



Development of a urinary biomarker of human exposure to deoxynivalenol

F.A. Meky^a, P.C. Turner^a, A.E. Ashcroft^b, J.D. Miller^c, Y.-L. Qiao^d, M.J. Roth^e,
C.P. Wild^{a,*}

^aMolecular Epidemiology Unit, Epidemiology and Health Services Research, School of Medicine, University of Leeds, Leeds LS2 9JT, UK

^bDepartment of Biochemistry and Molecular Biology, University of Leeds, Leeds LS2 9JT, UK

^cDept of Chemistry, Carleton University, Ottawa, Ontario, Canada K1S 5B6

^dDepartment of Epidemiology, Cancer Institute, Chinese Academy of Medical Sciences, Beijing, 100021, China

^eCancer Prevention Studies Branch, Center for Cancer Research, NCI, Bethesda, MD 20892, USA

Accepted 7 January 2002

Abstract

Deoxynivalenol (DON) is a mycotoxin frequently found as a contaminant of cereal crops and may be etiologically associated with adverse health effects in developing countries where considerable quantities of contaminated crops are consumed. We investigated the metabolism of DON in rats as a basis to establish methodology for a candidate biomarker of human exposure to this toxin and tested this methodology on urine samples from a potentially highly exposed population. Sprague–Dawley rats received a single dose of [¹⁴C]DON (5.0±0.1 mg/kg body weight, 5.5±0.1 µCi/kg) and the distribution of DON in body fluids was investigated over 72 h. DON and its metabolites were detectable in the plasma of rats with the highest levels at 8 h, at which time approximately 9% was bound to plasma protein. A total of 37% of the administered DON was excreted in the urine and DON-glucuronide was implicated as the major urinary metabolite based on reverse-phase HPLC analysis of β-glucuronidase- and sulphatase-treated samples. An immunoaffinity column (IAC)–HPLC method was subsequently developed to measure urinary metabolites, with a view to establishing a urine-based human biomarker. Urine samples were collected from female inhabitants of Linxian County, China, a high risk region for oesophageal cancer (OC) and an area of potentially high DON exposure, and Gejiu, a low risk region in China. DON was detected in all 15 samples following β-glucuronidase treatment and IAC enrichment with the identity of DON being confirmed by mass spectrometry. The mean levels of DON from the suspected high and low exposure regions of China were 37 ng/ml (range 14–94 ng/ml) and 12 ng/ml (range 4–18 ng/ml), respectively. This is estimated to correspond to daily exposures of 1.1–7.4 µg/kg/day and 0.3–1.4 µg/kg/day, respectively. This is the first reported measurement of a urinary biomarker for DON in both animals and humans and should facilitate epidemiological studies of disease associations with this mycotoxin.

© 2003 Elsevier Science Ltd. All rights reserved.

Keywords: Deoxynivalenol; Biomarkers; Human exposure

1. Introduction

Deoxynivalenol (DON), a trichothecene mycotoxin produced by *Fusarium graminearum* and *F. culmorum* (WHO, 1990), is a frequent contaminant of cereal crops (wheat, barley, corn and oats) throughout the world

(Scott, 1989; IARC, 1993; Placinta et al., 1999). Owing to its stability to processing and cooking (Gilbert, 1989) human exposure levels can be high (Li et al., 1999). In animal models, DON causes feed refusal, decreased weight gain, cardiotoxicity and teratogenicity (Rotter et al., 1996; Prelusky, 1997). DON also induces IgA nephropathy in mice (Bondy and Pestka, 2000), though its role in the human form of this disease remains undetermined. DON has also been shown to affect cell mediated and humoral immunity in several animal species (Bondy and Pestka, 2000), and to suppress the proliferation of human lymphocytes in vitro at low concentrations (Meky et al., 2001).

Abbreviations: DON, deoxynivalenol; ESF, Ecolite scintillation fluid; IACs, immunoaffinity columns; OC, oesophageal cancer; PBS, phosphate buffered saline; SIR, Selected Ion Recording; TIC, Total Ion Chromatogram.

* Corresponding author. Tel.: +44-113-343-6601; fax: +44-113-343-6603.

E-mail address: c.p.wild@leeds.ac.uk (C.P. Wild).

Human toxicoses related to trichothecenes including DON have been reported in China, India, Japan and Korea (reviewed by Wild and Hall, 1996; Miller, 1998). These include several outbreaks of poisoning in China during a recent 30-year period (1960–1991), events that have been associated with the consumption of mouldy cereals. Analysis of a few of these cereal samples found high levels of DON (16 to 51,450 µg/kg), and lower levels of other trichothecenes such as nivalenol and T-2 toxin, and other mycotoxins, for example zearalenone and fumonisins (Li et al., 1999). In addition, a similar analysis of staple food samples found differences in the natural occurrence of mycotoxins, such as DON, between high and low risk oesophageal cancer (OC) regions in China, further suggesting that this exposure may be etiologically related to disease (Hsia et al., 1988; Luo et al., 1990).

To more effectively conduct epidemiological studies into the adverse health effects of DON, a reliable means of determining exposure at the individual level is required. Inaccuracies of dietary questionnaire data and the heterogeneous distribution of mycotoxins in food commodities restrict the usefulness of both questionnaires and dietary analyses in relating exposure to human disease. Alternatively, a more relevant and reliable indication of individual exposure may be provided by a biomarker. Potential markers include the parent compound (or metabolites), or macromolecular adducts (protein/DNA) following bioactivation, in biological fluids (e.g. urine/blood). Animal studies have identified a de-epoxide metabolite of DON (DOM-1) and a glucuronide conjugate (Cote et al., 1986; Prelusky et al., 1986a; Gareis et al., 1987; Lake et al., 1987; Worrell et al., 1989). However, to date none of these biomarkers of DON exposure have been examined in humans. This study extended a series of earlier rat metabolism and distribution experiments to identify potential biomarkers that could be used in humans. Based on this information a novel method was subsequently developed, validated and used to measure urinary concentrations of DON and its metabolites in humans with presumed environmental exposure to mycotoxins.

