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Two different clean-up procedures for liquid chromatographic determination of ochratoxin A in urine

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Abstract

This paper describes two different procedures for extraction of ochratoxin A (OTA) from urine samples: one using acidic chloroform–methanol mixture, followed by solid-phase extraction (SPE) clean-up and the other using commercial Chem Elut columns and a chloroform–formic acid mixture. The recovery of OTA using the procedure with silica gel columns was 82% with a R.S.D. $< 8.4\%$ and the detection and quantitation limits were 0.5 and 1.5 ng OTA/ml, respectively. The recovery of OTA in the second procedure with urine samples purified only on commercial Chem Elut columns was 95% with R.S.D. $< 4.0\%$, and detection and quantitation limits 0.3 and 0.9 ng/ml, respectively. Both procedures of OTA extraction effectively eliminate interfering substances and give reliable and repeatable results. However, the procedure with Chem Elut columns gave higher recovery and lower detection and quantitation limits. It was successfully applied in determining OTA in human urine samples.
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1. Introduction

Ochratoxin A (OTA) is a secondary fungal metabolite produced by certain strains of *Aspergillus* and *Penicillium* species. It contaminates cereals, cereal products and plant-derived products such as herbs, spices, grapes, coffee, wine and beer. It occurs in food of animal origin such as pork and poultry, sausages and eggs. Animal experiments have shown that OTA targets the kidney, but it is also carcinogenic, genotoxic and immunotoxic. OTA was classified by IARC as Group 2 B (possible human carcinogen) [1].

Humans are mostly exposed to OTA by ingestion of contaminated food. OTA is a compound with unusually long serum half-life as a consequence of its binding to plasma proteins [2], its enterohepatic circulation [3], and its re-absorption from urine [4]. OTA is eliminated via bile and urine [5].

Due to the morphological and functional similarities in kidney changes between pigs treated with OTA and humans suffering from endemic nephropathy, it was assumed that OTA was involved in the aetiology of this disease [6]. Although inhabitants of endemic villages in Croatia had higher plasma concentrations of OTA than inhabitants of control villages [7], low concentrations of OTA were also found in the general, non-endemic population [8]. Numerous studies have shown that humans are exposed to low levels of OTA in countries where endemic nephropathy is not known [9].

Valenta has given an extensive review of a number of methods for OTA analysis in blood (plasma), breast milk and kidney, which include thin-layer chromatography, spectrofluorimetry, high-performance liquid chromatography, radioimmunoassay and enzyme-linked immunosorbent assay [10]. Although OTA is excreted with urine and the collection of urine samples is not invasive, literature about OTA analysis in urine is scarce [11–13]. The aim of this study was to find a quick and reliable method for detecting low levels of OTA in urine, suitable for testing large numbers of human urine samples. To that end, we created new HPLC

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conditions and compared two different procedures for the extraction of OTA from urine.

2. Experimental

2.1. Chemicals

Water and silica gel Si-60 (15–40 μm) were obtained from Merck (Darmstadt, Germany). Methanol, acetic acid, hydrochloric acid, chloroform, sodium sulphate anhydrous crystal, and formic acid were supplied by Kemika (Zagreb, Croatia). All chemicals were of pro analysis grade. Water and methanol used for HPLC mobile phase were of HPLC grade.

2.2. Standards

A stock solution of OTA (98% purity, Sigma, St. Louis, MO, USA) was prepared in methanol (1.0 mg OTA/ml methanol). The concentration of stock solution was checked spectrophotometrically at 333 nm using $6640\,M^{-1}\,cm^{-1}$ as the extinction coefficient. The working standards were prepared by adding known amounts of the diluted stock solution to the HPLC mobile phase to give final concentrations from 1.0 to 10.0 ng OTA/ml. The working standards were freshly prepared each day.

For the recovery, spiked urine samples were prepared by adding the same diluted stock solution of OTA (1.0 mg/ml methanol) to OTA-free urine samples (1.0 or 3.0 ml) to give the same range of final concentrations. OTA was extracted and analysed as described in Section 2.5.

2.3. HPLC system

The high-performance liquid chromatograph used in the experiment consisted of a gradient pump (INERT 9012, Varian, Walnut-Creek, CA, USA), manual injector (Rheodyne 7125, Cotati, CA, USA) with 50 μl loop, and fluorescent detector (9075, Varian, Walnut-Creek, CA, USA). The guard column and analytical column were LiChrospher RP-18 (Merck, Darmstadt, Germany) with 5 μm particles and their size was 4.0 mm × 4.0 mm and 125.0 mm × 4.0 mm, respectively. Chromatographic data were collected and processed using Star Chromatography Workstation software (Ver. 5.0, Varian, Walnut-Creek, CA, USA).

