Food and Chemical Toxicology 62 (2013) 231–237

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Urinary analysis reveals high deoxynivalenol exposure in pregnant women from Croatia

Bojan Šarkanj [a,b,1], Benedikt Warth [b,1], Silvio Uhlig [c], Wilfred A. Abia [b,d,e], Michael Sulyok [b,*], Tomislav Klapec [a], Rudolf Krska [b], Ines Banjari [f]

[a] Subdepartment of Biochemistry and Toxicology, Department of Applied Chemistry and Ecology, Faculty of Food Technology, Josip Juraj Strossmayer University, Osijek, Croatia
[b] Center for Analytical Chemistry, Department for Agrobiotechnology (IFA-Tulln), University of Natural Resources and Life Sciences, Vienna (BOKU), Austria
[c] Section for Chemistry and Toxicology, Norwegian Veterinary Institute, Oslo, Norway
[d] Laboratory of Pharmacology and Toxicology, Department of Biochemistry, Faculty of Science, University of Yaounde I, Cameroon
[e] Department of Food Technology, Faculty of Science, University of Johannesburg, 2028, Doornfontein Campus, P.O. Box 17011, Gauteng, South Africa
[f] Subdepartment of Nutrition, Department of Food and Nutrition Research, Faculty of Food Technology, Josip Juraj Strossmayer University, Osijek, Croatia

ARTICLE INFO

Article history:
Received 24 May 2013
Accepted 20 August 2013
Available online 28 August 2013

Keywords:
Mycotoxins
Deoxynivalenol glucuronide
Biomarker
Pregnant women
Human urine
Croatia

ABSTRACT

In this pilot survey the levels of various mycotoxin biomarkers were determined in third trimester pregnant women from eastern Croatia. First void urine samples were collected and analysed using a "dilute and shoot" LC–ESI–MS/MS multi biomarker method. Deoxynivalenol (DON) and its metabolites: deoxynivalenol-15-glucuronide and deoxynivalenol-3-glucuronide were detected in 97.5% of the studied samples, partly at exceptionally high levels, while ochratoxin A was found in 10% of the samples. DON exposure was primarily reflected by the presence of deoxynivalenol-15-glucuronide with a mean concentration of 120 μg L^{-1}, while free DON was detected with a mean concentration of 18.3 μg L^{-1}. Several highly contaminated urine samples contained a third DON conjugate, tentatively identified as deoxynivalenol-7-glucuronide by MS/MS scans. The levels of urinary DON and its metabolites measured in this study are the highest ever reported, and 48% of subjects were estimated to exceed the provisional maximum tolerable daily intake (1 μg kg^{-1} b.w.).

1. Introduction

Mycotoxins are secondary metabolites produced by toxigenic fungi that commonly contaminate agricultural products worldwide. Based on frequency of occurrence and toxic effects exerted on animals, aflatoxins (AFs), ochratoxins (OTs), deoxynivalenol (DON), zearalenone (ZEN) and fumonisins (FBs) are the most relevant mycotoxins worldwide. Once humans are exposed to mycotoxins via contaminated food, singly and or in combinations, these toxins might pose multiple threats to human health such as teratogenicity, immunosuppression and carcinogenicity (Binder et al., 2007).

The trichothecene DON is the most frequently encountered mycotoxin addressed by regulation in Europe (Binder et al., 2007). Due to its stability during processing (Jackson and Bullerman, 1999), a high level of exposure in humans is expected. Krstanović et al. (2005) determined a high contamination of barley with DON-producing *Fusarium* species in Croatia, whilst Pleadin et al. (2012a) confirmed high DON contamination of Croatian maize samples harvested in 2010., 85% of the investigated samples ($n = 40$) were DON contaminated with an average concentration of 2150 μg kg^{-1} (range 15–17,290 μg kg^{-1}). Contamination of maize from the same harvest with T-2 toxin (T-2) and FB's has also been reported (Pleadin et al., 2012b). Of the analysed samples, 67.4% were contaminated with FBs with an average concentration of 4509 μg kg^{-1} and a maximum concentration of 25200 μg kg^{-1}; 24.4% of the samples were contaminated with T-2 with average and maximum levels of 110 μg kg^{-1} and 210 μg kg^{-1}, respectively. Since high levels of mycotoxins were detected in cereals, it is expected that the potential exposure of humans is reflected by the presence of appropriate urinary biomarkers. There is limited information on DON exposure and metabolism during pregnancy in humans. Piekkola et al. (2012) reported the co-occurrence of DON and aflatoxin M_1 (AFM_1) in urine form pregnant women in Egypt in 18.3% of the analysed samples. The range of reported DON in Egypt was 0.5–59.9 ng mg^{-1} creatinine, while Hepworth et al. (2011) reported urinary DON concentrations in pregnant individuals from Bradford, UK, with a mean urinary concentration of 10 ng mg^{-1} creatinine and a maximum concentration of 117 ng mg^{-1} creatinine (Turner et al., 2012a).

