Journal of Chromatography B, 878 (2010) 2623–2629

Contents lists available at ScienceDirect

Journal of Chromatography B

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

Simultaneous analysis of ochratoxin A and its major metabolite ochratoxin alpha in plasma and urine for an advanced biomonitoring of the mycotoxin☆,☆☆

Katherine Muñoz*, Meinolf Blaszkewicz, Gisela H. Degen

Leibniz Research Centre for Working Environment and Human Factors, Ardeystraße 67, D-44139 Dortmund, Germany

ARTICLE INFO

Article history:
Received 28 August 2009
Accepted 26 November 2009
Available online 3 December 2009

Keywords:
Mycotoxins
Ochratoxin A
Plasma
Urine
Biotransformation
HPLC

ABSTRACT

Ochratoxin A (OTA) is a frequent mycotoxin contaminant found worldwide in foods and feedstuffs. Biomonitoring has been used to assess internal OTA exposure resulting from dietary intake and from other sources. Mycotoxin levels in blood and/or urine provide good estimates of past and recent exposure since OTA binds to serum proteins and is also partly excreted via the kidney. But, measuring OTA alone does not reflect its biotransformation. In light of scarce data on its metabolites in humans, it was the aim of this study to develop a method that allows analysis of OTA and its detoxication product ochratoxin alpha (OTα) in urine and in blood plasma. The method involves enzymatic hydrolysis of conjugates, liquid–liquid extraction, and analysis of sample extracts by liquid chromatography with fluorescence detection. Application of the validated method in a pilot study with 13 volunteers revealed the presence of OTA and OTα in all samples (limit of quantification: 0.05 ng/mL in urine, and 0.1 ng/mL in plasma). In line with negative findings of others, an OTA glucuronide was not detected, neither in urine nor in plasma. By contrast, conjugates of OTα (glucuronide and/or sulfate) are major products in these samples. This was confirmed by mass spectrometry detection. As OTα represents a large fraction of ingested mycotoxin, we propose to include analyses of this metabolite in future biomonitoring studies, also in light of the observed variations for urine OTα-levels that suggest different interindividual abilities for OTA-detoxification in humans.

© 2009 Elsevier B.V. All rights reserved.

1. Introduction

Ochratoxin A (OTA), N-[(5-chloro-3,4-dihydro-8-hydroxy-3-methyl-1-oxo-1H-2-benzo-pyran-7-yl) carbonyl]-,(R)-L-phenylalanine (Fig. 1a), is a secondary fungal metabolite produced by several species of the genus *Aspergillus* and *Penicillium*. This mycotoxin is found worldwide as a contaminant of many food commodities and animal feeds [1,2]. OTA is a well-known nephrotoxin in various species, and it was classified by IARC as possible human carcinogen (group 2b). Long-term exposure to OTA has been implicated in Balkan Endemic Nephropathy and associated with urinary tract tumors because of rather high OTA levels detected in food samples and in blood or urine from affected persons [3,4].

Toxicokinetics are an important determinant in the toxicity of OTA [5,6]. OTA is well absorbed from the gastrointestinal tract and binds to blood proteins, with considerable variations in serum half-lives across species that are apparently dependent on the affinity and extent of protein binding [7]. Also reabsorption of OTA from the intestine, enterohepatic recirculation, and reabsorption in the kidney proximal and distal tubules favour its accumulation in the organism [6]. In humans, OTA is rather persistent, with a serum half-life of about 35 days, due to unfavourable elimination kinetics [8]. The long biological half-life in human blood facilitates biomonitoring studies, and investigations in a number of countries have documented the presence of OTA in blood (e.g. [9–11]). In the population of Western European countries, with a low dietary OTA intake, internal mycotoxin levels are also rather low [2,12,13]. Much higher OTA blood levels (up to 100 ng/mL) were reported from some areas with a high prevalence of Balkan Endemic Nephropathy, and in Tunisian patients suffering from chronic interstitial nephropathy of unknown cause [4,9].

OTA levels in blood (plasma or serum) are suitable biomarkers of exposure in the general population and in occupational settings [11,14–16]. But, it is worth noting that blood samples are collected by an invasive procedure that involves medical personnel. Since urine samples are easier to obtain, analysis of OTA in this matrix has been used also in several studies (reviewed by Scott [11]), and appears to be a promising non-invasive alternative for OTA biomonitoring [17–19]. Results from a diet duplicate study in the UK [17] suggested a better correlation between OTA urine levels and dietary mycotoxin intake than with plasma OTA levels. Whilst

☆ This paper is part of the special issue "Biological Monitoring and Analytical Toxicology in Occupational and Environmental Medicine", Michael Bader and Thomas Göen (Guest Editors).
☆☆ Part of this work has been presented at the 50th Conference of the DGPT [32] and at the 31st Mycotoxin Workshop in Münster [33].

* Corresponding author. Tel.: +49 231 1084397; fax: +49 231 1084403.
E-mail address: munoz@ifado.de (K. Muñoz).