2. Materials and methods

2.1. Chemicals

[¹⁴C]DON [1.09 µCi /mg (93% radiochemical purity)] was prepared by the method of Miller and Arnison (1986). The radiochemical purity of [¹⁴C]DON was verified by reverse-phase HPLC. Ecolite scintillation fluid (ESF) was purchased from ICN (Costa Mesa, USA) and [¹⁴C] internal standard from Wallace (Milton Keynes, UK). Acetone was from Vickers (West Yorkshire, UK), and other reagents, of at least analytical grade, were from Sigma (Poole, UK).

2.2. Animal study design

Male Sprague–Dawley rats (University of Leeds), weighing 150–162 g received Rat and Mouse Standard Expanded Diet No.1 (Beekay Universal, Grimston Aldbrough, Hull, UK) and water ad lib. They were acclimatized for 1 week in individual metabolic cages under a 12-h light/dark cycle. Sixteen rats were administered 0.4 ml of [¹⁴C]DON (5.0±0.1 mg/kg, 5.5±0.1 µCi /kg) in 15% aq. ethanol by gavage. Urine was collected for the periods 0–8, 8–24, 24–48 and 48–72 h and stored at –20 °C. Four animals were sacrificed by CO₂ overdose at the end of each time period. Prior to death, blood was collected by cardiac puncture in tubes containing anticoagulant and plasma was obtained by centrifugation.

2.3. Measurement of [¹⁴C]DON in rat plasma and urine

Plasma samples (0.1 ml) were mixed with 3 ml ESF (containing 100 µl 1 M HCl) and kept in the dark (1 h) prior to scintillation counting (Rackbeta, model 1217, LKB Wallac). In order to differentiate total plasma radioactivity from [¹⁴C]DON bound to plasma proteins, plasma samples (0.6–1 ml), cooled on ice for 30 min, were mixed with 2 volumes of cold acetone, placed at –20 °C for 1 h and centrifuged (1000 g, 10 min, 0 °C). The pellet was washed with 2 volumes of cold acetone and centrifuged as above. The pellet and the combined supernatants (dried in vacuo) were reconstituted in 1 ml water or acetone respectively, and analysed in duplicate for radioactivity. Urine samples were centrifuged and 1 ml supernatants analysed by scintillation counting, as described above.

2.4. HPLC analysis of radiolabelled DON metabolites in rat urine

The presence of urinary DON metabolites was determined by HPLC. Urine samples (0.5 ml) were mixed with β-glucuronidase (*Escherichia coli* type IX-A, Sigma-Aldrich) in 200 mM sodium phosphate (pH 5.0) at 7000 U/ml, or 100 U/ml of sulfatase (from *limpets, patella vulgata*, type V, Sigma-Aldrich) or buffer only. The sulfatase incubation included 20 mM D-saccharic acid 1,4-lactone to inhibit residual β-glucuronidase activity in the sulfatase preparation. Urine samples from both treated and untreated rats were incubated with or without enzyme overnight at 37 °C. HPLC analysis (Spectra System, ThermoSeparation products, USA) was on a Supelcosil C18-RP column (25 cm×4.6 mm, 5 µm particle size), 100 µl injection, with a gradient from 10 to 20% aq. MeOH over 45 min using a flow rate of 1 ml/min, fractions were collected (1-min fractions) for determination of radioactivity.

2.5. HPLC analysis of human urine spiked with rat urine

Additional experiments were performed to test the effectiveness of enzyme hydrolysis of DON metabolites in the presence of human urine by mixing human urine with an equal volume of rat urine (8–24 h time period) from a rat treated with [¹⁴C]DON. These mixed urine samples were treated with β-glucuronidase (7000 U/ml) or buffer alone overnight at 37 °C. Samples (50–100 μl) were then injected onto HPLC and fractions collected as above.

2.6. Immunoaffinity column enrichment

Initially the binding of DON in PBS to immunoaffinity columns (IACs) (Labtech International Limited, East Sussex, UK) was investigated. These columns were used since they do not retain closely related tricothecenes (Cahill et al., 1999). IAC columns were loaded, by applying gentle pressure, with 1 ml PBS containing [¹⁴C]DON, using a range of concentrations up to 6.9 μg DON/ml. Columns were washed with 4 ml water, and DON eluted with 4 ml MeOH. The radioactivity in the eluates was determined by liquid scintillation counting.

Secondly, the capacity of IACs to bind DON in human urine was investigated. Human urine (5 ml) was spiked with 0.03, 0.05, 0.10 and 0.50 μg/ml [¹⁴C]DON, buffered to pH 6.5 with 1 ml 0.01 M phosphate buffered saline (PBS) pH 7.4. DON residues were isolated from samples using IAC columns, as described above, and radioactivity in the eluates was measured by scintillation counting.

Thirdly, the IAC binding of DON and DON-conjugates in rat urine was investigated. β-glucuronidase enzyme (7000 U/ml), or buffer, was incubated overnight at 37 °C with 0.25 ml rat urine from a rat treated with [¹⁴C]DON (8–24-h time period). [¹⁴C]DON metabolites were isolated from enzyme-treated samples and buffer-treated samples containing, 3.1 ± 0.4 μg (*n* = 4) and 3.3 ± 0.4 μg (*n* = 2) [¹⁴C]DON metabolites, respectively, using IACs as previously described. The radioactivity in the eluates was determined by liquid scintillation counting.

Finally, the capacity of IACs to bind DON metabolites in human urine was investigated. Human urine samples (4 ml) were spiked with 0.2 ml of rat urine from [¹⁴C]DON-treated animals (8–24-h time period). These samples were treated with β-glucuronidase, or buffer only, and extracted by IAC as previously described. To aid identification of DON and DON metabolites eluates were dried in vacuo, reconstituted in 250 μl 10% aq. ethanol, and 100 μl injected on HPLC, under the conditions described above.