* Corresponding author. Address: Center for Analytical Chemistry, Department of Agrobiotechnology (IFA-Tulln), University of Natural Resources and Life Sciences, Vienna (BOKU), Konrad Lorenzstr. 20, A-3430 Tulln, Austria. Tel.: +43 2272 66280 409.
E-mail address: michael.sulyok@boku.ac.at (M. Sulyok).
[1] These authors contributed equally to this work.

0278-6915/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.fct.2013.08.043

DON has been shown to pass the placental barrier in sows, and it was linked with lower birth weight (Tiemann et al., 2008) and immunosuppression (Jakovac-Strajn et al., 2009). Due to its ability to cross the placental barrier during *in vivo* studies in animals (Goyarts et al., 2007; Tiemann et al., 2008), and *ex vivo* studies in humans (Nielsen et al., 2011), DON might cause toxicity in the foetus. Time periods of high cereal contamination with DON were associated with induced labour at an early stage of pregnancy (Pestka and Smolinski, 2005). Therefore, DON is assumed to constitute a hazard to both pregnant women and their foetus, especially since the foetus has a less developed detoxification capacity. However, it is not known how the observed and other anticipated changes in metabolism, distribution and excretion of toxicants during pregnancy affect the potential risk (Anderson, 2005). A major detoxification route for DON in animals and humans is via glucuronidation (Pestka and Smolinski, 2005; Maul et al., 2012; Meky et al., 2003; Turner et al., 2011, 2012a; Warth et al., 2011, 2012a, 2013a), though the specific uridine-diphosphate glucuronosyltransferases (UGTs) for DON have not as yet been identified. It has been proposed that during the last two trimesters of human pregnancy at least one specific UGT, UGT1A4, activity is increased. However, in rats glucuronidation was reduced for some substrates (Inoue et al., 2005) but alterations to efflux transporter expression resulting in increased urinary excretion of glucuronides (Cao et al., 2002).

A DON glucuronide was first suggested by Meky et al. (2003) and Turner et al. (2008), and subsequently both DON-3-GlcA and DON-15-GlcA have been identified and characterised in human urine and using human liver microsomes (Maul et al., 2012; Warth et al., 2012a). The structure of a suggested third species, DON-7-GlcA, in highly contaminated human urine samples (Warth et al., 2013a) and from microsomal assays (Maul et al., 2012), awaits further confirmation; whilst the structural elucidation of three DON glucuronides formed using rat liver microsomes included the DON-3-GlcA, DON-15-GlcA, and a novel DON-8-GlcA (Uhlig et al., 2013). Klapec et al. (2012) determined OTA and OTα in the first void urine samples of Croatian pregnant women, and according to food frequency questionnaire data, the greatest contributors to dietary OTA intake were cereal products and fruit juices. Samples from that survey were re-examined in the study at hand to assess multiple mycotoxin exposures using a newly developed multi-biomarker LC–MS/MS method (Warth et al. 2012b).

In humans, many mycotoxins and their metabolites are effectively excreted via the urine which enables the estimation of exposure through urinary concentrations (Solfrizzo et al., 2011; Warth et al., 2013b) provided that a dose–response relationship has been established. Following easy, non-invasive sampling, urine analysis requires sensitive methodology due to low levels. Considering the limited data on mycotoxin levels in pregnancy (Hepworth et al., 2011; Klapec et al., 2012; Piekkola et al., 2012), the aim of this pilot study was to investigate mycotoxin exposure in 40 healthy third trimester pregnant women from eastern Croatia. Special emphasis was given to the tentative identification of a recently discovered third DON glucuronide.

