Toxicology Letters 275 (2017) 19–26

Contents lists available at ScienceDirect

Toxicology Letters

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

Ochratoxin A and its metabolites in urines of German adults—An assessment of variables in biomarker analysis

Nurshad Ali[a,1], Katherine Muñoz[b], Gisela H. Degen[a,*]

[a] Leibniz Research Centre for Working Environment and Human Factors (IfADo), Ardeystr. 67, D-44139 Dortmund, Germany
[b] Institute of Environmental Sciences, University Koblenz-Landau, Fortstr. 7, D-76829 Landau in der Pfalz, Germany

ARTICLE INFO

Keywords:
Biomarkers
Exposure
Mycotoxins
Ochratoxin A
Urine

ABSTRACT

Ochratoxin A (OTA), a mycotoxin known for its nephrotoxic and carcinogenic properties, is a worldwide occurring contaminant in a variety of food commodities. Biomonitoring (*i.e.* analysis in biological fluids) can serve to assess human internal exposure from all consumed foods and beverages. We now determined the concentration of OTA and its metabolite ochratoxin alpha (OTα) in plasma and in urine of two male volunteers with different food habits, in order to assess intra-individual temporal fluctuations and inter-individual differences in their biomarker levels. Moreover, the urinary levels of both OTA and OTα were analyzed in a cohort of German adults (23 males, 27 females) on their regular diet. All samples were subjected to an enzymatic hydrolysis of biomarker conjugates prior to clean-up by liquid–liquid extraction and HPLC-FD analysis. The profile in the first individual showed small fluctuations over time: mean levels in plasma were 0.42 and 0.45 ng/mL for OTA and OTα, respectively, and in urine means of 0.06 ng/mL for both analytes. The other individual had mean levels of 1.64 and 0.20 ng/mL for OTA and OTα in plasma, and 0.24 and 2.22 ng/mL for these analytes in urine. It is concluded that inter-individual differences in biomarker levels reflect dissimilar dietary exposure and/or disposition of ingested mycotoxin, with an apparently more efficient detoxification of OTA to OTα in the second individual. In the German cohort (n = 50), analytes were detected in 100% (OTA: range 0.02–1.82 ng/mL mean level 0.21 ± 0.31 ng/mL) and 78% (OTα: range 0.01–14.25 ng/mL, mean level 1.33 ± 2.63 ng/mL) of all urines. Parameters such as gender, age and body mass index did not show a significant association with urine biomarker levels. This study indicates frequent exposure to OTA among German adults. The new results are discussed in the context of biomarker data from other countries and some methodological issues.

1. Introduction

Ochratoxin A (OTA) is a mycotoxin produced by a number of *Aspergillus* and *Penicillium* species, and well known for its carcinogenicity in rodents and pronounced nephrotoxicity in many species (EFSA, 2006; FAO/WHO, 2008). OTA is found worldwide as a contaminant in many foodstuffs of plant origin, mainly cereals and cereal products, as well as coffee, cacao, beer, grape juice, raisins, wine and spices, or in animal derived products such as sausage and blood pudding (FAO/WHO 2001; Ostry et al., 2013). As OTA toxicity is related to dose, it is important to collect information on human exposure to this mycotoxin for a risk evaluation. Exposure is often assessed on the basis of both, surveys on contaminant levels in relevant food commodities, and data on dietary habits and food consumption in various groups of the population (examples for OTA in: SCOOP, 2002; EFSA, 2006; Kuiper-Goodman et al., 2010). But, this approach requires a rich database that is nowadays not available in many countries. Another (complementary) approach is biomonitoring, *i.e.* the analysis of biomarkers of exposure in human biological fluids. Biomarker analysis covers intake from all dietary sources and possible additional exposure by other exposure routes (Degen, 2011; Fromme et al., 2016). Studies conducted for OTA in blood, breast milk or urine samples from several countries provide valuable information on exposure scenarios in different populations, including breastfed infants (Fromme et al., 2016; Warth et al., 2016).