2.7. Biomarkers for human exposure to DON

This study was approved by the institutional review boards of the National Cancer Institute, Bethesda,

Maryland, and the Cancer Institute, Chinese Academy of Medical Sciences, Beijing, China. Participants were selected from eligible non-smoking volunteers between the ages of 19 and 75 years and included urine samples from 15 females; 11 were inhabitants of Linxian County, Henan Province, where the staple diet consists of corn and wheat, and four were inhabitants of Gejiu, Yunnan Province, where the staple diet consists primarily of rice. Informed consent was obtained from each participant. Each subject was given a sterile container, and up to 100 ml of first-voided morning urine was collected and placed in a light protective bag. The urine samples were then kept frozen until analysis. Initially six of these urine samples (4 ml) adjusted to pH 6.5, were treated with β-glucuronidase (7000 U/ml) or buffer only, and incubated at 37 °C overnight. Samples were centrifuged (2000 g, 10 min), added to an equal volume of PBS pH 7.4 and DON residues were isolated by IAC as previously described. Eluates were dried in vacuo, reconstituted in 250 μl 10% aq. ethanol, and 100 μl injected on HPLC with UV_{220nm} detection.

2.8. Mass spectrometry (MS) confirmation of DON in human urine

Urine samples were analysed by electrospray ionisation-MS operating in positive ionisation mode on a Platform II single quadrupole mass spectrometer (Micromass UK Ltd, Manchester, UK) equipped with an HP1100 HPLC system. All 15 Chinese urine samples (treated with β-glucuronidase and IAC enriched) or DON standards (70–640 ng/ml in 10% aq. ethanol) were injected (100 μl) on HPLC under the conditions described above. A post-column T-piece allowed 100 μl/min flow into the MS (capillary voltage 3.5 kV, counter electrode 0.5 kV). The sampling cone was set at 25 V and N₂(g) was used as both the drying (300 l/h) and nebulising (15 l/h) gas. Data acquisition was by Selected Ion Recording (SIR) monitoring the three molecular-related ions of DON (MH⁺, MNa⁺ and MK⁺ at *m/z* 297, 319, 335, respectively). The cycle time was 1 s for the three ions. The limit of detection was 4 ng DON per ml urine.

3. Results

3.1. Measurement of [¹⁴C]DON radioactivity in rat plasma and urine

The amount of [¹⁴C]DON recovered in plasma of Sprague–Dawley rats following a single oral dose of [¹⁴C]DON was highest (291 ± 47 ng/ml, 0.367% of dose) at 8 h, decreased rapidly to 14% of the 8 h concentration by 24 h (41 ± 10 ng/ml), and further decreased until 72 h (12 ± 5 ng/ml, 0.015% of dose). The binding of

[^{14}C]DON to plasma proteins was approximately 9% of the total plasma DON as determined by measuring the precipitated plasma cell pellet at the 8-h time point only. Later time points were not examined due to the low absolute levels of radioactivity in this experiment.

The concentration of urinary DON/DON metabolites based on scintillation counting of the urine from [^{14}C]DON dosed rats was 56 ± 21 $\mu\text{g}/\text{ml}$ (volume range 0.9–2.3 ml), 25 ± 14 $\mu\text{g}/\text{ml}$ (3.3–15.3 ml), 4 ± 3 $\mu\text{g}/\text{ml}$ (2.9–18.9 ml) and 1 ± 0.5 $\mu\text{g}/\text{ml}$ (5.8–11.6 ml), for time periods 0–8, 8–24, 24–48 and 48–72 h, respectively. The total amount of DON/DON metabolites was calculated from the concentration of metabolites and the volume of urine (see above) and is expressed as the rate of excretion ($\mu\text{g}/\text{h}$) and percentage of total administered dose excreted in each time period (see Fig. 1). The excreted radioactivity peaked from 0 to 24 h and then decreased rapidly. The mean amount of radioactivity excreted in urine over the 72-h period was 280 μg DON equivalent (37% of dose).

3.2. HPLC analysis of DON metabolites in rat urine

Rat urine was analysed by HPLC for [^{14}C]DON and its metabolites at each collection time (0–8, 8–24 and 24–48 h). Two main peaks were observed for all urine collection times; a major peak (approx. 6 min) comprised approximately 80% of total radioactivity, and a minor peak (approx. 16 min) co-eluted with [^{14}C]DON standard and comprised about 8% of the total radioactivity. Treatment of the 8–24-h urine samples with β -glucuronidase or sulphatase, resulted in a decrease in the size of the major peak (6 min) with the former, but not the latter, enzyme. The concentration of β -glucuronidase giving a maximum decrease in this peak was

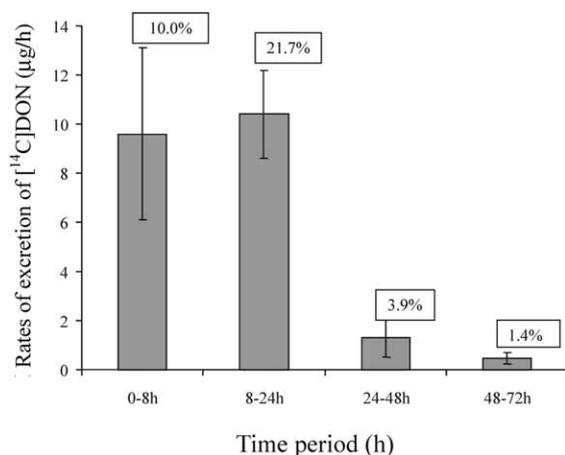


Fig. 1. Excretion profile of radioactivity in the urine of Sprague–Dawley rats following a single oral dose of [^{14}C]DON (5 mg/kg; 5.5 $\mu\text{Ci}/\text{kg}$). Data points from 0–8, 8–24, 24–48, 48–72 h are the mean \pm S.D. of the DON excreted per hour. The percentage of the administered dose excreted over each time period is given in boxes for each period.

optimised (data not shown) to that detailed in the methods section. At this concentration of enzyme the major peak (6 min) post-hydrolysis contained only 30% of the total radioactivity. This was associated with an increase in the minor peak (16 min; co-eluting with DON) to 43% of the total radioactivity and also the appearance of an additional later eluting peak (29 min), containing 9% of the total radioactivity (see Fig. 2). Treatment of the urine sample with sulfatase or buffer alone resulted in no change in the rat urine radiochromatograms.

