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Assessment of deoxynivalenol metabolite profiles in UK adults

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Abstract

Deoxynivalenol (DON) requires no activation for toxicity, though susceptibility may reflect individual variations in detoxification. This study reports the measurement of un-metabolised urinary DON (free DON) and DOM-1 in samples previously analysed for the combined measure of free DON+DON-glucuronide (fD+DG), with a concentration >5ng/ml, for 34 UK adults. Four consecutive daily urine samples were analyzed from twenty-two individuals, whilst from 12 individuals only a single sample was analysed. The mean (median) concentration of urinary fD+DG in this subset was 17.8ng/ml (13.8ng/ml), range 5.0–78.2ng/ml. In 23/34 (68%) individuals, free DON was detected, mean 2.4ng/ml; range 0.5–9.3ng/ml. Urinary DOM-1 was detected in 1/34 (3%) of individuals; present at ~1% of urinary fD+DG concentration for that individual. The concentration of fD+DG combined was significantly correlated with urinary free DON ($p<0.001$, $R^2=0.65$), but not with the percentage of free DON to fD+DG ($p=0.615$, $R^2=0.01$), suggesting that the level of DON exposure did not affect the metabolism to DG within the range observed. In this survey most individuals had no detectable urinary DOM-1 and 68% did not detoxify all of the ingested DON to DON-glucuronide. This study needs to be extended to understand whether the ratio provides a phenotypic measure of DON susceptibility.

Keywords

biomarker; deoxynivalenol; diet; metabolism; urine; UK

1. Introduction

Deoxynivalenol (DON) is a frequently occurring *Fusarium* mycotoxin, predominantly contaminating wheat, maize and barley in temperate regions of the world (CAST 2003). In animals DON has a myriad of proven toxicities, including feed refusal, decreased weight

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Declaration

Authors declare they have no competing financial interest

gain, gastroenteritis, cardiotoxicity, teratogenicity and immune toxicity (Amuzie and Pestka 2010; Pestka and Smolinski 2005; Rotter et al. 1996). Consumption of DON contaminated cereals has been associated with numerous poisoning incidents in China between 1961 and 1991 (Luo 1994), and a major incident in India (Bhat et al., 1989), affecting thousands of individuals. In these outbreaks typical symptoms were similar to those observed in animals, notably a rapid onset, nausea, vomiting, abdominal pain, diarrhea, headache, dizziness and fever. In one well documented incident DON contamination of wheat ranged between 0.3–92.8mg DON/kg (reviewed by Pestka and Smolinski 2005), suggesting that acute toxicity may occur at exposures estimated in the low $\mu\text{g/kg bw/day}$ range.

Given the common dietary exposure to DON, it is important to understand the potential health risks. Whilst dose is clearly critical in this respect, it is also relevant to study individual variations in xenobiotic metabolism, and thus the profile of activated and detoxification metabolites (Their et al., 2003). Such an understanding was central to the development and use of exposure biomarkers for aflatoxins (Groopman et al., 1993; Wild et al., 1992) in order to understand the role of the parent toxin in human disease (IARC 2002). For DON the research area is not as far advanced. Unlike the aflatoxins, DON is directly toxic via an epoxide moiety (Rotter et al., 1996). Thus the focus with DON is on the balance between the toxicity of the parent compound and any detoxification metabolites. In animals detoxification capacity varies by species, and one of the critical metabolic steps occurs prior to uptake from the intestinal tract, by non host enzymes (Pestka and Smolinski, 2005). DON can be metabolised within the intestinal lumen by gut microbiota, generating the less toxic de-epoxy metabolite known as DOM-1 (Swanson et al., 1988; Worrell et al., 1989; Sundstøl Eriksen et al., 2004). Thus a mixture of both DON and DOM-1 are absorbed from the gut (Pestka and Smolinski 2005). Further metabolism of DON to a less toxic metabolite involves addition of glucuronic acid, catalysed by UDP-glucuronyltransferase (Wu et al., 2007). The glucuronide-conjugated form of DON (DON-G) has been reported in most animal species, and in cows DOM-1-glucuronide (DOM-G) was additionally observed (reviewed by Pestka and Smolinski 2005). The organ site(s) of conjugation remain poorly defined, but certainly includes the liver and possibly the intestine and kidneys (Rotter et al., 1996, Goyarts and Danicke 2006).