2.4. Urine samples

Samples of human urine were collected from 35 apparently healthy persons from a village in eastern Croatia in July 2000 and kept frozen for two months at −80 °C, when analysed. There are no data on the stability of OTA in naturally contaminated human biological material, but some authors did not find changes in OTA concentration in milk and in pig kidney tissue stored for 6 and 8 weeks, respectively [14,15].

2.5. Sample preparation

We used two different procedures to extract OTA from urine samples. The first procedure relied on the method of Breitholtz-Emanuelsson et al. [16], previously used for OTA extraction from cow's milk. A urine sample (1.0 ml) was diluted with 5.0 ml of methanol, acidified with 1 M HCl (0.5 ml), and extracted with chloroform (5.0 ml) by turning the vial gently for 10 min. The mixture was centrifuged at 10,000 × *g* for 10 min. Methanol was removed by washing the chloroform phase twice with redistilled water (2.0 ml). This liquid-liquid extraction (LLE) was followed by solid-phase extraction (SPE) performed on an 8-mm diameter polypropylene column. Filter paper was placed on the bottom of the column to support 200 mg silica gel and 300 mg Na_2SO_4 placed on the layer of the silica gel. The column was placed on Vacuum Manifold system Visprep-DL (Supelco, Bellefonte, USA) and conditioned with 1.5 ml of chloroform. An aliquot of chloroform extract (3.0 ml) was transferred onto the column. The column was washed with chloroform (3.0 ml), and then OTA was eluted with 3.0 ml chloroform–formic acid (95 + 5 v/v). The solvent was evaporated under a gentle stream of nitrogen in a water bath at 60 °C.

The second procedure of OTA extraction was performed using disposable Chem Elut CE 1005 columns (Varian, Harbor City, CA, USA), containing high purity inert diatomaceous earth. These columns designed for LLE should not be conditioned before use. Urine samples (3.0 ml) were transferred to Chem Elut columns and extracted twice with 5.0 ml of organic solution (chloroform and formic acid, 9 + 1 v/v). The eluate was collected and evaporated under a stream of nitrogen in water bath at 60 °C.

The residues of both clean-up procedures were kept at +4 °C until analysed. Prior to HPLC analysis, residues were dissolved in 300 μl of the mobile phase.

2.6. HPLC analysis

The conditions of HPLC analysis were the same for both purification procedures. The mobile phase consisting of methanol, water and acetic acid (700:300:20), pH 3.0, was sonicated before use for 15 min in an ultrasonic bath to remove air bubbles. The flow-rate was 0.5 ml/min. The excitation wavelength of the fluorescence detector was set at 336 nm, and the emission wavelength was 464 nm. The injection volume was 50 μl, and the analysis was performed at room temperature. Under these conditions, the retention time of OTA was about 8 min.

3. Results and discussion

In our preliminary study, OTA was extracted from urine samples using two methods previously described for plasma

[8] and for urine [17]. Neither method was able to eliminate interfering substances due to a number of water-soluble compounds in urine. This is why we checked SPE clean-up on a silica gel cartridge after LLE procedure as described by Breitholtz-Emanuelsson et al. [16]. This method was compared with LLE clean-up procedure on the commercially available Chem Elut columns, suitable for purification of aqueous samples. We found that the whole purification procedure described by Breitholtz-Emanuelsson et al. can be substituted by the use of Chem Elut columns only. In both procedures, OTA was eluted from columns with chloroform–formic acid.

OTA was separated from the purified extract on a 125.0 mm long HPLC RP-18 column and quantified using a florescence detector set on 336 nm for excitation and 464 nm for emission. The analysis time was 15 min.

In OTA HPLC analysis, most authors use a mobile phase containing a mixture of either acetonitrile or methanol with water [10]. We used methanol because it is less toxic and less expensive, yet equally effective as acetonitrile in OTA resolution [18]. The HPLC separation of OTA on column with methanol–water–acetic acid as mobile phase gave good separation of OTA that was eluted as a sharp peak with retention time of about 8 min. Blank urine samples (OTA-free) extracted with both procedures showed no interferences at that retention time.

