

## Simple, Fast and Economic Method for Extraction and Determination of Ochratoxin A in Serum by High Performance Liquid Chromatography (HPLC)

Hussam Ahmed El-Gayar

Urology and Nephrology Center, Mansoura University, Mansoura, Egypt

hgayaar@yahoo.com

---

### ARTICLE INFO

Alumina,  
HPLC, Ochratoxin A,  
Serum

---

### ABSTRACT

**Objective:** A simple high performance liquid chromatographic method for the determination of ochratoxin A (OTA) in human blood serum is described. **Method:** The extraction procedure was simple and short, and liquid chromatographic analysis was carried out isocratically on reversed-phase C18 column, with acetonitrile : 0.08M phosphoric acid : isopropanol (45:45:10) at a flow rate of 1.5ml/min, and fluorescence detection (excitation at 330 nm and emission at 460 nm). **Results:** The mean recovery from authentic contaminated samples (n=5) spiked at 0.3, 1, 2, 4 ng/ml OTA was 97.98% (s.d=2.03). Thirty seven Egyptian serum samples were naturally contaminated with OTA in the range 0.1-2.88 ng/ml. **Conclusions:** The method is technically simple, specific, cost effective, time saving, suitable for large sample and requires small amount of sample and reagents. It fulfills the criteria for routine method and could be a suitable tool for surveying OTA in human blood serum.

**Patent No: 33182**

---

## INTRODUCTION

Ochratoxin A (OTA) is a mycotoxin of wide natural abundance as produced by several species of *Aspergillus* and *penicillium* fungi during storage of foods and feeds (1), (2). It causes multiple toxicities in animal and human including nephrotoxic, hepatotoxic, immunotoxic, teratogenic, and carcinogenic effects which represent serious health risks to livestock and general population (3). Our previous study in Egypt (4), as well as, studies in Bulgaria, Croatia, Rumania, and Serbia (5), the Czech Republic (6), Turkey (7), Italy (8), Bangladesh (9), Algeria (10), and Tunisia (11) have found significantly higher serum or plasma levels of OTA in patients with kidney disorders compared to healthy peoples. Health regulation authorities define maximal tolerable daily intake levels of OTA of 5ng/kg body weight (12). As a result of relatively long half-life of OTA in human blood of 35 days due to its high binding to plasma proteins (13), (14), OTA determination in plasma represents a good parameters for evaluating the exposure at the population level (15), (16).

High performance liquid chromatography with fluorescence detector (HPLC-FD) is the most commonly method used for determination of OTA at the sub nanogram concentration level. Removing interferences from the samples must be carried out before HPLC separation, so that several chromatographic methods are widely used for extraction and clean-up of OTA in serum including liquid - liquid extraction (LLE), solid phase extraction (SPE) columns; C18, immune-affinity, molecular imprinted polymer,

and combination of LLE with different types of SPE (17), (18), (19), (20). Most of these methods require a long processing time and a large amount of serum. For the best of our knowledge, this is the first time to use alumina in extraction of OTA from human serum. The aim of the present study is to develop an HPLC method which is simple, rapid, time-saving, as well as reducing the amount of solvent and volume of sample for extraction of OTA to use it in determination of OTA in human serum populations.

## Experimental

### Instrumentation

This study was approved by the ethical guidelines of Mansoura University. The following equipment was used: an 110 pump (Beckman, USA), model 7752 Rheodyne injector, fitted with 50 µl sample loop, a model LP-21 pulse dampner (SSI, USA), a fluorescence detector (RF-10 AXL Shimadzu, Kyoto, Japan), a reversed phase C18 column 5µm analytical column (250 X 4.6 mm I.D.) from Beckman, and analytical data were collected, stored and treated using the software Eurochrom 2000 for windows (Knauer, Germany).

### Chemicals and reagents

Ochratoxin A was obtained from Sigma chemical Co (St. Louis, MO, USA). Acetonitrile, isopropanol, ethanol, and methanol were obtained from Lab-scan chemical Co (Labscan Ltd, Dublin, Ireland). Ethanol was obtained from Merck (Merck, Germany). All solvents were of HPLC grade. Alumina was obtained from BDH (BDH, England), orthophosphoric acid, acetic acid and hydrochloric acid were obtained from BDH (BDH, England). Deionized water,

---

purified by MilliQ system (Millipore, Milford, MA, USA), was used throughout the study. Stock solutions of OTA were prepared at 50ng/ml in methanol. All stock solutions were protected from light and stored at 2-8 °C. The stock solutions were further diluted with methanol to give a series of working standards. The working solutions for spiking blank human serum samples were prepared fresh daily.