Urinary DON was observed in a small series of urine samples from Chinese women (Meky et al., 2003), however, pre-incubation of the urine samples with β -glucuronidase, but not sulphatase, increased the amount of DON measured, indicating the presence of DON-G. The β -glucuronidase pre-treatment allows the combined measure of urinary un-metabolised or free DON and DON-G (fD+DG) in one step, and because it should better represent the total burden of DON intake, the combined measure was proposed as a biomarker of DON exposure. Following assay refinement by inclusion of $^{13}\text{C}_{15}$ -DON as internal standard and optimisation of β -glucuronidase assay conditions (Turner et al., 2008a), urinary fD+DG has frequently been observed in UK adults; levels have been correlated with both cereal intake (Turner et al., 2008b, 2009) and with estimates of dietary DON consumption (Turner et al., 2010a). The latter survey provided the first quantitative relationship between DON intake and the urinary measure.

To date the capacity to produce the DON detoxification metabolites in exposed people remains unexamined. In this study we assessed the frequency and amounts of urinary free DON in a subset of samples previously analysed for fD+DG combined (Turner et al., 2010a). Multiple urine samples over several days were available, providing a potential opportunity to assess the temporal relationship between urinary DON and its metabolites. In addition we tested all samples for the presence of urinary DOM-1.

2. Material and methods

2.1 Study design

The study design was described previously by Turner and colleagues (Turner et al., 2010a). Ethical approval was obtained from the Leeds Teaching Hospitals NHS Trust Research Ethics Committee. Informed consent from all individuals was obtained prior to initiating the study. In brief, 35 volunteers from the University of Leeds, UK, 17 male, 18 female, aged 21–59 years were recruited. First morning urines were collected from each individual over two weeks on Monday-Friday only of each week. Urine was stored frozen at -20°C prior to analysis. One hundred of the samples previously analysed for urinary fD+DG combined (Turner et al., 2010a) were selected to undergo a mock digestion (no β -glucuronidase) such that free DON only would be measured. In order to avoid a large number of non-detects (LOQ 0.5ng/ml) only sample with a concentration greater than 5ng/ml were selected to assess urinary free DON. In brief, the assay involved spiking the urine sample with an internal standard (IS) $^{13}\text{C}_{15}$ -DON (Sigma-Aldrich Ltd, Poole, Dorset, UK), a mock digestion step overnight at 37°C , ie no addition of β -glucuronidase, prior to immunoaffinity enrichment and quantification using LC-MS, as described in detail by Turner et al., (2008a, 2010a). Samples were extracted in batches of 20 with two blanks and two quality controls (QC) per batch. The blank was PBS spiked with the IS and the QC was a urine sample containing 10ng DON/ml urine. The limit of quantitation was 0.5ng DON/ml urine; no signal was detectable for the blank (extracted PBS) and the mean QC was 9.8ng/ml (SD 0.5; n=10). Urinary DOM-1 was additionally measured according to Turner et al., (2010b), including a blank in quadruplicate and two QC samples (4 and 20ng DOM-1/ml urine) in quadruplicate. DOM-1 was not detected in the blank, and the mean of the QC samples were 3.3ng/ml (SD 0.4ng/ml) and 18.2ng/ml (SD 0.9ng/ml), respectively. No IS was available for DOM-1. The limit of quantitation was 0.06ng DOM-1/ml urine.

2.2 Statistical analysis

The Student t-test was used to compare the levels of urinary fD+DG between samples positive for free DON and non detectable for free DON. Regression analysis was used to compare levels of urinary free DON with fD+DG, and the percentage free DON to fD+DG with fD+DG, using STATA version 9.0 (STATA Corp, Texas, USA).

3 Results

The original study measured urinary fD+DG combined in multiple samples from 35 individuals, n=348 samples (Turner et al., 2010a). The sub-set analysed here (n=100) was selected on the basis of samples where this combined measure was $>5\text{ng/ml}$ and included 22 individuals with four consecutive urine samples and 12 individuals with one sample. The mean (median) concentration of urinary fD+DG in this subset was 17.8ng/ml (13.8ng/ml), range 5.0–78.2ng/ml. DOM-1 was only quantified in two of 100 (2%) urine samples. Both were from the same male subject who provided four samples for this analysis; urinary fD+DG and DOM-1 levels were 57.9ng/ml and 0.8ng/ml, 61.8ng/ml and 0.5ng/ml, 11.6ng/ml and non detectable, and 24.5ng/ml and non detectable, for samples 1, 2, 3 and 4, respectively. Thus in the two positive samples urinary DOM-1 represented about 1% of the amount of urinary fD+DG. Samples from four other individuals had a trace signal that co-eluted with the DOM-1 standard but these could not be quantified.