3.3. HPLC analysis of human urine spiked with rat urine

Prior to the analysis of human urine samples from environmentally exposed individuals the ability of β -glucuronidase to hydrolyse DON-glucuronide in human urine was verified by mixing equal volumes of human urine with rat urine from [^{14}C]DON-dosed animals, and determining the radioactivity in HPLC fractions pre- and post-hydrolysis. The HPLC elution peaks of the

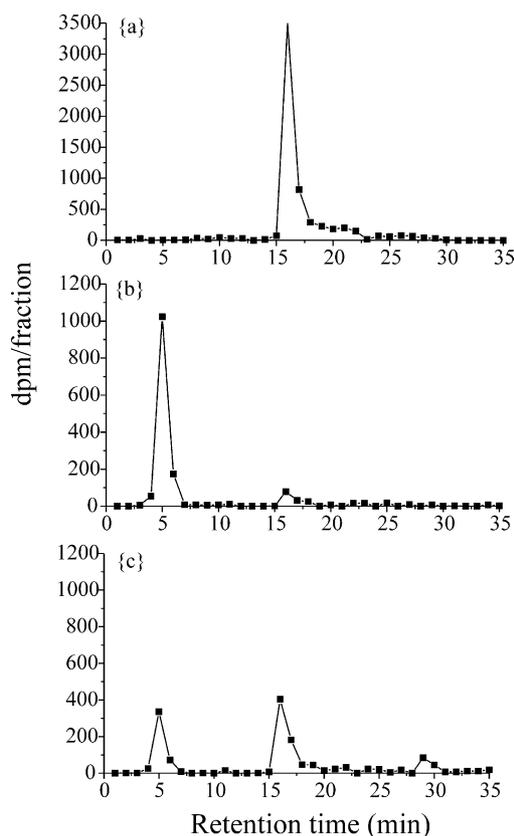


Fig. 2. Representative radiochromatograms of [^{14}C]DON standard and a urine sample (8–24 h) from a rat receiving [^{14}C]DON by gavage (5 mg/kg; 5.5 $\mu\text{Ci}/\text{kg}$) with and without β -glucuronidase treatment: (a) [^{14}C]DON standard; (b) 8–24-h urine sample treated with PBS buffer pH 5 only; (c) 8–24-h urine sample treated with β -glucuronidase enzyme (7000 U/ml PBS buffer pH 5) overnight at 37 $^{\circ}\text{C}$. The peak at 29 min in panel (c) has approximately the same elution time as DOM-1.

human/rat urine mixture, with or without treatment, were at identical times to those obtained from the analysis of the urine from [¹⁴C]DON-treated rats alone (as shown in Fig. 2). The percentage of the total eluate present in the major peak (identified at 6 min) before enzymatic hydrolysis was 87% and the percentage in the minor peak (co-eluting with DON) was 7%. After enzymatic hydrolysis the major peak decreased to 40% of the total eluate while the minor peak increased to 37%. These data suggest that β-glucuronidase enzyme was not inhibited by any of the components present in human urine, where equal mixtures of human and rat urine were used.

3.4. Immunoaffinity column enrichment

A series of spiking experiments were conducted to determine the capacity of the IAC columns for DON and its metabolites:

3.4.1. IAC recovery of [¹⁴C]DON spiked into PBS and human urine

The recovery of [¹⁴C]DON in PBS using commercially available IAC columns was 88% when up to 2.2 μg DON was applied, but decreased when greater quantities of DON were used (data not shown). Human urine (5 ml) was also spiked with 0.03, 0.05, 0.10 and 0.50 μg/ml [¹⁴C]DON, and following IAC extraction the recovery of radioactivity was determined (Table 1). Recoveries were greater than 69% for DON spiked at up to 0.10 μg/ml (0.5 μg total DON), but was less than 40% when the highest concentration of DON (2.5 μg total applied) was used, indicating that recovery of [¹⁴C]DON is less efficient from urine than from PBS.

3.4.2. IAC recovery from urine of [¹⁴C]DON-treated rats ± β-glucuronidase treatment

The amount of radioactivity isolated by IAC from urine of [¹⁴C]DON-treated animals (8–24-h time period) was 0.35 μg (0.30 and 0.40; *n* = 2), representing 10.5% of the total applied to the column. Following β-glucuronidase treatment the amount of radioactivity recovered increased to 1.95 ± 0.13 μg (mean ± S.D., *n* = 4), representing 62.9% of the total DON metabolites applied

supporting the notion that the IAC primarily retains the parent DON, and not its polar metabolites.

3.4.3. IAC recovery from human urine spiked with urine from [¹⁴C]DON-treated animals

Human urine samples (4 ml) were spiked with 0.2 ml of rat urine from [¹⁴C]DON-treated animals (8–24-h time period) for a final urine concentration of 0.52 μg DON metabolites/ml (2.2 μg total). These were incubated with or without β-glucuronidase, subject to IAC purification and injected onto HPLC with scintillation counting of fractions in order to identify the metabolites retained on IAC. With or without β-glucuronidase treatment, no [¹⁴C] material was detected at the elution time of the major hydrophilic DON metabolites (elution time approx. 6 min). Without β-glucuronidase treatment the peak co-eluting with DON represented 10% of the total radioactivity applied to the IAC, and this increased to 23% for samples treated with β-glucuronidase. This is lower than the approximate 40% recovered by HPLC analysis, without prior IAC enrichment, of the β-glucuronidase-treated mixture of human and [¹⁴C]DON-treated animal urine (see Section 3.3). This suggests that the early HPLC eluting DON metabolites were not retained by IAC, and that the IAC columns may be less efficient at retaining DON in the presence of human urine than in rat urine or PBS.

3.5. Biomarkers for human exposure to DON

To ascertain and subsequently characterize the pattern of DON and its metabolites found in urine samples obtained from people who may be environmentally exposed to DON, a series of six Chinese urine samples were treated with β-glucuronidase, subject to IAC purification and injected onto HPLC with UV detection as previously described. In agreement with the data on human urine spiked with urine from [¹⁴C]DON-treated rats, the increase in parent DON recovered following enzyme treatment ranged from 1.2- to 2.8-fold (see Fig. 3). This provided preliminary evidence that DON and its metabolites are present in human urine and confirmation of these data was therefore sought by HPLC with mass spectrometry (MS).