#### **Blood samples:**

Five milliliters of blood samples were withdrawn from healthy individuals from out-patient, Urology and Nephrology center, Mansoura University, Mansoura, Egypt. Sera were separated by centrifugation at 860 Xg for 20 min at 4°C and then quickly frozen at – 20°C until analysis.

#### **Extraction procedure**

Human serum was spiked with concentrations ranging from 0.3 to 4ng/ml of OTA. OTA in serum was extracted as follow: A 0.5-ml portion of serum was pipette into a 5 ml tube and adjusted to pH 4.5 by acetic acid followed by adding 2ml methanol. The mixture was vortexed for 1min and centrifuged for 5min at 2500 rpm. The supernatant was transferred into 5 ml clean tube. 150 mg Al<sub>2</sub>O<sub>3</sub> (pH=3.4) were added. The tube was vortex for 6 min and centrifuged for 2 minutes. After the supernatant was discarded and 2 ml of 0.08 M phosphoric acid was added, the mixture was vortexed for 3 minutes and centrifuged at 2500 rpm for another 2 minutes. The supernatant was then transferred into a 15 ml clean tube and 8 ml chloroform was added. The mixture was vortexed and centrifuged for one minute at 2500 rpm, the chloroform

extract was evaporated and the residue was redissolved in 150 microlitre methanol. 50 microlitres were injected into the high performance liquid chromatography (HPLC) under specified conditions.

#### **Results and Discussion**

Results of mean recovery of ochratoxin A from artificial contaminated human serum samples are shown in Table (1). Serum samples (n=5) were artificially contaminated with 0.3, 1.0, 2.0, and 4ng/ml OTA. The overall mean recovery was 97.98% (s.d=2.03) with a relatively low coefficient of variation (Table 1). Representative chromatograms of blank and spiked human serum are shown in Figure (1). Figure (2) shows 1, 2, and 4 ng/ml standard ochratoxin A respectively. Under the described conditions, the retention time was 5.3 min. the LOD was 0.08 ng/ml and LOQ was 0.28 ng/ml. Regression analysis was performed on the calibration curve in plasma. The obtained calibration curve for five independent runs were linear within the studied range between 1 to 4 ng/ml based on peak area. The regression equation describing the calibration run was  $con = 0.02(a) - 0.05$  ( $R=0.999$ ), where (a) is the area under peak (AUP). The recovery of OTA within the concentration range of the assay is 97.98% and ranged from 96.67% to 101%. It is comparable to other methods in human serum: 85% (21), 70-79% (22), in swine serum: 86.8% (20) and in ewes plasma: 89% (23). The reason for better recovery was the simpler extraction procedure. Additionally, a comparing of amount of serum sample, time and the amount of chloroform required for sample

---

preparation to the other methods. The present method requires 0.5 ml serum, ~ 30min and 8 ml chloroform compared to 0.8 ml serum and 4-48h for sample preparation (20), 2ml serum and 10 ml chloroform (24), 1ml serum (21). Meanwhile, the present method does not require the usage of immunoaffinity column as described in the procedures of (20) and (24) and consequently the procedure cost is significantly reduced.

#### **Analysis of real samples**

The developed method was applied to the determination of OTA in serum samples. Blood samples from thirty seven individuals were analyzed for OTA. OTA was detected in all serum samples, ranging from 0.1 to 2.88 ng/ml. Among the Thirty seven blood samples analyzed, twenty six samples were contaminated with OTA (70.3%) ranged from 0.10 to 0.19 ng/ml, six samples were contaminated with OTA (16.2%) ranged from 0.20 to 0.28 ng/ml, three samples were contaminated with OTA (8.1%) ranged from 0.34 to 0.37 ng/ml, one sample was contaminated with OTA (2.7%) at 0.70 and 2.88 ng/ml, respectively (Table 2). The chromatograms obtained (Figure 1e) indicate that the OTA peaks are not interfered by other matrix components.

#### **Conclusions**

In conclusion, an HPLC method for determination of OTA in human serum has been developed. Sample preparation is easy and rapid. The validation parameters permit OTA in serum to be determined in course of screening studies.

#### **References**

- 1- [Soto JB](#), [Ruiz MJ](#), [Manyes L](#), [Juan-García A.](#), 2016. [Blood, breast milk and urine: potential biomarkers of exposure and estimated daily intake of ochratoxin A: a review.](#) *Food Addit Contam Part A Chem Anal Control Expo Risk Assess.* 33(2):313-28.
- 2- Yu JC and Lai EP., 2010. [Molecularly imprinted polymers for ochratoxin a extraction and analysis.](#) *Toxins (Basel).* 2(6):1536-53.
- 3- El Khoury A. and Atoui A., 2010. [Ochratoxin a: general overview and actual molecular status.](#) *Toxins (Basel).* 2(4):461-93.
- 4- Wafa [EW](#); [Yahya RS](#); [Sobh, MA](#); [Eraky I](#); [el-Baz, M.](#); [El-Gayar, HA](#); [Betbeder AM](#); [Creppy, EE](#), 1998. Human ochratoxicosis and nephropathy in Egypt: a preliminary study. *Hum Exp Toxicol.* 17(2):124-9.
- 5- Fuchs R and Peraica M., 2005. Ochratoxin A in human kidney diseases. *Food Addit Contam.* 22 Suppl 1:53-7.
- 6- Malir F, Ostry V, Pfohl-Leszkowicz A, Roubal T., 2012. Ochratoxin A exposure biomarkers in the Czech