In the absence of the β -glucuronidase digestion step, free DON was detected in at least one of the four samples for 15/22 (68%) of individuals who provide four samples for this analysis, and for 8/12 (66%) of the individuals who provided a single urine sample; overall free DON was observed in 11/16 (69%) males and 12/18 (67%) females. The mean

concentration of urinary fD+DG combined was higher for those samples where urinary free DON was detected (24.4ng/ml; 95%CI: 19.5, 29.2ng/ml, $p<0.001$) compared to the samples where urinary free DON was not detected (12.2ng/ml; 95%CI: 9.7, 14.7ng/ml). Urinary free DON was only detected in 11/49 samples where the urinary fD+DG was less than the median value for fD+DG, but in 35/51 samples where urinary fD+DG exceeded the median value. Among the samples positive for free DON, the mean concentration was 2.4ng/ml; range 0.5 – 9.3ng/ml.

For those individuals providing four urine samples, free DON was detected in 4/4, 3/4, 2/4, 1/4 and 0/4 samples, for three, five, three, five and six individuals, respectively. For each individual with a mixture of detectable and non detectable free DON, the mean (or single value) for urinary fD+DG in the samples with detectable free DON was always greater than the mean (or single value) for urinary fD+DG in samples without detectable free DON. For those individuals with two or more samples positive for free DON, there was little variation for a given individuals multiple samples in the percentage of free DON to fD+DG, (Figure 1). Because of the lack of variation in the percentage of free DON to fD+DG, both the mean free DON (or single value) and the percentage free DON to fD+DG were compared with the fD+DG concentration (Figure 2 and Figure 3, respectively). The concentration of free DON was significantly positively associated with fD+DG ($p<0.001$, $R^2=0.65$), whilst the percentage free DON to fD+DG was not significantly associated with fD+DG ($p=0.615$, $R^2=0.01$). For samples with detectable free DON the mean percentage free DON to fD+DG was 8.9% (range 1.8 to 15.5%); though this may be an slight overestimate based on a number of individuals having no free DON detected, but for whom the fD+DG concentration and the limit of quantitation would suggest that the percentage for these individuals would be less than the mean reported above.

4 Discussion

The toxicokinetics of DON are based predominantly on animal data (reviewed by Pestka and Smolinski, 2005). Species susceptibility in part reflects uptake capacity and metabolism to less toxic compounds. Metabolism of DON is somewhat unusual in that intestinal microbiota contribute significantly via the formation of DOM-1, which predominates in animals that are less sensitive to DON toxicity. In ruminants, only a small percentage of DON remains unmodified by intestinal microbiota (reviewed by Pestka and Smolinski, 2005), whilst up to 80% of the ingested DON is absorbed unmodified in swine (Prelusky et al., 1988; Rotter et al. 1996). Indeed DOM-1 and DOM-G predominate in biofluids obtained from dairy cows compared to the parent DON; ruminants are relatively resistant to DON toxicity, whilst swine are one of the more sensitive species (Rotter et al., 1996; Pestka and Smolinski 2005).

Data on DOM-1 formation in humans is limited. A lack of de-epoxidase activity in human fecal samples ($n=10$) suggested that humans may be sensitive to DON toxicity based on this parameter (Sundstol-Eriksen and Petterson 2003). In the current study urinary DOM-1 was only quantified for one male volunteer, and represented about 1% of the total urinary fD+DG; whilst a trace but non quantifiable peak was observed for samples from four additional individuals. The individual with a quantifiable measure for DOM-1 had a high average urinary fD+DG, and two of the two highest urinary measures (57.9ng/ml and 61.8ng/ml) in this survey. These data suggest that DOM-1 was not a major metabolite in these individuals or that DOM-1, if formed, was not significantly excreted via the urine. DOM-1 analysis involved measuring samples following treatment with β -glucuronidase thus this assay should capture both DOM-1 and a DOM-1-glucuronide, if formed in humans. The observations in this study support the data from a separate study of 22 UK adults who were positive for urinary fD+DG but for whom DOM-1 was non-detectable (Turner et al., 2010b).