3.6. MS confirmation of DON in human urine

HPLC–MS detection was used to confirm the structural identity of the β-glucuronidase-treated and IAC isolated metabolites from human urine. The mass spectrum for these metabolites identified [DON + H]⁺ (*m/z* = 297), [DON + Na]⁺ (*m/z* = 319), and [DON + K]⁺ (*m/z* = 335) (see Fig. 4). The presence of DON in these human urine samples was established by monitoring these three ion species combined at the co-elution time for the DON standard. Initially two samples were

Table 1
Recovery of [¹⁴C]DON by IAC from human urine

Spiked [¹⁴ C]DON μg/ml (total μg)	Recovered ^a [¹⁴ C]DON μg (two values)	% Recovered
0.03 (0.15)	0.11 (0.10, 0.12)	81
0.05 (0.25)	0.17 (0.18, 0.16)	72
0.10 (0.50)	0.34 (0.36, 0.32)	69
0.50 (2.50)	0.97 (0.73, 1.2)	39

^a Mean of duplicate samples were used for each.

quantified by HPLC–MS with or without enzymatic treatment. The Total Ion Chromatogram (TIC) for the three ions monitored by SIR HPLC–MS for a typical urine sample following β -glucuronidase treatment and IAC enrichment is shown in Fig. 5. There was an increase in DON of 1.7- and 2.2-fold after β -glucuronidase treatment, a similar order of magnitude to the HPLC–UV measures reported above. The IAC linked with HPLC–MS was then used to quantify all 15 Chinese urine samples following β -glucuronidase treatment. The peak areas from the SIR HPLC–MS experiments were calculated using the MassLynx software supplied with the mass spectrometer and this was compared with measurements from a series of standard DON samples for quantification. All 15 Chinese urine samples contained detectable levels of DON (>4 ng/ml). The mean levels of urinary DON were 37 ng/ml (range 14–94 ng/ml)

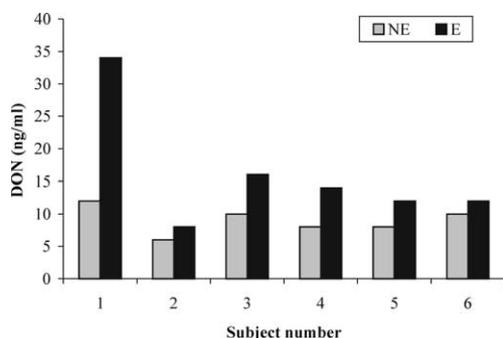


Fig. 3. The concentration of DON (ng/ml) in urine from six Chinese females with (E) or without (NE) treatment with β -glucuronidase. DON was extracted using IAC and injected onto HPLC with UV detection.

and 12 ng/ml (range 4–18 ng/ml) for Linxian and Gejiu, respectively (see Fig. 6). Although mean levels were significantly ($P=0.006$; Mann–Whitney test) higher in the high-risk area of China than the low risk area, this comparison is limited by the small number of samples analysed.

4. Discussion

In this study, the distribution of DON in body fluids of rats administered a single dose of [14 C]DON was examined and the results suggest that urinary analysis of DON and its metabolites might be a promising approach to human exposure assessment. An IAC/HPLC–MS technique was subsequently developed and used to demonstrate the presence of DON in urine of Chinese individuals who may be environmentally exposed to this mycotoxin.

The distribution of DON in body fluids was initially investigated in Sprague–Dawley rats following dosing with [14 C]DON (5 mg/kg body weight). DON and DON metabolites were detectable in plasma over the 72-h time period, with highest levels detectable at 8 h, a time point when DON was significantly associated with plasma proteins (approx. 9% of total plasma radioactivity). The low absolute levels of DON recovered in plasma after 8 h restricted further analysis of this binding. This is concordant with previous studies in swine, sheep and chickens that identified a peak in plasma DON levels after the first few hours post oral dosing with DON (Prelusky et al., 1985, 1986b, 1988). Also similar to our rat studies was the finding that DON was mostly cleared from plasma within 20–30 h in these species.

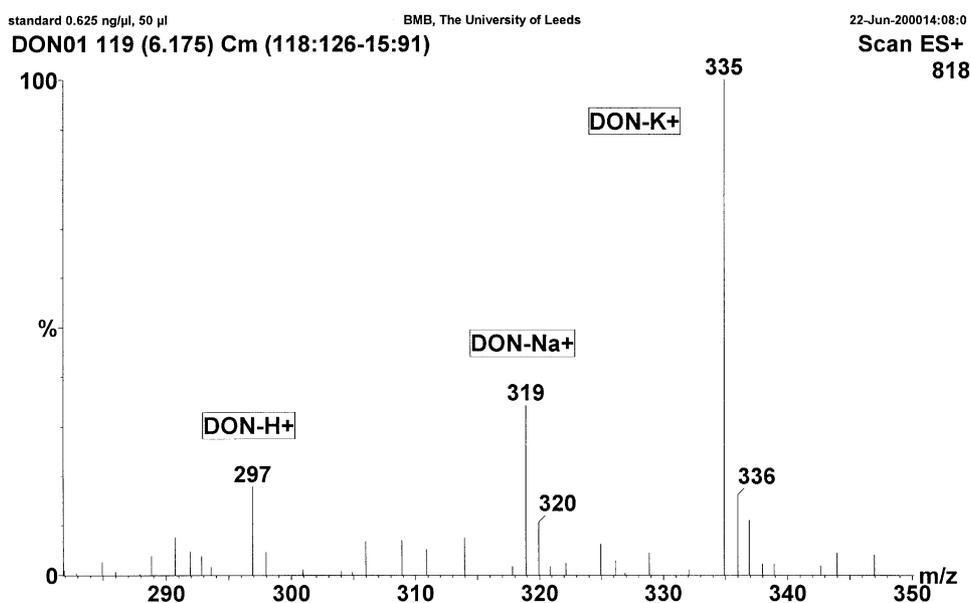


Fig. 4. HPLC–MS for DON standard (640 ng/ml) identifying DON-H $^{+}$ ($m/z=297$), the DON-Na $^{+}$ ($m/z=319$), and the DON-K $^{+}$ ($m/z=335$). MS capillary voltage 3.5 kV, counter electrode 0.5 kV. The sampling cone was set at 25 V and N $_2$ (g) was used to dry (300 l/h) and nebulise (15 l/h).