2. Materials and methods

2.1. Chemicals and reagents

Methanol (LC gradient grade) and glacial acetic acid (p.a.) were purchased from Merck (Darmstadt, Germany), acetonitrile (ACN; LC gradient grade) from VWR (Leuven, Belgium). Creatinine was from Sigma (Schnelldorf, Germany). Deoxynivalenol-3-*O*-glucuronide (DON-3-GlcA) and zearalenone-14-*O*-glucuronide (ZEN-14-GlcA) were synthesised by optimised procedures and the structures were confirmed by nuclear magnetic resonance (Fruhmann et al., 2012; Mikula et al., 2012). Deoxynivalenol-15-*O*-glucuronide (DON-15-GlcA) was separated from a naturally contaminated human urine sample to determine its MS response relative to that of DON-3-GlcA as described elsewhere (Warth et al., 2012a). Other mycotoxin standards were purchased from Romerlabs (Tulln, Austria) (DON, de-epoxy-deoxynivalenol (DOM-1), nivalenol (NIV), T-2, HT-2, OTA, AFM_1, FB_1 and FB_2) and Sigma (ZEN, α- and β-zearalenol (α- and β-ZEL)). Solid standards were dissolved in pure methanol (DON-3-GlcA, NIV) or ACN (DON, ZEN-14-GlcA, ZEN, α- and β-ZEL). Pre-dissolved standards were delivered in ACN or ACN/H_2O (FB_1 and FB_2) and stored at −20 °C. A combined multi-standard working solution containing 10.0 mg L^{-1} DON, DON-3-GlcA, DOM-1, NIV and HT-2, 5.0 mg L^{-1} FB_1 and FB_2, 2.5 mg L^{-1} ZEN-14-GlcA, α-ZEL, β-ZEL and T-2, 1.0 mg L^{-1} ZEN and 0.125 mg L^{-1} AFM_1 and OTA was prepared in ACN according to Warth et al. (2012b).

2.2. Participants and sample collection

During February 2011, 40 healthy non-smoking pregnant women who all reside in the eastern area of Croatia (from and around the city of Osijek; age: 26–33 years old), all in their final trimester of gestation, voluntarily participated in this study. Detailed description of the study design has been published before (Klapec et al., 2012) in a work focusing on urinary OTA and OTα. In order to expand the knowledge on the simultaneous exposure with other mycotoxins, especially to DON, which was assumed to be the main contaminant in Croatian cereals, additional analyses were performed at the Centre for Analytical Chemistry, (BOKU, Austria). The samples were kept at −20 °C and later transported in frozen condition to Austria.

2.3. Multi-mycotoxin biomarker analysis and LC–ESI–MS/MS parameters

2.3.1. Sample preparation

Urine samples were allowed to reach ambient temperature. Each urine sample was thoroughly mixed, 1 mL transferred into an Eppendorf tube and centrifuged for 3 min at 5600*g*. An aliquot of the supernatant (100 μL) was mixed with 900 μL of dilution solvent (ACN/H_2O = 10/90). Five microlitres (5 μL) of the diluted sample were injected into the LC–ESI–MS/MS system.

2.3.2. Analysis of urine samples & LC–ESI–MS/MS conditions

Sample analysis was performed using an AB Sciex QTrap® 5500 LC-MS/MS system (Foster City, CA) equipped with a TurboIonSpray electrospray ionization (ESI) source interfaced with an Agilent 1290 series HPLC system (Waldbronn, Germany). The optimised dilute and shoot approach described by Warth et al. (2012b) was applied for measurements of urinary biomarkers. This method

Table 1
Key performance parameters of the LC–MS/MS–ESI method applied for urine analysis (Warth et al., 2012b).

	LOD matrix [μg L^{-1}][a]	LOQ matrix [μg L^{-1}][b]
DON	4	13
DON-3-GlcA	6	20
DON-15-GlcA	3	11
OTA	0.05	0.17

[a] LOD based on a *S*/*N* ratio of 3:1 in spiked urine sample. Values correspond to concentration in urine, taking the 1:10 dilution into account.
[b] LOQ based on a *S*/*N* ratio of 10:1 in spiked urine sample. Values correspond to concentration in urine, taking the 1:10 dilution into account.