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Short communication

Determination of ochratoxin A in human urine by solid-phase microextraction coupled with liquid chromatography-fluorescence detection

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Abstract

A new method for the determination of ochratoxin A (OTA) in human urine samples has been developed using solid-phase microextraction (SPME) interfaced with liquid chromatography-fluorescence detection (LC-FD). This method is simpler and cheaper compared to the most widely adopted clean-up procedures for OTA extraction from urine (usually based on immunoaffinity columns).

Briefly, urine samples, diluted 1:5 with phosphate buffer (10 mM, pH 3), were partitioned against chloroform and the aqueous phase extracted by a polydimethylsiloxane/divinylbenzene (PDMS/DVB) fiber. The fiber was then “statically” desorbed, through a SPME interface, into a LC system operating in isocratic conditions.

The linear range investigated in urine was 0.01–1 ng/ml. Within-day R.S.D.% in urine spiked at 0.1 and 1 ng/ml were 3.9 and 1.9, respectively, whereas the between-days R.S.D.% were 5.5 and 3.0, respectively.

The limits of detection (LOD) and quantitation (LOQ) calculated at a signal-to-noise ratio of 3 and 10 (noise calculated peak to peak on a blank chromatogram at the OTA retention time) were 0.01 and 0.05 ng/ml, respectively.
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1. Introduction

Ochratoxins are a group of structurally related secondary metabolites produced by some toxigenic fungi such as *Penicillium verrucosum*, *Aspergillus ochraceus*, and *Aspergillus niger* [1]. Among them, ochratoxin A (OTA, 7-(L-α-phenylalanylcarbonyl) carboxyl-5-chloro-8-hydroxy-3,4-dihydro-3*R*-methylisocumarin) is the one of major toxicological concern. It possesses nephrotoxic, carcinogenic and immunosuppressive properties and causes kidney and liver tumours in mice and rats [2].

As far as humans are concerned, the International Agency for Research on Cancer (IARC) classified OTA in Group 2B (possible carcinogen to humans) [3]. With regard to its nephrotoxicity, OTA is considered to be involved in severe kidney pathology (the Balkan endemic nephropathy—BEN), possibly linked to urinary tracts tumors [4]. It is also a compound with unusually long serum half-life [5] due to binding to plasma proteins [6], enterohepatic circulation [7], and re-absorption from urine [8]. OTA is eventually eliminated via bile and urine [9].

OTA is generally found in several food commodities [10] such as cereals, oleaginous seeds, green coffee, wine, meat, cocoa, spices, etc., at concentration levels that depend upon both environmental and processing conditions. The intake of OTA by contaminated feed may lead to residues in blood, kidney and liver of pigs and poultry and to a lesser extent in muscles, adipose tissue and eggs. Thus, also products of animal origin can contribute to the OTA-intake of humans [11].

Measurement of OTA in urine could be definitely a good marker of human exposure to this mycotoxin and studies are less invasive than blood analysis [12]. Notwithstanding, literature about OTA analysis in urine is relatively scarce [13–17],

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likely because the low concentration of OTA in urine requires accurate and sensitive methods for its qualitative and quantitative determination.