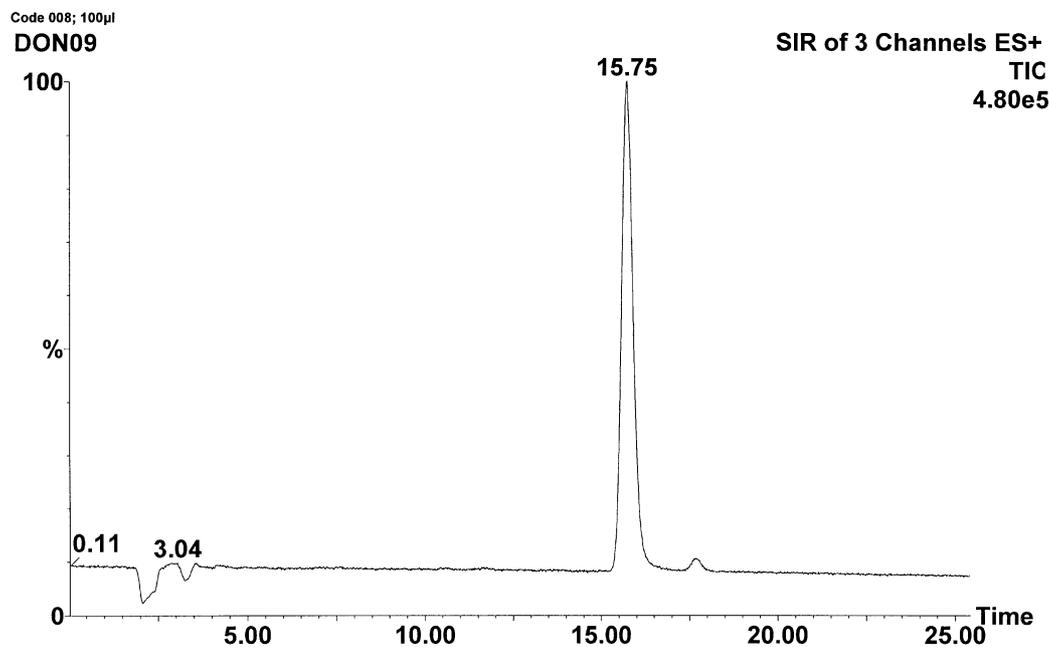


Fig. 5. HPLC–MS trace of DON found in Chinese urine sample (94 ng/ml) following β -glucuronidase treatment and IAC enrichment using Selected Ion Recording to monitor the three related ions of DON.

Plasma protein binding, of 9–11% of the total plasma radioactivity, has been previously reported only for sheep and swine (Prelusky et al., 1987, 1988), but the nature of this binding is unknown. Despite representing a low percentage of total plasma radioactivity, if the binding of DON to plasma proteins were stable, it may be a suitable biomarker of DON exposure.

The urinary excretion of DON was examined with respect to potential candidate biomarkers. Thirty seven percent of the administered DON was recovered in the rat urine over the 72-h study period. A similar earlier study reported 25% recovery of a 10 mg/kg [^{14}C]DON dose, 72 h post-dosing, of male PVG rats, but a detailed analysis of metabolites was not performed (Lake et al., 1987). In order to establish a useful biomarker we wished to further examine the DON species found in the urine of the dosed animals using HPLC and enzyme digests. When urine from [^{14}C]DON-treated rats was analysed by HPLC with liquid scintillation counting, 8% of the DON was excreted as the parent compound and 80% was eluted at an earlier time point consistent with the presence of polar metabolites. Treatment with β -glucuronidase, but not sulfatase, was accompanied by a decrease in the putative polar conjugate(s) (the 6-min major peak on HPLC), and an increase in the quantity of parent DON recovered (identified as the 16-min minor peak on HPLC). A DON-glucuronide conjugate has previously been identified in perfused male Wistar rat liver (Gareis et al., 1987) and also in the urine of dairy cows (Cote et al., 1986) and sheep (Prelusky et al., 1986a). Although a sulphate conjugate was not identified in our animal model this metabolite has been

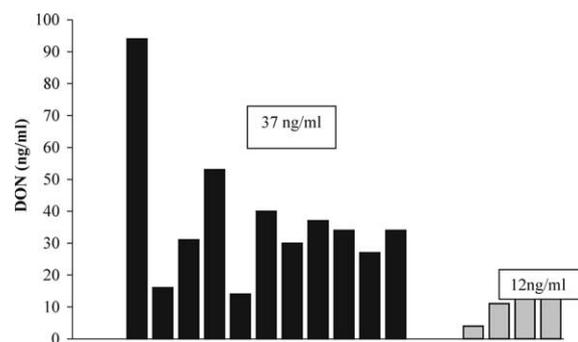


Fig. 6. The concentration of DON ng/ml urine detected by HPLC–MS from 15 Chinese; 11 from a high risk (black bars) and four from a low risk area (shaded bars), following β -glucuronidase treatment. Mean levels of DON for the two groups are given in the boxes. DON was extracted using IAC and injected onto HPLC–MS with SIR monitoring.

detected in sheep (Prelusky et al., 1987). Based on our study and previous data, a major urinary metabolite in rats therefore appears to be a DON-glucuronide. The above-mentioned study in PVG rats also identified DOM-1 (Lake et al., 1987), a later eluting metabolite on reverse-phase HPLC. In our analysis, prior to β -glucuronidase treatment, no putative DOM-1 peak was detected. However, following treatment with this enzyme, a urinary metabolite with a retention time similar to DOM-1 (29 min) was observed (Fig. 2), though insufficient authentic DOM-1 standard prevented full characterisation. Thus, this evidence suggests that DOM-1-glucuronide may be present in the urine of [^{14}C]DON-exposed rats. We went on to spike human urine samples with urine from [^{14}C]DON-treated rats and hydrolysed

these samples with β -glucuronidase. Hydrolysed samples had an increase from 7 to 37% in the amount of parent DON recovered. This suggests that DON-glucuronide can be hydrolysed to DON in the presence of human urine and this approach was therefore pursued in human samples.

