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Assessment of human deoxynivalenol exposure using an LC–MS/MS based biomarker method

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ABSTRACT

The *Fusarium* toxin deoxynivalenol (DON) is one of the most abundant mycotoxins worldwide and poses many adverse health effects to human and animals. Consequently, regulatory limits and a provisional maximum tolerable daily intake (PMTDI) for this important type B-trichothecene were assigned. We conducted a pilot survey to investigate the level of DON exposure in Austrian adults by measurements of DON and its glucuronide conjugates (DON-GlcA's), as biomarkers of exposure, in first morning urine. The average concentration of total DON (free DON + DON-GlcA's) was estimated to be $20.4 \pm 2.4\ \mu g\ L^{-1}$ (max. $63\ \mu g\ L^{-1}$). Surprisingly, we found that one third of the volunteers ($n = 27$) exceeded the established PMTDI when consuming regular diet. DON-GlcA's were directly quantified by LC–MS/MS and the results were compared with indirect quantification after enzymatic hydrolysis and confirmed the suitability of the direct method. Moreover, we investigated the *in vivo* metabolism of DON in humans and were able to determine two closely eluting DON-GlcA's in naturally contaminated urine samples for the first time. In contrast to previous findings we have tentatively identified DON-15-glucuronide as a major DON metabolite in human urine based on the analysis of these samples. About 75% of total glucuronides were derived from this metabolite while DON-3-glucuronide accounted for approximately 25%. The reported new findings clearly demonstrate the great potential of suitable biomarkers to critically assess exposure of humans and animals to DON.

1. Introduction

Deoxynivenol (DON) is a toxic secondary metabolite produced by several *Fusarium* species in various types of grain. It is the most frequent occurring type-B trichothecene and can be found world-wide. DON is also known under its synonym "vomitoxin" and has been associated with many different diseases in both, human and animals (reviewed by Pestka, 2010a). Acute exposure causes emesis, e.g. in pigs while chronic effects can lead to anorexia, growth retardation, immunotoxicity and interferences with reproduction and development. In humans, DON has been linked with gastroenteritis but chronic effects were not established so far. The primary mode of action of DON is inhibition of protein biosynthesis, indirectly also effecting DNA and RNA synthesis, inflammatory responses (ribotic stress response) and neurological processes (Pestka, 2010a,b; WHO, 2001). Thus, DON is an issue concerning public health and regulations have been introduced in many countries. In the European Union the following concentrations were assigned as maximum levels in 2005: unprocessed durum wheat, maize and oats $1750\ \mu g\ kg^{-1}$, other unprocessed cereals $1250\ \mu g\ kg^{-1}$, cereal flour and pasta $750\ \mu g\ kg^{-1}$, bread, pastries and cereals $500\ \mu g\ kg^{-1}$ and processed infant foods $200\ \mu g\ kg^{-1}$ (European Commision, 2005). Furthermore, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) conducted a detailed risk assessment of DON and established a provisional maximum tolerable daily intake (PMTDI) of $1\ \mu g\ kg^{-1}$ bodyweight in 2001 (WHO, 2001). The Scientific Committee on Food (SCF) established the same limit which was referred to as full TDI (SCF,

Abbreviations: D3GlcA, deoxynivalenol-3-glucuronide; D15GlcA, deoxynivalenol-15-glucuronide; DON, deoxynivalenol; DON-GlcA, deoxynivalenol-glucuronide; DOM-1, de-epoxy deoxynivalenol; PMTDI, provisional maximum tolerable daily intake; JECFA, Joint FAO/WHO Expert Committee on Food Additives; SCOOP, Scientific Cooperation on Questions Relating to Food; ACN, acetonitrile; IS, internal standard; PBS, phosphate buffered saline; LC–MS/MS, liquid chromatography tandem mass spectrometry; ESI, electrospray ionization; EPI, enhanced product ion scan; SRM, selected reaction monitoring; CE, collision energy; DP, declustering potential.