The most widely used technique for OTA quantitation in various matrices is reversed phase liquid chromatography with fluorescence detection (LC-FD) after a suitable sample extraction/clean-up step. Domijan et al. [18] have recently described two different clean-up procedures for LC-FD determination of OTA in urine. The first one is based on liquid–liquid extraction followed by purification of the extract by solid-phase extraction (SPE). In the second procedure, urine samples were loaded onto a ChemElut disposable column and extracted with acidic chloroform. The SPE and ChemElut eluates were evaporated to dryness and reconstituted in mobile phase prior to LC-FD analysis. The two procedures gave limits of quantitation (LOQ) of 1.5 and 0.9 ng/ml, respectively, that appears not adequate considering the OTA urinary levels normally found (see later). Slightly lower LOQ (i.e. 0.2 ng/ml) were reported by Jonsyn-Ellis [19] using solvent extraction followed by extract clean-up on silica gel column. Pascale and Visconti [20] extended the use of a clean-up step by immuno affinity column (IAC), normally employed for the analysis of several mycotoxins in food, to OTA determination in urine. Human urine were diluted with 5% $NaHCO_3$ solution, filtered and loaded on IAC. After a washing step, OTA was eluted, the eluate evaporated to dryness and reconstituted with mobile phase before LC-FD. Due to the selective preconcentration provided by IAC, a limit of detection (LOD) of 0.005 ng/ml was claimed (recovery ranged from 88 to 93% at OTA levels ranging from 0.05 to 1 ng/ml). A solvent ($CHCl_3$) extraction step was introduced by Petkova-Bocharova et al. [21] prior to IAC clean-up: in spite of the larger preconcentration factor achieved, the improvement in LOQ was quite limited while the recovery (at OTA levels of 0.4 ng/ml) was lowered to about 70%. The method described by Pascale and Visconti [20] has been recently employed by Pena et al. [14] for the estimation of OTA occurrence in urine of Coimbra (Portugal) inhabitants. A LOQ (S/N = 10) of 0.02 ng/ml (corresponding to 1.3 ng/ml or 65 pg injected on column) was claimed that was apparently conflicting with the OTA chromatogram relevant to the injection of 10 ng/ml of an OTA standard (500 pg on column). Calibration curve was generated using OTA standard solutions in the range 1–10 ng/ml (corresponding to 0.015–0.15 ng/ml in urine). Beside the doubtful correctness of this calibration mode, the upper limit of the linearity range appears inadequate for inhabitants from areas with Balkan Endemic Nephropathy [21] or patients affected by karyomegalic interstitial nephritis [20].

As it can be argued after the paper of Pascale and Visconti [20] no new and effective methodological approach has been presented for OTA determination in human urine. The attempt of Domijan et al. [18] to avoid the use of IAC's was practically unsuccessful due to inadequate LOQ and the poor extent of sample purification achieved (see for instance chromatograms in Fig. 1 of the relevant reference).

A possible alternative to IAC for OTA extraction from urine could be represented by solid-phase microextraction (SPME) [22]. This solventless extraction technique has been mainly applied in combination with GC; however, a growing interest for SPME coupled to LC was observed in the past few years [23], including some applications from our laboratory in the field of mycotoxins analysis in food [24–27] and drugs analysis in urine [28,29].

In this work, SPME coupled to LC-FD has been applied to the determination of OTA in human urine samples. LOD and LOQ values comparable to those obtained by IAC can be achieved. Steps such as solvent evaporation and residue reconstitution in a low volume of mobile phase (that in absence of an internal standard become critical points) are avoided. One fiber can be reused for more than hundred determinations thus minimizing the cost per analysis.

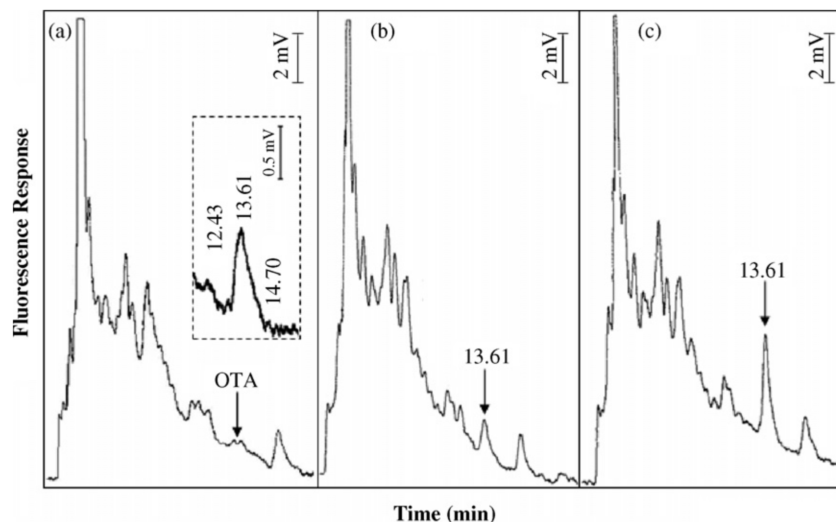

Fig. 1. SPME–LC-FD chromatograms relevant to three human urine samples: (a) unspiked sample, (b) and (c) samples spiked with OTA at the concentration level of 0.01 and 0.03 ng/ml, respectively. The inset shows an LC-FD chromatogram obtained on the same urine sample of (a) after a clean-up step by immunoaffinity column. For chromatographic and detection conditions see Section 2.