The specificity of the method was checked by adding OTA working standard solution to positive urine samples, which resulted in an increase in peak height without any shoulders or interference.

3.1. Linearity and precision

The standard curve was based on results from the analyses of samples with OTA added to the mobile phase. Four to six replicates of each point of calibration curve were chromatographed. The selected concentrations of standards covered the expected concentration range of samples. The linearity of the method was tested by calculating the regression of the calibration curve in the concentration range from 1.0 to 10.0 ng OTA/ml of mobile phase. The parameters of the curve were $r^2 = 0.998$, R.S.D. of the slope 2.6%, and the y-intercept was not significantly different from zero.

The linearity of the curves obtained from the peak areas of OTA-spiked urine samples before the both extraction

Table 1
Recovery of ochratoxin A (OTA) from spiked urine samples. R.S.D. for method using silica gel columns was between 3.0 and 8.4%, and when Chem Elut columns were used between 1.0 and 4.0%

Added (ng OTA/ml urine)	Recovery (%) (*N*)	
	Silica gel columns	Chem Elut columns
1.0	98 (3)	92 (5)
2.0	93 (3)	94 (3)
3.0	93 (3)	96 (3)
4.0	73 (3)	93 (3)
5.0	68 (3)	94 (5)
10.0	67 (3)	99 (3)
Mean	82	95

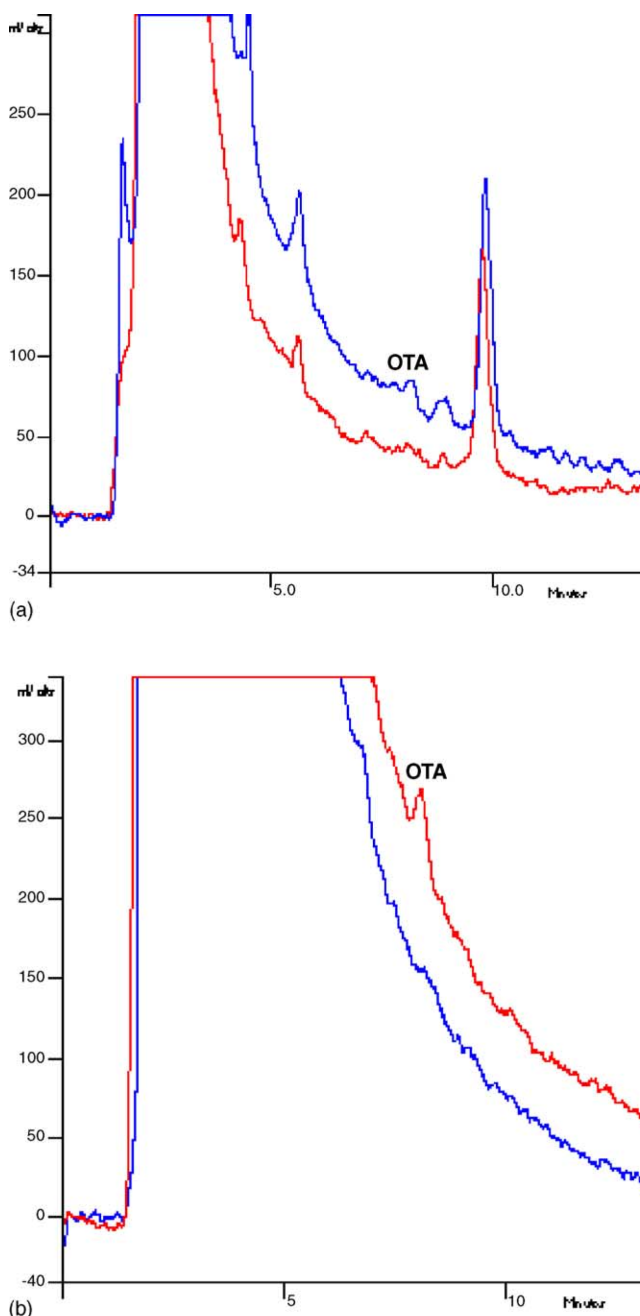

Fig. 1. Chromatograms of blank and spiked urine samples (concentration 1.0 ng OTA/ml urine) extracted: (a) with acidic chloroform–methanol mixture and cleaned up on polypropylene column filled with 200 mg silica gel and 300 mg Na_2SO_4; (b) Chem Elut disposable columns. The mobile phase consisted of methanol, water and acetic acid (700:300:20), pH 3.0; the flow-rate was 0.5 ml/min; $\lambda_{exc.}$ was 336 nm, and $\lambda_{em.}$ was 464 nm; the retention time of OTA was about 8 min.

