTS

- The metabolism of deoxynivalenol-3-glucoside (D3G) in rats was studied.
- Urine and feces were analyzed by a validated LC–MS/MS biomarker method.
- D3G was readily hydrolyzed to deoxynivalenol (DON) during digestion.
- Most D3G was metabolized by the gut microbiota and recovered in feces.
- D3G is of considerably lower toxicological relevance than DON, at least in rats.

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ABSTRACT

Deoxynivalenol-3-β-D-glucoside (D3G), a plant metabolite of the *Fusarium* mycotoxin deoxynivalenol (DON), might be hydrolyzed in the digestive tract of mammals, thus contributing to the total dietary DON exposure of individuals. Yet, D3G has not been considered in regulatory limits set for DON for foodstuffs due to the lack of *in vivo* data. The aim of our study was to evaluate whether D3G is reactivated *in vivo* by investigation of its metabolism in rats. Six Sprague-Dawley rats received water, DON (2.0 mg/kg body weight (b.w.)) and the equimolar amount of D3G (3.1 mg/kg b.w.) by gavage on day 1, 8 and 15, respectively. Urine and feces were collected for 48 h and analyzed for D3G, DON, deoxynivalenol-glucuronide (DON-GlcA) and de-epoxy deoxynivalenol (DOM-1) by a validated LC–tandem mass spectrometry (MS/MS) based biomarker method. After administration of D3G, only 3.7 ± 0.7% of the given dose were found in urine in the form of analyzed analytes, compared to 14.9 ± 5.0% after administration of DON, and only 0.3 ± 0.1% were detected in the form of urinary D3G. The majority of administered D3G was recovered as DON and DOM-1 in feces. These results suggest that D3G is little bioavailable, hydrolyzed to DON during digestion, and partially converted to DOM-1 and DON-GlcA prior to excretion. Our data indicate that D3G is of considerably lower toxicological relevance than DON, at least in rats.

1. Introduction

The mycotoxin deoxynivalenol (DON), a secondary metabolite of several *Fusarium* species, is one of the most important mycotoxins in cereal crops worldwide, and the most frequently occurring type B trichothecene in Europe (SCOOP, 2003). DON inhibits protein synthesis and modulates immune responses (reviewed by Pestka, 2010). In animals, toxicity symptoms include feed refusal, vomiting and growth depression (summarized by Pestka, 2007). Furthermore, DON causes inhibition of germination and growth retardation in plants (reviewed by Rocha et al., 2005). However, plants can metabolize DON to a variable extend through enzymatic conjugation to glucose (Berthiller et al., 2009b; Lemmens

Abbreviations: D3G, deoxynivalenol-3-β-D-glucoside; DON, deoxynivalenol; JECFA, Joint FAO/WHO Expert Committee on Food Additives; DOM-1, de-epoxy deoxynivalenol; DON-GlcA, deoxynivalenol-glucuronide; DOM-1-GlcA, DOM-1-glucuronide; b.w., body weight; SPE, solid phase extraction; MeOH, methanol; ACN, acetonitrile; HPLC, high performance liquid chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry; SRM, selected reaction monitoring; DP, declustering potential; CE, collision energy; R_A, apparent recovery; SSE, signal suppression/enhancement; R_E, recovery of the extraction step; LOD, limit of detection; LOQ, limit of quantification; Z14G, zearalenone-14-β-D-glucoside.

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et al., 2005; Poppenberger et al., 2003). The resulting "masked" mycotoxin deoxynivalenol-3-β-D-glucoside (D3G) affects protein biosynthesis to a far lower extent than DON *in vitro* and is therefore regarded as a detoxification product of DON in plants (Poppenberger et al., 2003).