1570-0232/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2009.11.044

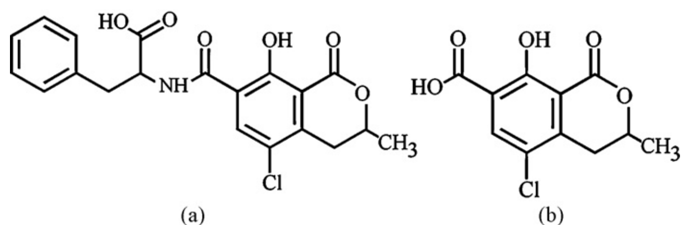

Fig. 1. Structures of ochratoxin A (a) and ochratoxin alpha (b).

concentrations of OTA excreted in urine are considerably lower than circulating OTA levels in blood, the sample volumes available for analysis are higher. In addition to faecal and urinary excretion of the mycotoxin, lactating humans (and other mammals) also excrete OTA in their milk [6,11].

Biomonitoring for OTA requires suitable methods of sample clean-up for the different matrices [11,20]. In this context, it is important to consider also the biotransformation of OTA in the organism, and the question whether the particular method used in sample preparation will allow detection of mycotoxin metabolites in subsequent analysis. So far, information of OTA metabolites in human blood or urine is scarce.

OTA metabolites detected in animals or *in vitro* studies indicate several routes of biotransformation (reviewed in [3,6]): only a small percentage of OTA is hydroxylated, mainly by hepatic cytochrome P540 enzymes of several species, to 4(R/S)-OH-OTA (Fig. 4 in [6]). This detoxication product was not detected in human urine samples, although the method developed was suitable for the simultaneous analysis of OTA and this metabolite [18]. Phase I reactions could also yield OTB, the less toxic dechlorination product of OTA [6]. Another reaction involves opening of the isocoumarin ring and results in a ring-opened lactone form of OTA, a product of similar toxicity detected so far only in rats by special analytical procedures [21]. A major pathway is apparently hydrolysis of the amide bond resulting in phenylalanine and ochratoxin alpha (OTα) as cleavage products (Fig. 1b). In ruminants, the greater resistance to the toxic effects of OTA has been attributed to the capacity of the rumen microflora to degrade the toxin to OTα, a non-toxic metabolite [6], although excretion of OTα as main metabolite was also detected in rats, a species sensitive to OTA toxicity [21,22]. No data are available on the gastrointestinal hydrolysis of OTA and OTα excretion in humans.

In contrast to metabolic studies on phase I reactions, little is known on the formation of conjugates, i.e. phase 2 metabolites of OTA or its derivatives. Acyl glycosides (*O*-labile ester conjugates of OTA with pentose and hexose) were detected in rat urine and in cultures of rat and human hepatocytes [22,23]. Studies in rats and pigs suggested also the presence of OTA glucuronide and/or sulfate conjugates in urine and bile [21,24], and recently Pena et al. [19] reported indirect evidence for OTA glucuronide in human urine samples.

In light of scarce data on OTA detoxication, it was the aim of this study to develop a method suitable for analysis of OTA and its metabolite OTα in human urine and blood samples. An enzymatic hydrolysis step with β-glucuronidase/sulfatase was included in sample preparation to account also for conjugates, and two methods for clean-up (immunoaffinity columns and liquid–liquid extraction) were tested for recovery of parent compound and of OTα.

2. Experimental

2.1. Standards, reagents and biochemicals

Methanol, chloroform, isopropanol, acetic acid, sodium hydrogen carbonate ($NaHCO_3$) and phosphoric acid were obtained from Merck (Darmstadt, Germany). Solvents used as mobile phase were HPLC and LC–MS grade, respectively. OTA (purity 98%) was purchased from Sigma–Aldrich (Taufkirchen, Germany), and OTα standard (purity 98.9%; 11.9 μg/mL acetonitrile) from Biopure (Tulln, Austria). OTA in methanol was calibrated spectrophotometrically at 333 nm using the molar extinction coefficient 6400 M^{-1} cm^{-1} [25]. The working standard solutions were prepared weekly by dilutions in methanol/water (1:1, v/v) in a range from 0.10 to 10 ng/mL. Stock solutions of 50 ng/mL of OTA and OTα in methanol/water (1:1, v/v) were used for the spiked samples. Enzyme β-glucuronidase/arylsulfatase (β-Gluc/ArylS) from *Helix pomatia* (with specific activity 5.5 U/mL β-glucuronidase, 2.6 U/mL arylsulfatase at 37 °C) was purchased from Roche (Mannheim, Germany), and used with 10-fold hydrolysis buffer (13.6 g sodium acetate hydrate, 1.0 g ascorbic acid, 0.1 g EDTA in 100 mL deionised water, adjusted to pH 5.0 with acetic acid 98%) for the enzymatic treatment of plasma and urine samples.

2.2. Sample collection

In this pilot study, healthy volunteers were asked to provide blood and urine samples on the same day. Samples were collected from the group of seven women and six men (ages ranged between 20 and 57 years) in October 2008 in Dortmund, Germany. All participants signed an informed consent before the sampling. Blood samples were collected in the late morning. On the same day urine samples were collected. Plasma (3 mL) was obtained from whole blood by centrifugation at 3500 rpm and kept frozen at −20 °C until analysis. Urine samples (20 mL) were immediately frozen and also stored at −20 °C.