Using the information gathered from our rat experiments, commercially available DON IAC columns were used to develop an enrichment method for human urine samples so that the concentration of DON and its metabolites may be analysed using HPLC. These columns have not previously been used with biological fluids, rather they were developed for purification of DON from cereal crops. We found that when human urine was spiked with [^{14}C]DON the IAC recoveries were lower than when [^{14}C]DON was spiked in PBS. Despite this, recoveries were reasonably high (>69%) when 0.5 μg or less of DON in 5 ml of urine was loaded (Table 1). However, the HPLC analysis of human urine spiked with urine from [^{14}C]DON-treated rats, with or without β -glucuronidase treatment, failed to contain the early HPLC eluting peak associated with DON-glucuronide or other polar metabolites suggesting that the IAC columns are unable to bind these compounds in this setting.

When urine from [^{14}C]DON-treated rats was subject to β -glucuronidase treatment and IAC purification, a five- to six-fold increase in the amount of parent DON was observed. The Chinese urine samples were subsequently analysed by the same method but the β -glucuronidase treatment resulted in only a 1.2–2.8 fold increase in the recovery of DON. One possible explanation for this difference is that the proportion of parent DON to glucuronide conjugated DON in human urine is different than the proportion found in rats. However, a relatively low level increase in parent DON post-IAC of β -glucuronidase-treated urines was also observed when small volumes of rat urine (0.2 ml) from the [^{14}C]DON-treated rats were mixed with human urine (4 ml), suggesting a possible methodological explanation. This may include component(s) in human urine that hinder the efficiency of either the β -glucuronidase hydrolysis or the purification of DON on IAC. We believe the former explanation to be unlikely given that the degree of hydrolysis of DON-conjugates to parent DON was similar when urine from [^{14}C]DON-treated rats was analysed alone or combined with human urine. This point requires further study before any conclusions can be drawn concerning possible variations in DON metabolism between individuals.

To our knowledge, this represents the first report of DON found in human urine samples. Eleven of these samples were obtained from Linxian County, a rural Chinese population with a staple diet of wheat and corn and thus represents a population that may be environmentally exposed to high levels of mycotoxins such as

DON. Linxian County is known for its high rates of OC and studies suggest that this disease may be etiologically associated with mycotoxin exposure (Yang 1980; Hsia et al., 1988; Luo et al., 1990). The levels of DON in these samples were quantified and compared to those detected in samples obtained from a low OC risk region, Gejiu, whose major dietary component consists of rice. DON metabolites were found in all 15 urine samples by HPLC–MS with SIR. The presence of DON in human urine samples was established by the following criteria: first the specificity of the monoclonal antibody based IAC for DON; second, the HPLC–UV analysis of urine eluted from IAC gave rise to a single peak that co-elutes with the same retention time as authentic DON standard; third, when urine samples were incubated with β -glucuronidase there was a consistent increase in the quantity of DON detected and finally, structural identification of this compound using HPLC–MS demonstrated a compound with the same molecular mass and retention time as an authentic DON standard.

The mean levels of DON detected in the human urine samples were 37 ng/ml (range 14–94) and 12 ng/ml (range 4–18 ng/ml) from the high- and low-risk areas for DON exposure and OC, respectively. In the 4 ml of urine applied to the IAC the quantity of DON (16–382 ng) should not exceed the capacity of the columns but clearly optimisation of column capacity for human biomarker studies would be valuable. Although the total number of urine samples was small, the results are interesting given that the high risk region's staple diet consists of wheat and corn, foods frequently associated with mycotoxin contamination, whereas the low risk region's diet consists predominantly of rice, a food that is reportedly less commonly contaminated. We used these preliminary results to roughly estimate the daily cumulative individual DON exposure. Given that approximately 30% of the total DON consumed is excreted during a 24-h period in the animal model and assuming a 60-kg person produces 1 l urine/day, and a 40% recovery of DON in human samples, the levels detected in our high- and low-risk populations may represent a daily exposure ranging from 1.9 to 13.0 and 0.6 to 2.5 $\mu\text{g}/\text{kg}/\text{day}$, respectively. These calculated daily exposures are consistent with previous studies on the concentration and consumption of contaminated wheat and corn. For example, a study in Linxian County found wheat and corn DON levels to range from 9–309 $\mu\text{g}/\text{kg}$ and 17–3505 $\mu\text{g}/\text{kg}$, respectively (Luo et al., 1990). Presuming a daily intake of a 60-kg person to be 250 g of wheat or 200 g of corn the daily exposure to DON would range from 0.04 to 1.3 and 0.06 to 11 $\mu\text{g}/\text{kg}/\text{day}$ for wheat and corn respectively, a range not dissimilar to that determined from our urinary analysis. The ease of use for this methodology makes it highly suitable for screening of larger numbers of samples in studies of DON exposure.

In conclusion, we have identified the glucuronide conjugate as one of the major DON metabolites in the urine of exposed rats. This finding suggests that DON or one of its hydrolysis products may be a measurable, and potentially useful biomarker in human exposure studies. We also present a unique protocol for IAC enrichment of β -glucuronidase-treated DON metabolites, and subsequent HPLC analysis that permitted the analysis of human urine samples from a population where exposure to DON has been previously reported. Further studies are needed to optimise analytical conditions, particularly the capacity of the IACs. Despite the preliminary nature of the results, this method enabled us to verify the presence of DON in human urine by IAC-HPLC with MS analysis and approximate an individual's daily exposure. Additional validation and risk assessment studies of this biomarker using this protocol are needed.

Acknowledgements

FAM gratefully acknowledges the financial support of the Egyptian government. CPW was supported by the National Institute of Environmental Health Sciences, USA, grant no. ES06052. The authors thank Geraldine Fox for final preparation of the manuscript.