OTA analysis in blood samples reveals widespread exposure across the globe, albeit at variable levels (data reviewed by Coronel et al., 2010; Duarte et al., 2011; Märtlbauer et al., 2009). For example, OTA concentration in serum of the adult German population can vary between 0.06 and 2.03 ng/mL (mean 0.27 ng/mL), internal levels that result from a low dietary exposure to the mycotoxin (Rosner et al.,

Abbreviations: OTA, ochratoxin A; OTα, ochratoxin alpha

* Corresponding author at: Leibniz–Institut für Arbeitsforschung an der TU Dortmund (IfADo), Ardeystr. 67, D-44139 Dortmund, Germany.
E-mail address: degen@ifado.de (G.H. Degen).
[1] Present address: Department of Biochemistry and Molecular Biology, Shahjalal University of Science and Technology, Sylhet-3114, Bangladesh.

http://dx.doi.org/10.1016/j.toxlet.2017.04.013
Received 16 March 2017; Received in revised form 18 April 2017; Accepted 19 April 2017
Available online 23 April 2017
0378-4274/

2000). Similar ranges and mean values for OTA in plasma or serum have been reported for healthy adults in other Western European countries and some Asian and South American countries (Märtlbauer et al., 2009; Ali et al., 2014). Considerably higher OTA levels (up to 50 ng/mL) have been found in persons from areas of Bulgaria and Croatia known for a high prevalence of Balkan Endemic Nephropathy (BEN), and in Tunisian patients suffering from chronic interstitial nephropathy (CIN) of unknown aetiology (Table 1 in Aslam et al., 2012). Chronic exposure to high dietary mycotoxin levels has been suggested as putative cause of renal disease (Castegnaro et al., 2006; Peraica et al., 2008); yet the aetiological role of OTA in BEN and CIN is still a controversial issue, also since aristolochic acids are considered a main risk factor for endemic nephropathy and associated urothelial cancer in BEN regions (Stiborova et al., 2016). Nonetheless, because of OTA's known adverse effects (mainly nephrotoxicity), continued mycotoxin analysis in foods and human biomonitoring is important and indicated (EFSA, 2006).

Considerations in the context of biomarker analysis are ease of collecting biological specimen (invasive or non invasive), the kinetics of OTA in the organism and methodological aspects. OTA has a long half-life in human blood (about 35 days) due to its extensive binding to serum proteins, and only a small free fraction undergoes glomerular filtration and urinary excretion (Studer-Rohr et al., 2000; Ringot et al., 2006). OTA levels in plasma or serum reflect exposure during a longer period (> 1 month) whilst urinary levels better reflect recent OTA intake (Gilbert et al., 2001; Castegnaro et al., 2006); OTA occurrence in these matrices is thus a good indication of past and present exposure (Duarte et al., 2011). Yet, an analytical focus on OTA alone does not reflect its biotransformation: OTα – formed by hydrolysis in the gastrointestinal tract – is the major metabolite of OTA in humans (Ringot et al., 2006). The detoxication product OTα is further metabolized to a glucuronide (and possibly sulfate); these polar metabolites are far more efficiently cleared from the circulation than OTA and readily excreted with urine (Duarte et al., 2011). Thus, after enzymatic hydrolysis with ß-glucuronidase/arylsulfatase, OTα is often found in considerably higher concentrations in urines than the parent compound, and may serve as additional biomarker of mycotoxin exposure (Muñoz et al., 2010; Coronel et al., 2011; Klapec et al., 2012). Also the presence of OTA conjugates has been demonstrated recently in several urine samples (Muñoz et al., 2017) which indicates that enzymatic hydrolysis of phase II metabolites is highly advisable in human biomonitoring studies.

As variations in OTA intake over time and between individuals are expected, the present study was conducted with two aims: (i) biomarker profiles were determined repeatedly in plasma and in urine of two male volunteers with rather different food habits to gain further insight into fluctuations over time; (ii) urinary levels of OTA and its metabolite OTα in a cohort of German adults (23 males, 27 females) were analyzed to characterize the range of biomarker levels in persons on their regular diet. These new results will be compared to available data from several other countries, and current approaches in OTA biomonitoring will be discussed in terms of methodological issues.