D3G was first detected in naturally contaminated wheat and maize in 2005 (Berthiller et al., 2005). Since then, the worldwide occurrence of D3G in different cereal crops has been reported (Berthiller et al., 2009a; De Boevre et al., 2012; Desmarchelier and Seefelder, 2011; Li et al., 2011; Sasanya et al., 2008). The molar percentages of D3G/DON varied strongly in these studies, but reached maximum levels of 46% (Berthiller et al., 2009a). This percentage may increase in the future as a consequence of plant breeding efforts to enhance *Fusarium* head blight resistance by introgression of resistance loci (Lemmens et al., 2005). Considerable amounts of D3G were found in foodstuffs such as breakfast cereals, snacks and beers (Kostelanska et al., 2009; Malachova et al., 2011). Despite its frequent occurrence, the toxicological relevance of D3G in humans and animals has not yet been evaluated. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) stressed the possibility that D3G is hydrolyzed in the digestive tract of mammals (JECFA, 2011). Although this assumption is not yet supported by *in vivo* data, a recent study showed that certain intestinal bacteria are capable of cleaving D3G to DON *in vitro* (Berthiller et al., 2011).

Numerous studies have examined the toxicokinetics of DON *in vivo*, revealing two major metabolic pathways: de-epoxidation by anaerobic bacteria and conjugation to glucuronic acid. De-epoxy deoxynivalenol (DOM-1), which is at least 50-fold less toxic than DON (Sundstøl Eriksen et al., 2004), is formed by anaerobic ruminal or intestinal microbes (summarized by Zhou et al., 2008). DOM-1 can be excreted *via* the feces or it can be absorbed and detected in different biological samples of animals, like urine, plasma (reviewed by Rotter et al., 1996), and milk (Seeling et al., 2006). The ability to detoxify DON to DOM-1 in the upper gastrointestinal tract is considered a major cause for the differences regarding the susceptibility to DON among species (Pestka, 2007; Rotter et al., 1996).

The main metabolic pathway of mammals to detoxify resorbed DON is glucuronidation, a phase II reaction which reflects one of the most important mechanisms to inactivate xenobiotics by enhancing their polarity and excretability. Studies in different animal species showed that deoxynivalenol-glucuronide (DON-GlcA) is the major DON metabolite in plasma and urine (summarized by Wu et al., 2007). In humans, the measurement of urinary DON and DON-GlcA levels has been used successfully as a biomarker to assess DON exposure (Turner et al., 2011; Warth et al., 2012a). In addition, the formation of DOM-1-glucuronide (DOM-1-GlcA) in urine of rats has recently been reported (Lattanzio et al., 2011).

The presence of characteristic metabolites in urine and in feces allows conclusions regarding the absorption and metabolism of mycotoxins (Galtier, 1998). Studies determining the total recovery of orally administered DON in excreta of rats have been performed as early as in the 1980s (Lake et al., 1987; Worrell et al., 1989; Yoshizawa et al., 1983). Depending on whether DON was applied in its pure form or as a radiolabeled compound, observed recoveries ranged from around 15 to 89% of the applied toxin dose, respectively.

D3G has so far not been considered in the regulatory limits for cereal-based food established by the European Commission for DON (European Commission, 2006). Yet, JECFA stated that D3G might be an important contributor to dietary DON exposure and emphasized the need of *in vivo* data concerning the absorption, distribution, metabolism and excretion (ADME) in order to evaluate the potential health risk of D3G (JECFA, 2011).

The aim of the present study was to determine the fate of orally administered D3G in rats and to compare it with the pattern of DON metabolism. To this end, urine and feces of D3G and DON treated rats were analyzed for D3G, DON, DON-GlcA and DOM-1 by a validated LC–tandem mass spectrometry (MS/MS) based biomarker method. This study provides the first insight into the metabolism and excretion of D3G *in vivo*, thus contributing to the risk assessment of this masked mycotoxin.

2. Materials and methods

2.1. Chemicals and standard solutions

Methanol (MeOH), acetonitrile (ACN) (both LC grade) and glacial acetic acid (p.a.) were purchased from VWR International GmbH (Vienna, Austria). Water was purified with a with a Purelab Ultra system (ELGA LabWater, Celle, Germany).

DON and DOM-1 standards were obtained from Romer Labs GmbH (Tulln, Austria). D3G was previously purified from DON treated wheat plants (Berthiller et al., 2005) and DON-3-GlcA was chemically synthesized according to the method developed by Fruhmann et al. (2012). For use as analytical standards, solid compounds (DON, D3G, and DON-3-GlcA) were dissolved in ACN. A mixed stock solution, containing 100 μg/mL DON, D3G, DOM-1 and DON-GlcA, was prepared in ACN and stored at −20 °C. Further dilutions for spiking experiments and liquid standards were prepared in MeOH/water (20/80, v/v; feces samples) and ACN/water (10/90, v/v; urine samples).