2.3. Enzymatic cleavage of conjugates

To account for the possible presence of phase II metabolites, specifically glucuronide or sulfate conjugates, all urine and plasma samples were divided in aliquots: one aliquot (3 mL) was subjected to enzymatic treatment with β-Gluc/ArylS prior to further sample clean-up; the other aliquot (5 mL) was directly subjected to sample extraction (see following section). An increase in the chromatographic peak of OTA and/or OTα in the enzyme-treated sample compared with the aglycone levels measured in the non-hydrolysed (parallel) sample is then indicative of the presence of conjugates. Initially, different amounts of β-Gluc/ArylS enzyme solution were tested to ensure optimal conditions for the cleavage of analyte conjugates. Tests in plasma, with 10 or 20 μL of the enzyme solution gave similar results, with OTα concentration increasing from 0.08 ± 0.03 ($n = 3$) up to 0.21 ± 0.02 ng/mL ($n = 6$). In tests with urine samples, the highest values were obtained with addition of 40 μL enzyme, with increases in OTα levels from 0.12 ± 0.02 ($n = 3$) up to 1.31 ± 0.08 ng/mL ($n = 4$). Finally, the method was established as follows: Three mL urine were mixed with 0.25 mL hydrolysis buffer and 40 μL enzyme, and 1 mL plasma sample was mixed with 0.1 mL hydrolysis buffer solution and 10 μL enzyme. All samples were kept at 37 °C overnight prior to liquid–liquid extraction of total OTA and OTα.

2.4. Sample extraction

2.4.1. Liquid–liquid extraction (LLE)

Five milliliters of urine (non hydrolyzed urine) or 1 mL plasma were mixed with 5 mL of 1% $NaHCO_3$ in water, followed by a pH adjustment with 1 M phosphoric acid in a pH range between 3 and 4. Then OTA and OTα were extracted using 3 mL chloroform/isopropanol (97:3, v/v). The mixture was centrifuged at 4500 rpm for 15 min. In plasma samples, a second centrifugation was required in some cases to allow a complete separation of

the protein layer. The aqueous upper layer was aspirated with a pipette and discarded; then exactly 2 mL of the organic layer were transferred to a new vial, using a 2 mL volumetric pipette, and evaporated to dryness under a stream of nitrogen at 45 °C. The extract was reconstituted in 500 μL of methanol/water (1:1, v/v), and filtered through a 0.45 μm pore size Teflon syringe filter prior to HPLC analysis. The enrichment factors were 6.6 and 1.3 for urine and plasma, respectively.

2.4.2. Immunoaffinity column (IAC) extraction

With the aim to compare the LLE with another frequently used clean-up method for biological samples, IAC for OTA was used as clean-up step with urine samples, following the method described by Pena et al. [19] with some modifications. OchraTest® (Vicam®; Watertown, MA, USA) columns were used as IAC. Five milliliters human urine was diluted with 5 mL of a solution of 5% $NaHCO_3$ in distilled water, the sample was mixed in a rotary shaker for 15 min, and then quantitatively loaded on the OchraTest® column at a flow rate of 1 drop/s. The column was washed twice with 5 mL distilled water, then OTA was eluted (flow rate 1 drop/s) from the column using 3 mL of methanol, and the column was dried by passing air through the column. The eluate was evaporated to dryness under a stream of nitrogen at 55 °C, and the residue was dissolved in 500 μL methanol/water (1:1, v/v). Thus, the enrichment factor was 10.

2.5. HPLC analysis with fluorescence detection

Sample extracts and standards were analyzed using a HPLC Shimadzu system consisting of two LC-10AS pumps, RF-10Axl fluorescence detector, SIL-10AD Vp auto injector, CBM-20A communication module, and Shimadzu LC solution software. A Nucleosil 100 chromatographic column ($C_{18,}$ 250 mm × 3 mm, 5 μm) was used for separation of the analytes. The injection volume was 80 μL and the analysis was performed at a column temperature of 40 °C. The mobile phases used were the following: as phase A acetic acid 2%/methanol (66:34, v/v), and phase B methanol/isopropanol (90:10, v/v). The stepwise gradient was: 0–15 min 5% B, 15–16 min 5–40% B, 16–30 min 40% B, 30–31 min 40–95% B, 31–33 min 95% B, 33–34 min 95–5% B, and re-equilibration 34–45 min 5% B. The retention times for OTα and OTA were 10 and 22 min, respectively. The fluorescence detector was set at 333 nm excitation and 450 nm emission wavelengths [26]. Confirmation of OTα and OTA in positive samples was done by the standard addition method and in selected samples by formation of the methyl ester derivative (Fig. 2) [27]. The retention time was 20.5 min for OTα methyl ester (OTα-Me) and 32.8 min for OTA methyl ester (OTA-Me), using the same chromatographic conditions as for the mycotoxin and its metabolite.

2.6. LC–MS/MS analysis

Also liquid chromatography with mass spectrometry was used to measure OTA and its metabolites in plasma and urine samples. Detection was performed with a Varian 1200L Quadrupol MS/MS equipped with a Turbo Ion Spray electrospray ionization (ESI) source and a Prostar® Varian HPLC system. The following settings were used: nitrogen as drying gas (19 psi), gas temperature 250 °C, and argon used as collision gas (2.0 mTorr). Chromatographic separation was carried out at 21 °C on a Nucleosil® 100-5 C18 HD 125 mm × 3 mm column. Water (mobile phase A) and methanol (mobile phase B) were used as eluents in the following gradient: 0–2 min 10% B, 2–4 min 40% B, 4–9 min 60% B, 9–12 min 100% B (column wash), 12–14 min 100–10% B, and from 14 to 20 min 10% B (re-equilibration). The flow rate was 0.3 mL/min. ESI-MS/MS was executed in a multiple reaction monitoring (MRM) in negative ion mode. The specific transition of precursor ion and product ion were:

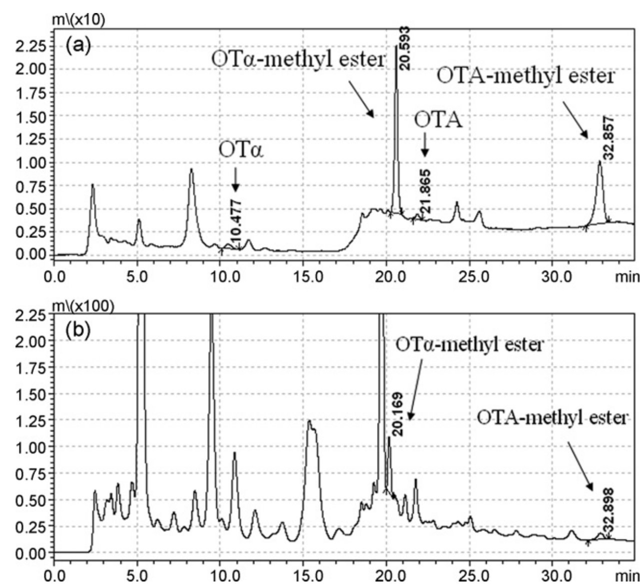

Fig. 2. Chromatogram of a standard (a) (2.5 ng/mL) and a urine sample (b) (spiked 0.5 ng/mL) after the methyl ester derivatization.

255 → 211 *m*/*z* for OTα, which represents the loss of the carboxyl group. The optimized collision energy (CE) was 16 eV. For OTA, the transitions of precursor and product ions were 402 → 358 *m*/*z* with an optimized CE of 16 eV. The retention time for OTα and OTA were 4.8 and 8.4 min.

3. Results and discussion

3.1. Validation parameters

The validation process was done using a blank urine sample with no detectable OTA; in the case of human plasma, one with a very low background (<0.1 ng/mL) was used. The calibration curves show a linear trend in the range of 0.10–10 ng/mL (quantification range), with a coefficient of determination (R^2) of 0.9999 and 0.9961 for OTα and OTA, respectively. Sensitivity was estimated by the minimum detected level in spiked samples: The limit of detection (LOD) and limit of quantification (LOQ) were determined based on the lowest quantity of analyte that can be clearly distinguished (LOD) from background (S/N = 3) or quantified (LOQ; S/N > 6) in both plasma and urine samples. For OTα and OTA the LOD in urine was 0.02 ng/mL and the LOQ was 0.05 ng/mL. The values for OTα and OTA in plasma were 0.07 ng/mL (LOD) and 0.1 ng/mL (LOQ). Recovery of analytes with LLE (Section 2.4) was assessed at three levels in plasma and urine samples (Table 1) with mean values of 88% and 94% for OTA in urine and plasma. For OTα the recovery levels were similar, with mean values of 78% and 104% in urine and in plasma. Reproducibility was determined in intra- and inter-day assays at a

Table 1
Recovery of OTA and OTα in plasma and urine samples with LLE.

Spike level (ng/mL)	OTα		OTA	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
Urine				
0.05 (*n* = 3)	89.9	6.8	85.6	9.5
0.5 (*n* = 3)	74.5	3.2	104	13
2.0 (*n* = 3)	71.2	5.1	74.4	4.2
Plasma				
0.1 (*n* = 3)	94	12	92	5.3
1.0 (*n* = 3)	117	12	85	9.3
2.0 (*n* = 3)	101	7.5	106	3.4

Table 2
Reproducibility intra- and inter-day.

Spike level	OTα			OTA		
	Mean ± SD	Recovery (%)	RSD%	Mean ± SD	Recovery (%)	RSD%
Urine intra-day (n = 10) 0.25 ng/mL	0.17 ± 0.01	66	8.6	0.20 ± 0.02	81	5.6
Urine inter-day (n = 10) 0.25 ng/mL	0.21 ± 0.04	83	8.8	0.33 ± 0.02	112	4.7
Plasma intra-day (n = 9) 1.0 ng/mL	0.52 ± 0.04	52	7.8	1.08 ± 0.08	103	7.9
Plasma inter-day (n = 9) 1.0 ng/mL	0.67 ± 0.09	67	13	0.78 ± 0.05	78	6.6

Table 3
Recovery for OTA with IAC clean-up.

Concentration (ng/mL)	Mean ± SD	Recovery (%)	RSD%
0.05 (n = 4)	0.04 ± 0.01	75	8.5
0.25 (n = 4)	0.22 ± 0.01	88	3.5
0.5 (n = 4)	0.46 ± 0.02	85	5.0
1.0 (n = 4)	0.85 ± 0.04	85	4.4

concentration of 0.25 ng OTα and OTA/mL (n = 10) for urine samples, and 1.0 ng/mL (n = 9) for plasma samples (Table 2). The RSD values for OTA and OTα were lower than 13% in urine and plasma.

The clean-up procedure with IAC Ochratest®, and analysis of extracts with HPLC and fluorescence detection (see Section 2.5), generated cleaner chromatograms, and no other interfering peaks were observed close to OTA. In relation to the validation parameters, the clean-up with IAC showed a lower variation for OTA than the LLE method (RSD <5%) whilst the recovery levels were similar or even inferior at low spike levels (Table 3). The validation values obtained in this study with IAC are similar to those reported by others [11,20]. But, IAC clean-up is only suitable for OTA analysis; OTα is not retained on the column.