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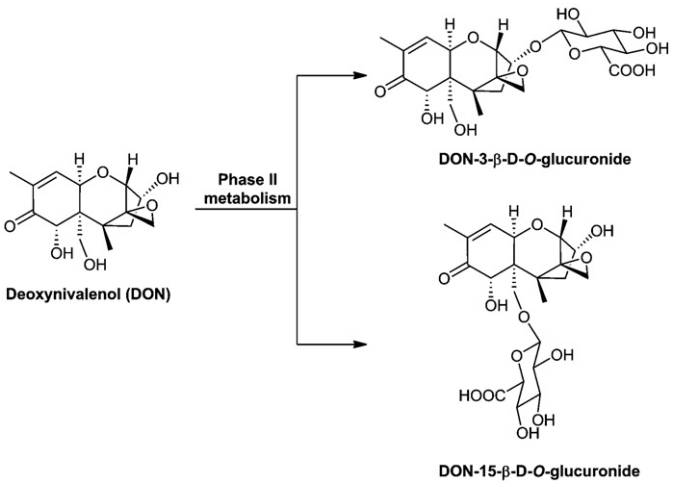

Fig. 1. Structure of DON and its glucuronide isomers.

2002). The JECFA Committee updated its evaluations recently and it was decided to transform the PMTDI to a group PMTDI of 1 μg kg^{-1} bodyweight to include the acetylated derivatives of DON (FAO/WHO, 2010). Its masked plant metabolite DON-3-glucoside was not included yet due to lack of data, although a recent study indicated its potential toxicological relevance for humans (Berthiller et al., 2011).

In a small scale human study about 91% (range 85–98%) of DON excreted via urine was conjugated with glucuronic acid to DON-glucuronide(s) (DON-GlcA's; Turner et al., 2011). However, it is not known to date which DON-GlcA isomer(s) are formed as a result of human phase II metabolism. We recently postulated that it is most likely that both, the C3-OH and the C15-OH groups are glucuronidated (Warth et al., 2011) to result in DON-3-glucuronide (D3GlcA) and deoxynivalenol-15-glucuronide (D15GlcA) as displayed in Fig. 1. Based on our recent study we assumed D3GlcA to be the main conjugation product of DON in humans at moderate exposure (Warth et al., 2011). This hypothesis was in line with the findings of Lattanzio et al. (2011), who reported only one DON-GlcA in human urine while two different glucuronides were detected in rat urine. However, those results were based on hypothetical masses of target molecules detected by LC–MS/MS without comparison to an authentic reference standard for unambiguous identification. A small quantity of one DON-GlcA was obtained using rat liver microsomes for toxicological evaluation (Wu et al., 2007). In this study the authors proposed the formation of D3GlcA by MS/MS fragmentation and demonstrated that the metabolite can be regarded as a detoxification product. Beside DON-GlcA also a de-epoxy metabolite (DOM-1), was described as a minor metabolite in the urine of French farmers (Turner et al., 2010a) although it has traditionally been regarded as a common metabolisation product in animals which is not formed in humans (Wu et al., 2010). Also, a current Italian study could detect DOM-1 in rat urine only (Lattanzio et al., 2011).