2. Experimental

2.1. Chemicals

OTA was obtained from Sigma (St. Louis, MO, USA). Stock standard solutions were prepared in methanol at approximately 1 mg/ml; the actual concentration was calculated by UV spectrophotometry assuming a molar absorption coefficient of 6640 M^{-1} cm^{-1} at 333 nm. All the other chemicals used were of analytical grade. More diluted solutions were prepared in phosphate buffer (10 mM, pH 3) before use.

Immunoaffinity columns (Ochraprep, Rhone Diagnostics) were obtained from OR-SELL (Carpi, Italy). β-Glucuronidase from EC 3.2.1.31 (type B-1 from bovine liver) was obtained from Sigma–Aldrich.

2.2. Apparatus

The SPME interface (Supelco, Bellefonte, PA), consisted [29] of a standard six-port Rheodyne valve equipped with a fiber desorption chamber (60 μl volume), installed in place of the sample loop. The LC apparatus consisted of a Dionex P680 LPG pump equipped with a vacuum membrane degasser, a Rheodyne 7125 injection valve (connected in series to the SPME interface) fitted with a 50 μl loop and a Supelcosil LC-18 DB (150 mm × 4.6 mm) chromatographic column. The fluorescence detector was a Jasco model FP-2020 Plus connected to a Hewlett & Packard 3395 computing integrator.

2.3. Chromatographic and detection conditions

The optimized mobile phase was a water/acetonitrile/acetic acid mixture (111 + 87 + 2, v/v/v). The flow rate was 1 ml min^{-1} and temperature was ambient. Fluorescence excitation and emission wavelengths were 332 nm (4 nm bandwidth) and 460 nm (18 nm bandwidth), respectively.

2.4. Solid-phase microextraction

Silica fibers (Supelco) with three different coatings, i.e. 85 μm thick polyacrylate (PA) film, 50 μm thick Carbowax/Template Resin (CW/TPR-100) film and 60 μm thick polydimethylsiloxane/divinylbenzene (PDMS/DVB) film, were employed for comparative studies. A manual SPME device (Supelco) was used to hold the fibers. The extraction was carried out (on human urine samples processed as described in Section 2.5) under magnetic stirring for 60 min at room temperature. OTA desorption into the SPME–LC interface was performed in the "*static desorption*" mode by soaking the fiber in mobile phase for 60 s. Then, the injection valve was changed to the inject position and the fiber was exposed for 10 s to the mobile phase stream. In order to avoid possible memory effects the fiber was fully desorbed, before the next extraction, under stirring in 3 ml of fresh mobile phase and flushing it with distilled water.

2.5. Human urine samples

Human urine samples from healthy donors ($n = 10$) were centrifuged for 15 min at 5000 rpm, followed by filtering through a 0.45 μm Millex-HV type filter (Millipore). NaOH 1 M was added to reach a final pH ≥ 8. Samples (2.5 ml) were first partitioned against $CHCl_3$ (5 ml) in order to reduce matrix interferences. The mixture was, then, centrifuged at 5000 rpm for 15 min and the organic phase (chloroform) discarded. The aqueous phase was acidified with 37% HCl and finally diluted 1:5 with phosphate buffer (10 mM, pH 3). A 3 ml aliquot was transferred into a 5 ml clear vial (Supelco), the vial sealed with hole caps and Teflon-faced silicone septa (Supelco) and subjected to SPME (extraction time 1 h).

Recoveries were calculated as peak area ratio of OTA (standard solution in phosphate buffer, 10 mM, pH 3)/OTA (spiked urine samples). Urine samples were spiked with OTA in order to reach 0.1 and 1 ng/ml concentration levels.

Calibration curves in urine were constructed using OTA-free urine samples spiked with variable amounts of the toxin in order to obtain the following concentration levels: 0.01, 0.05, 0.1, 0.25, 0.5 and 1 ng/ml. Three replicates for each concentration were performed.

The within-day ($n = 5$) and between-days ($n = 3$ over 5 days) coefficients of variation were calculated on urine samples spiked at 0.1 and 1 ng/ml.

Clean-up by immunoaffinity columns was performed as previously described [18]; briefly, urine sample was diluted 1 + 1 (v + v) with a water solution of 5% $NaHCO_3$. Ten millilitres of diluted sample was loaded onto a Ochraprep column, the column washed and finally eluted with 2 ml of MeOH. The methanolic extract was evaporated to dryness, reconstituted with 500 μl of mobile phase and 20 μl injected.