According to this result, the liquid–liquid extraction (LLE) method offers more attractive possibilities for biomonitoring in urine and in plasma, since also more polar metabolites such as OTα are extracted in parallel with OTA. Finally, the method achieved a recovery of 114% for OTα and 104% for OTA in a range between 0.05 and 2.0 ng/mL (n = 9) in urine with a RSD 14% and 4.8%, respectively. In plasma, the mean recovery was 90% and 91% for OTα and OTA (n = 9, range: 0.1–1.0 ng/mL) with a RSD of 7.5% and 5.3%, respectively.

3.2. Pilot study

OTα and OTA were determined in biological samples from a small cohort of 13 volunteers. Urine and plasma were processed using the LLE method described above, with and without preceding conjugate hydrolysis of parallel samples. Quantification of the analytes was done by interpolation on the calibration curve. All urine and plasma samples (100%) were positive for OTA and OTα. As only 3 of 13 urine samples showed values between the LOD and LOQ for OTA, no statistical adjustment was applied to these values. Table 4 summarizes the concentrations of OTA and of OTα aglycone, determined without cleavage of conjugates, and also values for OTα total (aglycone plus conjugates) as measured upon enzymatic hydrolysis of sample extracts by HPLC with fluorescence detection (Section 2.5). As described in Section 3.3, we found no evidence of OTA conjugates; the concentrations after enzyme (glucuronidase/sulfatase) treatment were not higher than those of OTA measured upon direct extraction. By contrast to OTA, the levels of its metabolite OTα

Table 4
Analyte concentrations in plasma and urine samples.

	Mean ± SD (ng/mL)	Median (ng/mL)	Range (ng/mL)
Urine men (n = 6)			
OTA	0.08 ± 0.05	0.10	0.04–0.14
$OTα_{(Aglycone)}$	1.31 ± 1.68	0.50	0.05–4.70
$OTα_{(Total)}$	2.21 ± 1.34	2.80	0.74–4.06
Urine women (n = 7)			
OTA	0.05 ± 0.05	0.04	0.02–0.13
$OTα_{(Aglycone)}$	0.99 ± 0.37	1.00	0.42–1.54
$OTα_{(Total)}$	3.46 ± 2.75	5.27	0.49–7.12
All urine samples (n = 13)			
OTA	0.07 ± 0.05	0.05	0.02–0-14
$OTα_{(Aglycone)}$	1.14 ± 1.17	0.92	0.05–4.70
$OTα_{(Total)}$	2.88 ± 2.24	2.09	0.49–7.12
Plasma men (n = 6)			
OTA	0.26 ± 0.10	0.28	0.20–0.29
$OTα_{(Aglycone)}$	0.10 ± 0.05	0.09	0.07–0.17
$OTα_{(Total)}$	1.03 ± 0.37	1.01	0.65–1.64
Plasma women (n = 7)			
OTA	0.23 ± 0.03	0.23	0.19–0.27
$OTα_{(Aglycone)}$	0.08 ± 0.03	0.08	0.03–0.13
$OTα_{(Total)}$	0.88 ± 0.54	1.18	0.07–1.29
All plasma samples (n = 13)			
OTA	0.25 ± 0.03	0.24	0.19–0.29
$OTα_{(Aglycone)}$	0.09 ± 0.03	0.08	0.03–0.17
$OTα_{(Total)}$	0.95 ± 0.46	1.14	0.07–1.64

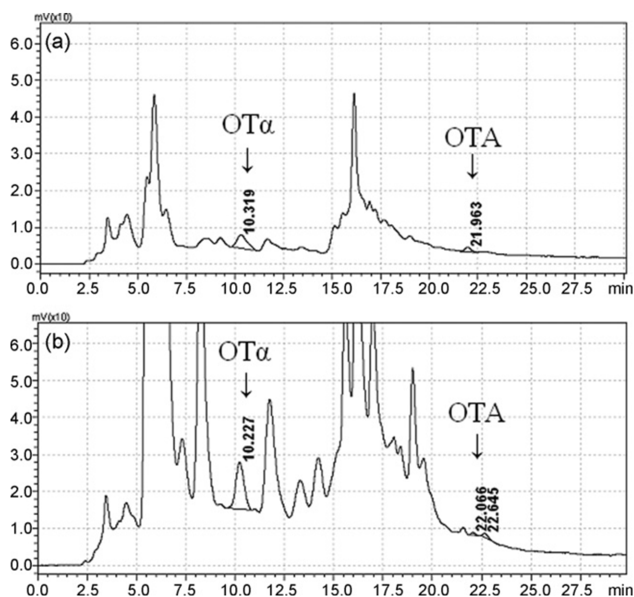

Fig. 3. Chromatogram of a urine sample extracted directly (a), and after treatment with β-glucuronidase/sulfatase (b) by HPLC/fluorescence analysis.

increased considerably upon conjugate hydrolysis, in both urine and plasma samples.