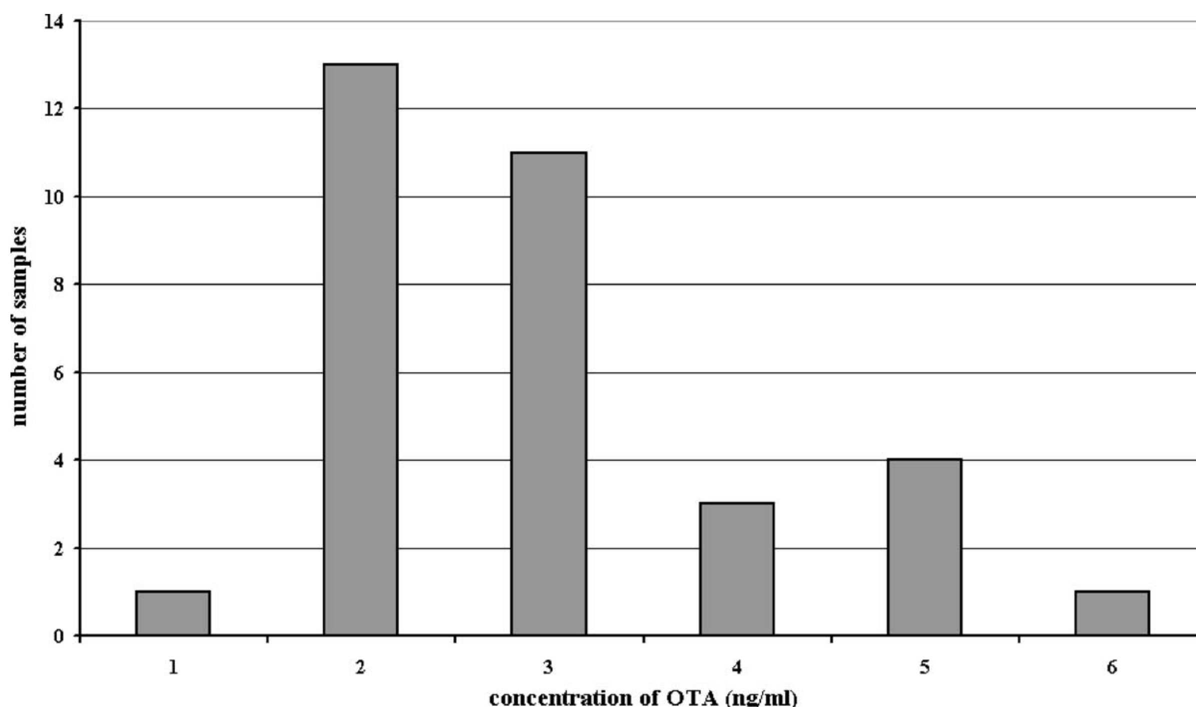

Fig. 2. Occurrence of OTA in human urine samples collected from healthy persons from eastern Croatia in summer 2000.

procedure was checked. In both procedures the y-intercept was not different from zero, and the r^2 and R.S.D. of the slope for silica gel and Chem Elut extraction were 0.991, 5.5% and 0.994, 4.4%, respectively.

The precision of the method was evaluated in a series as repeatability (within-day precision) and as reproducibility (between-day precision) of four to six replicates of standards prepared in mobile phase. R.S.D. for within-day precision was 2.0%, and for between-day precision 3.5%.

3.2. Limits of detection and quantitation

The limits of detection and quantitation of OTA were calculated by taking the average noise signal and adding 3 and 10 standard deviations of the noise, respectively. The detection limit of the procedure with silica gel purification was 0.5 ng OTA/ml, and the quantitation limit 1.5 ng OTA/ml. Limits of detection and quantitation of the procedure with Chem Elut were 0.3 and 0.9 ng OTA/ml, respectively.

3.3. Recovery

Recovery was calculated by comparing the area under the peak of OTA from spiked urine with the area under the peak of standards in mobile phase. Table 1 shows the recoveries of two different purification procedures. The overall OTA recovery of the clean-up with silica gel columns was 82% with R.S.D. below 8.4%, but the recovery decreased at higher OTA concentrations, which shows that the procedure is suitable only for the detection of small OTA concentrations. Chem Elut columns showed better OTA recovery (mean recovery was 95% with R.S.D. below 4.0%), which did not depend on OTA concentrations.