Enzymatic hydrolysis were performed as follows: 5 ml of acetate buffer (1 M, pH 5.0) containing 9000 U of β-glucuronidase were added to 10 ml of urine, and incubated overnight (ca. 17 h) at 37 °C. Then, 5 ml of the resulting mixture were processed as previously described.

3. Results and discussion

The effect of the most important parameters (e.g., extraction time, temperature, pH, ionic strength) influencing the extraction efficiency of OTA from beer and wine has been extensively discussed elsewhere [26,27]. A similar behavior was observed in the case of urine samples; the influence of each parameter is summarized as follows. Extraction time: significant (equilibrium reached at $t \geq 16$ h; time optimized for a satisfactory extraction: 1 h); ionic strength: significant (since an increase in the ionic strength was found to improve also the extraction of interfering compounds, no ionic strength adjustment was performed, i.e. the final ionic strength was that resulting from urine dilution with phosphate buffer—see Section 2); pH: significant (efficiency decreasing on increasing pH values; best extraction efficiency at pH ≤ 3); extraction temperature: significant (explored range from 25 to 50 °C; efficiency decreasing on increasing temperature; best extraction efficiency at 25 °C). As

for the choice of the fiber it was proved that the extraction efficiency of the PDMS/DVB coating was better than that of PA and CW/TPR-100. PDMS/DVB fibers were then chosen for further investigations. Desorption conditions: fiber soaking in mobile phase for 60 s ("*static desorption*" step), then fiber exposure to the mobile phase stream for 10 s ("*dynamic desorption*" step).

Peak areas obtained from spiked urine samples were lower (76.0 ± 3.5)% than those obtained, under the same experimental conditions, for extraction from a standard solution (OTA levels ranging from 0.1 to 1 ng/ml) indicating the presence of matrix effects. These effects can be likely explained considering that adsorption is the prevailing extraction mechanism in mixed coatings with a porous solid as the primary extraction phase; since adsorption is a competitive process, the presence of co-extracted interferences can reduce the amount of analyte extracted. Matrix effects could be compensated for by a calibration plot constructed using spiked urine samples as calibration standards (see later).

The response of the developed SPME–LC procedure was linear in the range 0.01–1 ng/ml and the unweighted regression line of peak area (arbitrary unit) versus [OTA] (ng/ml) was described by the following equation:

$$A = (0.278 \pm 0.17) + (22.42 \pm 0.39)C$$

with $R^2 = 0.999$ and a standard error of regression $s_{y/x} = 0.35$.

The within-day ($n = 5$) precision of the method, calculated on a urine sample spiked at a concentration level of 0.1 and 1 ng/ml, were 3.9 and 1.9%, respectively; the day-to-day ($n = 5$ on 5 days) precision of the method (calculated at the same concentration levels) were 5.5 and 3.0%, respectively.

The limits of detection (LOD) and quantitation (LOQ) calculated at a signal-to-noise ratio of 3 and 10 (noise calculated peak to peak on a blank chromatogram at the OTA retention time) were 0.01 and 0.05 ng/ml, respectively.

Fig. 1 reports the SPME–LC-FD chromatograms obtained on an unspiked urine sample (Fig. 1a), and on urine samples spiked with OTA at the concentration levels of 0.01 and 0.03 ng/ml (Fig. 1b and c, respectively). As apparent, the optimized mobile phase allowed a good separation of OTA from matrix components with a simple isocratic elution in less then 20 min.

As can be inferred, for instance, by the chromatogram of Fig. 1a, a peak eluting at the same retention time of OTA (but at a very low S/N ratio) was sometimes observed (in 7 of the 10 sample analyzed). The chromatographic behavior of this peak was identical to that of OTA, even under several gradient elution programs tested. So, it can be reasonably hypothesized that the peak in unspiked urine samples originates from OTA background level. The difficulty in finding a true blank sample when using method possessing low limits of detection (LOD) is not surprising [23]. The above hypothesis could be readily verified using a sample clean-up step by immunoaffinity column (see Section 2) that provides a 10-fold preconcentration factor. The inset in Fig. 1 (reporting the LC-FD chromatogram relevant to the same urine sample of Fig. 1a after the immunoaffinity column clean-up step) clearly shows that the peak at 13.61 min in Fig. 1a is to be likely ascribed to a natural OTA contamination of the analyzed urine.