In plasma samples (without conjugate hydrolysis), OTA was the predominant form in women (0.23 ± 0.03 ng/mL) and men (0.26 ± 0.10 ng/mL). OTA levels in men were slightly higher than those in women, but this difference was not statistically significant ($p < 0.05$). The OTα mean aglycone concentrations (0.10 ng/mL for men and 0.08 ng/mL for women) were lower than those for parent compound (Table 4). However, after pre-treatment with β-glucuronidase/sulfatase rather high concentrations of OTα total (1.03 ng/mL for men and 0.88 ng/mL for women) were detected. This is clear indication of considerable levels of OTα conjugates in blood, with OTα aglycone accounting for about 10% of OTα total (Table 4).

In urine samples (without conjugate hydrolysis), OTα was the major metabolite, with average aglycone concentrations (mean value 1.00 ± 0.37 ng/mL for women, and 1.31 ± 1.68 ng/mL for men), about 16–20 times higher than those for OTA (Table 4). No major differences were observed for OTA levels in urine from women (0.05 ± 0.05 ng/mL) and men (0.09 ± 0.05 ng/mL). Whilst pre-treatment with β-glucuronidase/sulfatase enzyme did not affect urinary OTA concentrations, the median levels of OTα were clearly increased (about fivefold) when comparing the aglycone and total concentration in urines of males and females (Table 4).

3.3. *Results with β-glucuronidase/sulfatase pre-treatment and analysis by LC–MS/MS*

Aliquots of all plasma and urine samples were incubated with conjugate cleaving enzyme (see Section 2.3). After enzymatic treatment and extraction of these matrices, the chromatograms showed a different pattern. Several new peaks were observed in urine and in plasma, and also clear increases in the peak intensity of the metabolite OTα, as illustrated in Fig. 2 for urine sample extracts before and after enzymatic cleavage. In the case of OTA no increase in peak intensity was observed after β-glucuronidase/sulfatase treatment, neither in urine nor in plasma. Peak identification, based on retention times, was confirmed by standard addition, and also by independent analysis with LC–MS/MS.

All samples were confirmed as positive for OTA and OTα by LC–MS/MS analysis (as described in Section 2.6). No 'false positives'

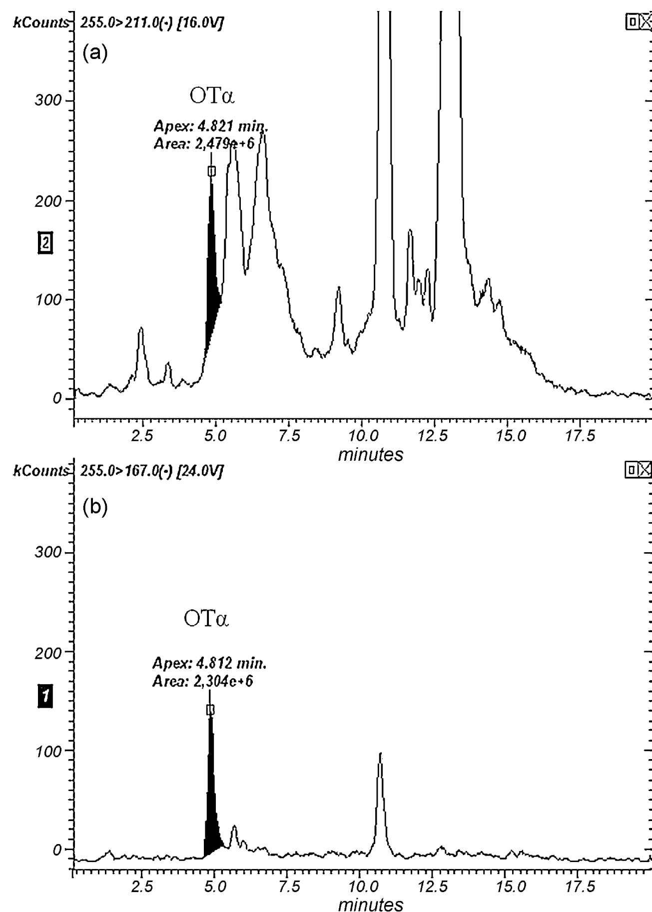

Fig. 4. LC–MS/MS analysis of a naturally contaminated urine sample (concentration: 0.44 ng OTα/mL) using mass transition (a) 255 → 211 *m*/*z* and (b) 255 → 167 *m*/*z*.

were observed, and the absence of OTA-glucuronide conjugate was also confirmed. When urine and plasma samples were analyzed with LC–MS/MS some interfering peaks were observed close to OTα, especially in urine samples (Fig. 3a). To avoid false positive results, a second product ion was used (255 → 167 *m*/*z*, CE 24 eV). With the second product ion (167 *m*/*z*), the analysis of OTα was more specific and no interferences are observed adjacent to OTα (Fig. 3b).

3.4. *Pattern of metabolites and implications for OTA biomonitoring*

The results of our pilot study are of interest in several respects: (i) the tested method for biomonitoring of OTA allowed the detection of parent compound and main metabolite OTα in all of urine and plasma samples from our small cohort; (ii) liquid–liquid extraction for clean-up and enzymatic pre-treatment of samples revealed the presence of relatively high levels of OTα. The occurrence of this detoxication product and its conjugate has not been described so far in humans.[1] Moreover, (iii) whilst Pena et al. [19] suggested the presence of OTA glucuronide in human urine (based on indirect evidence), this conjugate was not detected by others [20,28]. In line with the latter, we were unable to find convincing evidence for OTA glucuronide in our samples. Application of our method in a pilot study, with parallel sampling of blood and urine, provides not only novel data on levels of OTA/OTα in these samples, but also (iv)

[1] The major metabolite OTα would escape detection when immunoaffinity columns, specific for OTA, are used in sample clean-up.