2.2. Animals and study design

Male Sprague-Dawley rats were obtained from the breeding facility of the Medical University of Vienna (Himberg, Austria) and allowed to acclimatize for one week. The rats (5 months old, 250–280 g body weight (b.w.)) were housed individually in polycarbonate cages (Tecniplast, Hohenpeißenberg, Germany) under controlled conditions (24 ± 1 °C, humidity 50 ± 5%, 12 h light/dark cycle). Pelleted feed (R/M-H, Ssniff, Soest, Germany) and water were provided *ad libitum*. The rodent diet was analyzed for its concentrations of DON and D3G before the start of the experiment.

Using a repeated measures design, the rats ($n = 6$) received water, DON (2.0 mg/kg b.w.) and the equimolar amount (6.8 μmol/kg b.w.) of D3G (3.1 mg/kg b.w.) by gavage on days 1, 8 and 15 of the experiment, respectively. Stock solutions of 400 μg/mL DON and 619 μg/mL D3G were prepared by dissolving the solid standards in water. Thereof, volumes of 1.4–1.8 mL were administered to the rats according to their weight. Feed was withdrawn 12 h before the treatment. After administration, the animals were housed individually in polycarbonate metabolic cages (Tecniplast, Hohenpeißenberg, Germany) for 48 h. Urine and feces were collected for the periods 0–24 h and 24–48 h after dosing and volumetrically measured or weighted, respectively. The samples were frozen at −20 °C at the end of the 48 h period.

The study design was approved by both, the Ethics Committee of the Medical University of Vienna and the Austrian Ministry for Science and Research.

2.3. Sample preparation

Urine samples were centrifuged (10 min, 14,000 × *g*), acidified with 1% of acetic acid and cleaned up by solid phase extraction (SPE) on Strata C18-T cartridges (200 mg, Phenomenex, Aschaffenburg, Germany). After conditioning of the cartridges with 5 mL of MeOH and 5 mL of MeOH/water/acetic acid (5/94/1, v/v/v), 500 μL of urine samples containing 1% of acetic acid were applied. Subsequently, the cartridges were washed with 1 mL of MeOH/water/acetic acid (5/94/1, v/v/v). The analytes were eluted with 2 mL of MeOH/water/acetic acid (70/29/1, v/v/v) and the eluates were evaporated to dryness under compressed air. The residues were reconstituted in 5 mL of ACN/water (20/80, v/v) for LC–MS/MS analysis.

Feces samples were freeze-dried, homogenized and 250 mg aliquots were extracted three times (40/40/20 min) with MeOH/water (50/50, v/v, 3/2/2 mL) on a GFL rotary shaker (Burgwedel, Germany). 500 μL aliquots of the pooled raw extracts were combined with 500 μL cold MeOH. Subsequently, the solutions were vortexed for 15 s and centrifuged at 9000 × *g* for 10 min. Finally, 300 μL of the supernatants was evaporated to dryness under compressed air and re-dissolved in 300 μL of MeOH/water (20/80, v/v). The samples were vortexed for 30 s and clarified by centrifugation (10 min, 14,000 × *g*) for LC–MS/MS analysis.

Clean-up procedures for feces and urine as described above resulted in sample dilutions by a factor of 56 and 10, respectively.

2.4. HPLC–MS/MS parameters

Analysis was performed on an 1100 series high performance liquid chromatography (HPLC) system (Agilent Technologies, Waldbronn, Germany) coupled to a QTrap 4000 LC–MS/MS System (AB Sciex, Foster City, CA) equipped with a Turbo V electrospray ionization (ESI) source. Chromatographic separation was achieved on an Atlantis® T3 column (3.0 mm × 150 mm, 3 μm, Waters, Vienna, Austria) equipped with a 4 mm × 3 mm C_{18} security guard cartridge (Phenomenex, Torrance, CA, USA). Eluent A was composed of water/acetic acid (99.9/0.1, v/v) and eluent B of ACN/acetic acid (99.9/0.1, v/v). After an initial period of 2 min at 5% B, the proportion of B was