By contrast, DOM-1 was detected in 26/76 (34%) French farmers who were positive for urinary fD+DG (Turner et al., 2010b). In the French survey DOM-1 concentrations were low (median 0.2ng/ml), and predominated in cattle handlers, perhaps suggesting modest acquisition of microbiota with de-epoxidase activity through occupation. Overall, however, these data suggest that DOM-1 does not represent a major detoxification metabolite in humans.

DON-G has been observed in the serum and urine of swine (Goyarts and Danicke, 2006), ruminants (Prelusky et al. 1985, 1986; Cote et al. 1986) and the urine of rodents (Meky et al. 2003). DON and DON-G excretion in the urine represented 37% of the intake in rats dosed at 5mg/Kg bw (Meky et al., 2003), and 50% and 61% of the ingested DON in swine at a high (163µg/kg bw/day) and a more moderate dose (4µg/kg bw/day) respectively (Goyarts and Danicke, 2006).

In the current study, one hundred samples with DON $\geq$ 5ng/ml were analysed for DON metabolites. Samples with lower total urinary DON were not selected in order to avoid generating negative data simply due to the proximity of the measure to the limit of detection. Urinary free DON was observed in 68% of the individuals, and in those positive samples it represented on average approximately 9% of the total combined urinary measure of free DON and DON-G. Therefore on average around 91% of total urinary DON was in the glucuronide-conjugated form on entering the bladder. These data suggest that the majority of DON in this series of individuals was converted to the glucuronide at some point from ingestion to urinary excretion.

In animal studies the percentage of free DON in urine tends to be higher than that reported here for humans. However, caution is needed in these comparisons of ratios because of differences in the level and patterns of DON exposure in the animals compared to the dietary studies in UK adults. In the former situation, saturation of pathways may occur at high levels of exposure. It was notable that for each individual where multiple urine samples were measured, that there was little variation in the percentage of free DON to fD+DG within any given individuals samples, despite a two to five fold variation in the combined measure (fD+DG) for some individuals. In addition, the percent of free DON was not correlated with the fD+DG concentration, data suggesting that the level of DON intake in the range observed does not influence the percent of DON conjugated to glucuronide. DON is predominantly cleared from swine within 48 hours of a single dose (Eriksen et al., 2003); however our observations in the UK indicate that multiple exposures are likely during each day (Turner et al., 2008a, 2008b, 2010a). Therefore as conjugation to the detoxification product DON-G is incomplete systemic circulation of free DON is predicted for a significant portion of the day; fespicially for those individuals with higher levels of DON intake.

In summary DOM-1 does not appear to be a major route of detoxification and thus no or very limited protection will be afforded by this pathway. By contrast DON-G does appear to be a major metabolite, though the toxicokinetics of formation in humans is not defined as yet. Un-conjugated DON did persist and was excreted in the urine for 68% of the group, and in this limited survey the percentage of conjugation appeared independent of the dose. It will be important to understand variations in the ability to detoxify DON both by deepoxidation to DOM-1 and conjugation to DON-G in a larger population, and to consider differences by age, ethnicity, and populations at risk of higher levels of exposure. Genetic variations in the UDP-glucuronyl transferases involved in the glucuronidation pathway for DON should also be investigated.

Abbreviations

CI	confidence interval
DOM-1	deepoxy deoxynivalenol
DOM-G	DOM-1 glucuronide
DON	deoxynivalenol
DON-G	DON-glucuronide
LC-MS	liquid chromatography-mass spectrometry
IS	internal standard
PBS	phosphate buffered saline
QC	quality control

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Figure 1. Mean and SD of percentage urinary free DON to fD+DG for 11 samples with multiple measures

Urinary DON only measured in the absence of β -glucuronidase, urinary fD+DG measured using a pre-incubation with β -glucuronidase to convert any DON-G to DON. Sample ID arbitrarily numbered 1–11.

Figure 2. Scatterplot of urinary free DON only against fD+DG

Where multiple samples were measured for the same individuals the mean free DON and fD +DG were used. Where single measures only were available these were used. Urinary DON and DON-G measured as described for Figure 1

Figure 3. Scatterplot of percentage urinary free DON to fD+D against fD+DG

Where multiple samples were measured for the same individuals the mean free DON and fD+DG were used. Where single measures only were available these were used. Urinary DON and DON-G measured as described for Figure 1