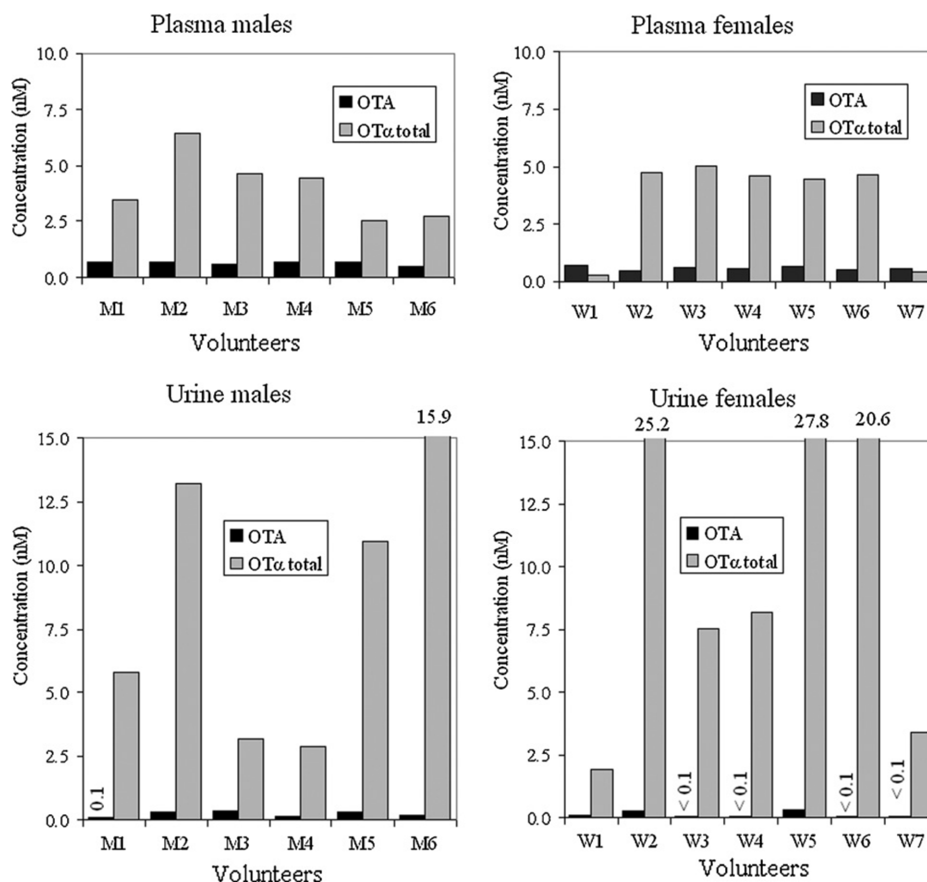

Fig. 5. Biomonitoring results of 13 volunteers: OTA and OTα levels in plasma and urine.

some clues on the interindividual variability of the analytes, and their relative amounts in the different biological matrices.

The results for all sample donors are depicted in Fig. 5. Levels detected in urine and plasma are given as nanomolar concentrations (more appropriate when ratios of parent compound and metabolite are considered). Because of the polar properties of OTα and its glucuronide conjugate, it is expected that these metabolites are more efficiently cleared from the circulation than OTA [6]. This view is in line with kinetic studies in rats [21,22], and our observations on the renal excretion of OTA and its metabolite in human volunteers.

Interestingly, the graphs show considerable interindividual variation in the levels of total OTα, especially in urines of males and females. This may be related to interindividual differences in OTA degradation by gastrointestinal microbial or mammalian hydrolytic enzymes, known to convert OTA to OTα [29,30,31]. As urinary OTA concentrations are rather low (in comparison to OTA concentrations in plasma), simultaneous analysis of the major metabolite OTα can increase sensitivity and facilitate future biomonitoring studies with non-invasive sampling. OTα may also serve as an additional biomarker in analyses of urine. Finally, in sample preparation the use of deconjugating enzymes which cleave phase 2 metabolites (glucuronide and/or sulfate conjugates) is recommended as this provides a more complete picture of mycotoxin biotransformation products present in biological samples (Fig. 5).

4. Conclusions

- The method presented here involves sample clean-up by liquid–liquid extraction (with or without preceding cleavage of conjugates) and HPLC analysis with fluorescence detection. It has been validated and allows sensitive detection of parent compound along with its detoxication product OTα in human urine and plasma samples. As the method does not require sophisticated lab equipment, it can be also used to monitor ochratoxin exposure in countries with no access to more expensive instruments.
- Application of this method in a pilot study (with 13 volunteers) revealed the presence of OTA and OTα in all samples. In line with negative findings of others [20,28], an OTA glucuronide was not detected, neither in urine nor in plasma. By contrast, conjugates of OTα are major products in these samples; most likely OTα-glucuronide and possibly also OTα-sulfate.
- From OTA concentrations measured in plasma and urine samples, it is concluded that the donors were exposed to low dietary mycotoxin levels since the values (Table 4) are in the range reported in the literature [11,13] for other cohorts with low OTA intake.
- This is the first report on the presence of OTα in urine or plasma of humans. As OTα represents a large fraction of ingested mycotoxin, we propose to include analyses of this metabolite in future biomonitoring studies, also in light of the observed variations for urine OTα-levels that suggest different interindividual abilities for OTA-detoxification in humans.