References

- Bondy, G.S., Pestka, J.J., 2000. Immunomodulation by fungal toxins. *Journal of Toxicology and Environmental Health Part B Critical Reviews* 3, 109–143.
- Cahill, L.M., Kruger, S.C., McAlice, B.T., Ramsey, C.S., Prioli, R., Kohn, B., 1999. Quantification of deoxynivalenol in wheat using an immunoaffinity column and liquid chromatography. *Journal of Chromatography (A)* 859, 23–28.
- Cote, L.M., Dahlem, A.M., Yoshizawa, T., Swanson, S.P., Buck, W.B., 1986. Excretion of deoxynivalenol and its metabolite in milk, urine, and feces of lactating dairy cows. *Journal of Dairy Science* 69, 2416–2423.
- Gareis, M., Bauer, J., Gedek, B., 1987. On the metabolism of the mycotoxin deoxynivalenol in the isolated perfused rat liver. *Mycotoxin Research* 3, 25–32.
- Gilbert, J., 1989. Current views on the occurrence and significance of Fusarium toxins. *Society of Applied Bacteriology Symposium Series* 18, 89. S-98S.
- Hsia, C.C., Wu, J.L., Lu, X.Q., Li, Y.S., 1988. Natural occurrence and clastogenic effects of nivalenol, deoxynivalenol, 3-acetyl-deoxynivalenol, 15-acetyl-deoxynivalenol, and zearalenone in corn from a high risk area of esophageal cancer. *Cancer Detection and Prevention* 13, 79–86.
- IARC, 1993. *IARC Monographs on the Evaluation of Carcinogenic Risks of Chemicals to Humans*. Vol. 56. Some Naturally-occurring Substances: Some Food Items and Constituents, Heterocyclic Aromatic Amines and Mycotoxins. International Agency for Research on Cancer, Lyon, pp. 245–395.
- Lake, B.G., Phillips, J.C., Walters, D.G., Bayley, D.L., Cook, M.W., Thomas, L.V., Gilbert, J., Startin, J.R., Baldwin, N.C., Bycroft, B.W., Dewick, P.M., 1987. Studies on the metabolism of deoxynivalenol in the rat. *Food and Chemical Toxicology* 25, 589–592.
- Li, F., Luo, X., Yoshizawa, T., 1999. Mycotoxins (trichothecenes, zearalenone and fumonisins) in cereals associated with human red-mold intoxications stored since 1989 and 1991 in China. *Natural Toxins* 7, 93–97.
- Luo, Y., Yoshizawa, T., Katayama, T., 1990. Comparative study on the natural occurrence of Fusarium mycotoxins (trichothecenes and zearalenone) in corn and wheat from high- and low-risk areas for human esophageal cancer in China. *Applied Environmental Microbiology* 56, 3723–3726.
- Meky, F.A., Hardie, L.J., Evans, S.W., Wild, C.P., 2001. Deoxynivalenol-induced immunomodulation of human lymphocyte proliferation and cytokine production. *Food and Chemical Toxicology* 39, 827–836.
- Miller, J.D., Arnison, P.G., 1986. Degradation of deoxynivalenol by suspension-cultures of the fusarium head blight resistant wheat cultivar Frontana. *Can. J. Plant Pathol. Rev. Can. Phytopathol.* 8, 147–150.
- Miller, J.D., 1998. Global significance of mycotoxins. In: Miraglia, M., van Egmond, H., Brera, C., Gilbert, J. (Eds.), *Mycotoxins and Phycotoxins—Developments in Chemistry, Toxicology and Food Safety*. Alaken Inc, Fort Collins, CO, pp. 3–16.
- Placinta, C.M., D'Mello, J.P., Macdonald, A.M., 1999. A review of worldwide contamination of cereal grains and animal feed with Fusarium mycotoxins. *Animal Feed Science and Technology* 78, 21–37.
- Prelusky, D.B., 1997. Effect of intraperitoneal infusion of deoxynivalenol on feed consumption and weight gain in the pig. *Natural Toxins* 5, 121–125.
- Prelusky, D.B., Hartin, K.E., Trenholm, H.L., Miller, J.D., 1988. Pharmacokinetic fate of [¹⁴C]-labeled deoxynivalenol in swine. *Fundamental and Applied Toxicology* 10, 276–286.
- Prelusky, D.B., Veira, D.M., Trenholm, H.L., Foster, B.C., 1987. Metabolic fate and elimination in milk, urine and bile of deoxynivalenol following administration to lactating sheep. *Journal of Environmental Science and Health B22*, 125–148.
- Prelusky, D.B., Veira, D.M., Trenholm, H.L., Hartin, K.E., 1986a. Excretion profiles of the mycotoxin deoxynivalenol, following oral and intravenous administration to sheep. *Fundamental and Applied Toxicology* 6, 356–363.
- Prelusky, D.B., Hamilton, R.M., Trenholm, H.L., Miller, J.D., 1986b. Tissue distribution and excretion of radioactivity following administration of ¹⁴C-labeled deoxynivalenol to White Leghorn hens. *Fundamental and Applied Toxicology* 7, 635–645.
- Prelusky, D.B., Veira, D.M., Trenholm, H.L., 1985. Plasma pharmacokinetics of the mycotoxin deoxynivalenol following oral and intravenous administration to sheep. *Journal of Environmental Science and Health B20* 603–624.
- Rotter, B.A., Prelusky, D.B., Pestka, J.J., 1996. Toxicology of deoxynivalenol (vomitoxin). *Journal of Toxicology and Environmental Health* 48, 1–34.
- Scott, P.M., 1989. The natural occurrence of trichothecenes. In: Beasley, V.R. (Ed.), *Trichothecene Mycotoxicosis: Pathophysiologic Effects*. CRC Press, Boca Raton, FL, pp. 1–26.
- WHO, 1990. *Selected Mycotoxins: Ochratoxin, Trichothecenes, Ergot*. Environmental Health Criteria No. 105. WHO, Geneva.
- Wild, C.P., Hall, A.J., 1996. Epidemiology of mycotoxin-related disease. In: Howard, D.H., Miller, J.D. (Eds.), *The Mycota VI. Human and Animal Relationships*. Springer-Verlag, Berlin, pp. 213–227.
- Worrell, N.R., Mallett, A.K., Cook, W.M., Baldwin, N.C., Shepherd, M.J., 1989. The role of gut micro-organisms in the metabolism of deoxynivalenol administered to rats. *Xenobiotica* 19, 25–32.
- Yang, C.S., 1980. Research on esophageal cancer in China: a review. *Cancer Res.* 40, 2633–2644.