Since DON is rapidly metabolized to its glucuronide metabolite(s), the combined measurement of free DON + DON-GlcA was proposed as a suitable biomarker to effectively assess human DON exposure (Meky et al., 2003). Due to a lack of authentic reference standards, a method for DON determination in urine after enzymatic deconjugation with β-glucuronidase was developed (Meky et al., 2003) and later modified by inclusion of an internal standard and creatinine normalization (Turner et al., 2008a). Therefore, combined measurement of free DON and its DON-GlcA's was achieved and the method was applied in several studies (Turner et al., 2010a,b, 2011, 2008b, 2009). These studies revealed the power of biomarker driven work when compared to traditional exposure assessment by analyzing food stuff. Furthermore, it was predicted that a few percent of U.K. adults are likely to exceed the PMTDI with children having a greater likeliness of exceedance (Turner, 2010). Other European surveys also detected DON in human urine recently (Lattanzio et al., 2011; Rubert et al., 2011; Solfrizzo et al., 2011). However, in all these studies it was not possible to differentiate between different conjugates since an enzymatic hydrolysis with β-glucuronidase followed by a time consuming clean up was employed which only allowed for the determination of DON (free DON + DON hydrolyzed from DON-GlcA's). We recently synthesized an authentic, NMR confirmed, deoxynivalenol-3-*O*-glucuronide reference standard (D3GlcA, Fruhmann et al., in press). Subsequently, we developed a method for the direct quantification of D3GlcA in human urine without the need for a sample clean up by selective and sensitive determination by LC–MS/MS (Warth et al., 2011). In that preliminary study we did not detect DON but quantified DON-GlcA as D3GlcA in two human urine samples at concentrations >30 μg L^{-1} which resulted in an estimated total daily exposure close to the PMTDI value. Austria has already been identified as a country with relatively high average DON exposure with a mean daily intake of 0.294 μg kg^{-1} body weight and 1.037 μg kg^{-1} body weight for the high exposure group (SCOOP, 2003). However, this varies from season to season.

In this paper we report on the first pilot survey to examine DON exposure levels in Austrian individuals by measurements of relevant biomarkers in urine. The determined concentrations were subsequently correlated to the established PMTDI value. Additionally, DON metabolism in humans was studied, and D15GlcA was tentatively identified as a major metabolisation product of DON in humans for the first time.

2. Experimental

2.1. Chemicals and reagents

D3GlcA was synthesized by an optimized Königs-Knorr procedure and the structure of the product was confirmed by nuclear magnetic resonance (Fruhmann et al., in press). Other mycotoxin standards were purchased from Romer Labs Diagnostic GmbH Tulln, Austria (DON, U-[$^{13}C_{15}$]-DON, DOM-1). A combined multi standard working solution for preparation of calibrants and QC samples was prepared in ACN containing 10 mg L^{-1} DON, D3GlcA, DOM-1 and 1 mg L^{-1} of the internal standard U-[$^{13}C_{15}$]-DON. Methanol (LC gradient grade) and glacial acetic acid (p.a.) were purchased from Merck (Darmstadt, Germany), ACN (LC gradient grade) from VWR (Leuven, Belgium). Creatinine was from Sigma (Schnelldorf, Germany).

2.2. Samples and preparation

For the pilot survey 27 volunteers (age 20–63) from Vienna and the provinces of Lower Austria and Styria were asked to donate first morning urine and provide information about their weight. Furthermore, the diet of the previous day was recorded qualitatively to identify potential main sources of dietary DON such as bread, breakfast cereals, pasta, or beer. After collection, the samples were immediately frozen at −20 °C until analysis. Blank urine for quality control samples was obtained from a 26-year-old male who avoided the consumption of presumably DON contaminated food such as cereal based products for 5 days. Only first morning urine samples from day two to five were combined to yield a pooled blank urine sample, after verification of mycotoxin absence in the single samples. Written consent was obtained of all volunteers and the study was permitted by the ethics commission of the government of Lower Austria.

Urine samples were allowed to reach room temperature and centrifuged for 3 min at 5600 × *g*. 100 μL of the supernatant was mixed with 900 μL of dilution solvent (ACN/H_2O: 10/90, v/v). 5 μl of diluted sample were injected into the LC–MS/MS system. Enzymatic hydrolysis of the samples was performed using β-glucuronidase from *Escherichia coli* (Type IX-A, Sigma). 500 μL of a urine sample were mixed with 500 μL PBS buffer (75 mM, pH 7.4) containing 3000 units of β-glucuronidase and incubated for 18 h at 37 °C. Digested samples were centrifuged and 200 μL of the supernatant were diluted with 800 μL dilution solvent to result in a total dilution factor of 10 like untreated samples.