Acknowledgements

The authors thank Gabi Baumhoer and Iris Glaeser for the technical help. This work was supported by a DAAD-CONICYT scholarship to K.M.

References

[1] JEFCA (Joint FAO/WHO Expert Committee on Food Additives), Ochratoxin A, Safety Evaluation of Certain Mycotoxins in Food; WHO Food Additives Series 47; FAO Food and Nutrition Paper 74, WHO, Geneva, Switzerland, 2001, p. 281.

[2] EFSA (European Food Safety Authority), Opinion of the scientific panel on contaminants in the food chain on a request from the commission related to ochratoxin A in food, EFSA J. 365 (2006) 1.
[3] IARC (International Agency for Research on Cancer), Monograph on the Evaluation of Carcinogenic Risk to Humans. Some Naturally Occurring Substances: Food Items and Constituents, Heterocyclic Aromatic Amines and Mycotoxins, IARC, Lyon, 1993, p. 489.
[4] M. Castegnaro, D. Canadas, T. Vrabcheva, T. Petkova-Bocharova, I.N. Chernozemsky, A. Pfohl-Leszkowicz, Mol. Nutr. Food Res. 50 (2006) 519.
[5] E. Petzinger, K. Ziegler, J. Vet. Pharmacol. Ther. 23 (2000) 91.
[6] D. Ringot, A. Chango, Y.-J. Schneider, Y. Larondelle, Chem. Biol. Interact. 159 (2006) 18.
[7] S. Hagelberg, K. Hult, R. Fuchs, J. Appl. Toxicol. 9 (1989) 91.
[8] I. Studer-Rohr, J. Schlatter, D.R. Dietrich, Arch. Toxicol. 74 (2000) 499.
[9] M. Aslam, A.E. Beg, M. Blaszkewicz, G.H. Degen, K. Golka, Mycotoxin Res. 21 (2005) 164.
[10] K. Muñoz, M. Vega, G. Rios, S. Muñoz, R. Madariaga, Food Chem. Toxicol. 44 (2006) 1884.
[11] P.M. Scott, Food Addit. Contam. 1 (2005) 99.
[12] H. Rosner, B. Rohrmann, G. Peiker, Arch. Lebensmittelhyg. 51 (2000) 104.
[13] European Commission, Directorate General-Health and Consumer Protection, Assessment of Dietary Intake of Ochratoxin A by the Population of European Union Members States, SCOOP Report of Experts Participating in Task 3.2.7, 2002, p. 18.
[14] G.H. Degen, M. Blaszkewicz, Y. Lektarau, C. Grüner, Mycotoxin Res. 19 (2003) 3.
[15] G.H. Degen, J. Meifort, M. Blaszkewicz, Mycotoxin Res. 21 (2005) 168.
[16] G.H. Degen, S. Mayer, M. Blaszkewicz, Mycotoxin Res. 23 (2007) 88.
[17] J. Gilbert, P. Brereton, S. MacDonald, Food Addit. Contam. 18 (2001) 1088.
[18] M. Castegnaro, V. Maru, T. Petkova-Bocharova, I. Nikolov, H. Bartsch, IARC Sci. Publ. 115 (1991) 165.
[19] A. Pena, M. Seifrtová, C. Lino, I. Silveira, P. Solich, Food Chem. Toxicol. 22 (2006) 1449.
[20] H. Valenta, J. Chromatogr. A 815 (1998) 75.
[21] S. Li, R.R. Marquardt, A.A. Frohlich, Food Chem. Toxicol. 38 (2000) 141.
[22] H. Zepnik, W. Völkel, W. Dekant, Toxicol. Appl. Pharmacol. 192 (2003) 36.
[23] K. Gross-Steinmeyer, J. Weymann, H.G. Hege, M. Metzler, J. Agric. Food Chem. 50 (2002) 935.
[24] I. Kühn, H. Valenta, K. Rohr, J. Chromatogr. B 668 (1995) 333.
[25] A.E. Pohland, S. Nesheim, L. Friedman, Pure Appl. Chem. 64 (1992) 1029.
[26] H. Xiao, S. Madhyastha, R.R. Marquardt, S. Li, J.K. Vodela, A.A. Frohlich, W. Kemppainen, Toxicol. Appl. Pharmacol. 137 (1996) 182.
[27] B. Zimmerli, R. Dick, J. Chromatogr. B: Biomed. Appl. 666 (1995) 85.
[28] R. Vatinno, A. Aresta, C.G. Zambonin, F. Palmisano, J. Pharm. Biomed. Anal. 44 (2007) 1014.
[29] M.J. Pitout, Biochem. Pharmacol. 18 (1969) 485.
[30] R.C. Doster, R.O. Sinnhuber, Food Cosmet. Toxicol. 10 (1972) 389.
[31] M.S. Madhyastha, R.R. Marquardt, A.A. Frohlich, Arch. Environ. Contam. Toxicol. 23 (1992) 468.
[32] Munoz, et al., Naunyn-Schmiedeberg's Arch. Pharm. Toxicol. 379 (Suppl. 1) (2009) 89 (#447).
[33] Munoz et al., 31st Mycotoxin Workshop, 15.-17.06.2009, Münster, Germany, Conference Abstracts, 2009 p. 50.