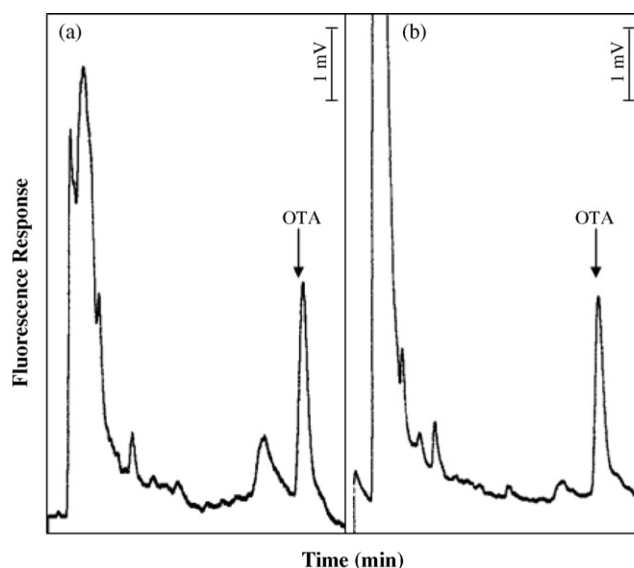

Fig. 2. LC-FD chromatograms relevant to two aliquots of the same urine sample, naturally contaminated with OTA, (a) before enzymatic hydrolysis and (b) after enzymatic hydrolysis. Both aliquots were subjected to a clean-up step by immunoaffinity column. For extraction, chromatographic and detection conditions see Section 2.

It is well known that xenobiotics [29], including mycotoxins, are typically transformed "in vivo", e.g. by glucuronidation, into more water-soluble compounds that can be readily excreted from the body in urine. As far as OTA is concerned, only two controversial works are available in the literature. Orti et al. [13] reported a solid-phase extraction of hydrolyzed human urine samples followed by LC-FD analysis for the determination of some mycotoxins in urine. While glucuronic conjugates of aflatoxins seemed to be present, no evidences of OTA glucuronidation was observed. On the contrary, the occurrence of OTA conjugates with glucuronic acid have been recently claimed for the first time by Pena et al. [14]. In the present work, enzymatic hydrolysis was performed as described in Section 2 in order to indirectly obtain information about the presence of OTA glucuronides, by observing the eventual increase of the chromatographic peak of the parent compound before and after the hydrolysis step. Fig. 2a and b, reports the LC-FD chromatograms relevant to two aliquots of the same urine sample, naturally contaminated with OTA, before and after enzymatic hydrolysis, respectively. Both aliquots were subjected to a clean-up step by immunoaffinity column. It was found that the OTA peak remained practically unchanged after enzymatic hydrolysis. These experimental evidence seems to suggest the absence of an OTA glucuronidation pathway.

4. Conclusions

A SPME–LC-FD method for the determination of OTA in human urine samples has been developed for the first time. The LOD and the LOQ of the method, i.e. 0.01 and 0.05 ng/ml, respectively, were definitely good compared to existing literature on the same topic (see Table 1). An isocratic elution permitted a simple (20 min) LC separation of the target mycotoxin. Finally it

Table 1
Comparison between different extraction clean-up methods for OTA determination in human urine by LC-FD

	LOD (ng/ml)	LOQ (ng/ml)	Linearity range (ng/ml)	Recovery (%)	Reference
IAC	0.005	n.a.	0.05–1	88–93% [OTA] ranging from 0.05 to 1 ng/ml	[20]
IAC	n.a.	0.02	0.015–0.15	91–96% at [OTA] ranging from 0.02 to 0.5 ng/ml	[14]
Solvent ($CHCl_3$) extraction followed by IAC		0.004		70% at [OTA] 0.4 ng/ml	[21]
LLE followed by SPE	0.5	1.5	n.a.	82%	[18]
Extraction with acidic $CHCl_3$ on a ChemElut disposable column	0.3	0.9	n.a	95%	[18]
Solvent extraction followed by clean-up of the extract on silica gel column	0.2			93%	[19]
Solvent ($CHCl_3$) partition followed by SPME	0.01	0.05	0.01–1		This work

is worth noting the simple and cost-saving sample pre-treatment and the minute amount of organic solvent required.

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