Chromatograms of spiked urine samples (1.0 ng OTA/ml) cleaned by means of silica gel and Chem Elut columns are presented in Fig. 1.

3.4. Analysis of human urine samples

Samples containing OTA above the detection limit of the method (0.3 ng/ml) were considered positive. Fig. 2 shows the frequency of positive OTA findings in 35 urine samples of healthy humans. Positive samples contained OTA in the range from 0.99 to 5.22 ng/ml. The mean OTA concentration for all samples was 2.39 ± 1.29 ng/ml (mean $\pm$ S.D.) with the median 2.11 ng/ml. OTA concentrations above the detection limit were found in 33 (94%) samples. Such a high frequency of OTA-positive samples is in accordance with our earlier investigation of OTA in blood of general population collected in Summer 1997 in eastern Croatia [8].

4. Conclusion

Exposure to OTA may be confirmed by its finding in urine. In contrast to blood sampling, the collection of urine is not invasive. LLE followed by SPE procedure with silica gel columns requires a small volume of urine, which makes it convenient for the determination of OTA in urine of laboratory animals. However, urine extraction on Chem

Elut columns without clean-up of the extracts is simpler, less time-consuming, and its recovery is higher regardless of the OTA concentration. It also enables the detection of low concentrations of OTA in human urine. Tested on human urine samples, this method has confirmed that humans are frequently exposed to OTA.

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References

[1] IARC, IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Some Naturally Occurring Substances: Food Items and Constituents, Heterocyclic Aromatic Amines and Mycotoxins, Vol 56, International Agency for Research on Cancer, Geneva 1993, pp. 489–521.
[2] K. Hult, R. Fuchs, in: P.S. Steyn, R. Vleggaar (Eds.), Mycotoxins and Phycotoxins, Elsevier Science Publishers, Amsterdam, 1986, p. 365.
[3] R. Fuchs, B. Radić, M. Peraica, K. Hult, R. Pleština, Period Biolo. 90 (1988) 39.
[4] A. Dahlmann, W.H. Dantlzer, S. Silbernagl, M. Gekle, J. Pharmacol. Exp. Therapeut. 286 (1998) 157.
[5] S. Li, R.R. Marquardt, A.A. Frolich, T.G. Vitti, G. Crow, Toxicol. Appl. Pharmacol. 145 (1997) 82.
[6] P. Krogh, in: A. Puchlev, V. Dinev, B. Milev, D. Doichinov (Eds.), Proceedings of the Second International Symposium on Endemic Nephropathy, Sofia, November 1972, Bulagarian Academy of Sciences, Sofia, 1974, p. 266.
[7] B. Radić, R. Fuchs, M. Peraica, A. Lucić, Toxicol. Lett. 91 (1997) 105.
[8] M. Peraica, A-M. Domijan, R. Fuchs, A. Lucić, B. Radić, Toxicol. Lett. 110 (1999) 105.
[9] M. Peraica, B. Radić, A. Lucić, M. Pavlović, Bull. WHO 77 (1999) 754.
[10] H. Valenta, J. Chromatogr. A 815 (1998) 75.
[11] D.L. Orti, R.H. Hill, J.A. Liddle, L.L. Needham, J. Anal. Toxicol. 10 (1986) 41.
[12] M. Castegnaro, V. Maru, G. Maru, M.D. Ruiz-Lopez, Analyst 115 (1990) 129.
[13] H. Valenta, I. Kühn, K. Rorh, J. Chromatogr. 613 (1993) 295.
[14] H. Valenta, M. Goll, Food Add. Contam. 13 (1996) 669.
[15] A.C. Entwistle, K. Jorgensen, A.C. Williams, A. Boenke, P.J. Farnell, Food Add. Conam. 14 (1997) 223.
[16] A. Breitholtz-Emanuelsson, M. Olsen, A. Oskarsson, I. Palminger, K. Hult, J. AOAC Int. 76 (1993) 842.
[17] S. Li, R.R. Marquardt, A.A. Frohlich, T.G. Vitti, G. Crow, Toxicol. Appl. Pharmacol. 145 (1997) 82.
[18] D. Beker, B. Radić, J. Chromatogr. 570 (1991) 441.