2. Materials and methods

2.1. Chemical and reagents

Chloroform, methanol HPLC-grade, isopropanol, acetic acid (96%), sodium hydrogen carbonate ($NaHCO_3$) and phosphoric acid (85%) were purchased from Merck (Darmstadt, Germany). OTA (purity > 98%) was obtained from Sigma-Aldrich (Taufkirchen, Germany), and OTα standard (purity 98.9%; 11.9 µg/mL acetonitrile) from Biopure (Tulln, Austria). OTA was dissolved in methanol and calibrated spectrophotometrically at 333 nm using the molar (M) extinction coefficient of 6400 $M^{-1}\,cm^{-1}$. The working standard solutions were prepared weekly as dilutions in methanol/water (1:1, v/v) in a range from 0.05 to 10 ng/mL. The β-glucuronidase/arylsulfatase (β-Gluc/ArylS) enzyme from *Helix pomatia* (specific activity 5.5 U/mL β-glucuronidase, 2.6 U/mL arylsulfatase at 37 °C) was purchased from Roche (Mannheim, Germany).

2.2. Human sample collection and cohort description

Blood plasma and urine samples were collected repeatedly in the mornings from two volunteers. From volunteer A (male, 30 years) samples were collected once a week over a period of 7 weeks in april and may 2013. Volunteer B (male, 60 years) provided samples (blood, urine) on 7 days within two weeks in november 2013. Blood samples in EDTA vials were centrifuged at 3000 rpm (about 2500 × *g*) for 15 min for separation of plasma, and then stored at −20 °C until analysis; urine samples were stored under the same conditions (at −20 °C). Both volunteers kept a food record: Person A (of Asian origin) consumed regularly bread, joghurt and fruits during the day, and rice with chicken, fish or spinach or sometimes pizza and salat for supper; he drank regularly milk, not more than one cup of coffee per day and no beer or wine. Person B (German) had muesli, milk, joghurt, cheese and egg sandwich, fruit juice and coffee for breakfast, no lunch during the test period, and bread with cold meat, cheese, tomatoes and fruits for supper; he drank regularly five cups of coffee with milk per day, and about two bottles of beer in the evening.

For the second part of the study, first morning urine samples (n = 50) were collected from IfADo employees (27 females and 23 males) into a non-sterile disposable container (approximately 80 mL) between July and August 2013. Urine donors were informed about the study and a written consent was obtained from all participants prior to inclusion in the study. The participants were of good health and they were asked to fill a short questionnaire for anthropometric information (age, gender, height, and weight), occupation and regular food habits. The participants consumed typical mixed German food (grain-based products, potatoes and vegetables, milk and milk-based products, eggs, meat and meat-products); three were vegetarians. Urine samples were immediately stored at −20 °C until analysis. This study was approved by the Institutional Internal Review Board of IfADo. The demographic characteristics of all study subjects (the two male volunteers and the German cohort) are provided in Supplementary Table 1. For further details see also Thesis (Ali, 2016) and the study on citrinin monitoring in the German cohort (Ali et al., 2015).

2.3. Creatinine analysis

Creatinine was determined in urine samples to correct for differences in urine dilution between individual spot urines by a modified Jaffe method (Blaszkewicz and Liesenhoff, 2012). Analysis of creatinine was carried out with a plate reader, Tecan Genios® (Salzburg, Austria). The creatinine values of urine donors are included in Supplementary Table 1; they were all in the range of reference values (0.5–2.5 g/L) for adults (Kommission Humanbiomonitoring, 2005).

2.4. Sample preparation and chromatographic analysis

Analysis of OTA and OTα in plasma samples was based on the method of Muñoz et al. (2010) with enzymatic hydrolysis of conjugates and carried out as described elsewhere in more detail (Ali et al., 2014). In line with previous results, recovery of OTA and OTα (assessed at three concentration levels in triplicate spiked samples) was about 90% for OTA and 72% for OTα. The LOD and LOQ for both analytes in plasma were 0.05 and 0.1 ng/mL, respectively.

Biomarkers in urine were analyzed by a validated method which applies liquid–liquid extraction for OTA and OTα aglycones and HPLC-FD detection (Muñoz et al., 2010) with slight modifications (Ali et al., 2016a): 3 mL of urine was enzymatically treated by adding 250 µL hydrolysis buffer (pH 5.0) and 40 µL of β-Gluc/ArylS and incubation at