- 
- Republic and comparison with foreign countries. *Biomarkers*.17(7):577-89.
- 7- Ozçelik N<sup>1</sup>, Koşar A, Soysal D., 2001. Ochratoxin A in human serum samples collected in Isparta-Turkey from healthy individuals and individuals suffering from different urinary disorders. *Toxicol Lett*. 121(1):9-13.
- 8- Breitholtz-Emanuelsson A, Minervini F, Hult K, Visconti A., 1994. Ochratoxin A in human serum samples collected in southern Italy from healthy individuals and individuals suffering from different kidney disorders. *Nat Toxins*. 2(6):366-70.
- 9- Ali N, Blaszkewicz M, Manirujjaman M, Perveen R, Al Nahid A, Mahmood S, Rahman M, Hossain K, Degen GH., 2014. Biomonitoring of ochratoxin A in blood plasma and exposure assessment of adult students in Bangladesh. *Mol Nutr Food Res*. 58(11):2219-25.
- 10- Scott PM. *Mycotoxins in Grain-Compounds Other than Aflatoxin*; Miller, J.D., Trenholm, H.L.,Eds.; Eagan Press: Saint Paul, MN, USA, 1997; p. 261.
- 11- Hmaissia Khelifa K, Ghali R, Mazigh C, Aouni Z, Machgoul S, Hedhili A., 2012. Ochratoxin A levels in human serum and foods from nephropathy patients in Tunisia: where are you now? *Exp Toxicol Pathol*. 64(5):509-12.
- 12- Walker R., 2002. Risk assessment of ochratoxin: current views of the European Scientific Committee on Food, the JECFA and the Codex Committee on Food Additives and Contaminants. *Adv. Exp. Med. Biol*. 504, 249–255.
- 13- [Mantle P](#), [Kilic MA](#), [Mor F](#), [Ozmen O.](#), 2015. [Contribution of organ vasculature in rat renal analysis for ochratoxin a: relevance to toxicology of nephrotoxins.](#) *Toxins (Basel)*. 24;7(4):1005-17.
- 14-Pfohl-Leszkowicz A. and Manderville RA., 2007. Ochratoxin A: An overview on toxicity and carcinogenicity in animals and humans. *Mol. Nutr. Food Res*. 51, 61–99.

- 
- 15- [Matsuda Y](#), [Wakai T](#), [Kubota M](#), [Osawa M](#), [Sanpei A](#), [Fujimaki S](#), 2013. [Mycotoxins are conventional and novel risk biomarkers for hepatocellular carcinoma](#). *World J Gastroenterol*. 19(17):2587-90.
- 16- di Giuseppe R, Bertuzzi T, Rossi F, Rastelli S, Mulazzi A, Capraro J, de Curtis A, Iacoviello L, Pietri A.,2012. Plasma ochratoxin A levels, food consumption, and risk biomarkers of a representative sample of men and women from the Molise region in Italy. *Eur J Nutr*. 51(7):851-60.
- 17- [Tolosa J](#), [Font G](#), [Mañes J](#), [Ferrer E](#)., 2016. [Multimycotoxin analysis in water and fish plasma by liquid chromatography-tandem mass spectrometry](#). *Chemosphere*. 145:402-8.
- 18- Lee TP., Saad B., Khayoon WS., Salleh B., 2012. [Molecularly imprinted polymer as sorbent in micro-solid phase extraction of ochratoxin A in coffee, grape juice and urine](#). *Talanta*. 15; 88:129-35.
- 19- Korn M, Frank O, Hofmann T, Rychlik M., 2011. Development of stable isotope dilution assays for ochratoxin A in blood samples. *Anal Biochem*. 15;419(2):88-94.
- 20- Curtui VG. and Gareis [A](#).,2001. [Simple HPLC method for the determination of the mycotoxins ochratoxin A and B in blood serum of swine](#). *Food Addit Contam*. 18(7):635-43.
- 21- [Zimmerli B](#) and [Dick R](#)., 1995. Determination of ochratoxin A at the ppt level in human blood, serum, milk and some foodstuffs by high-performance liquid chromatography with enhanced fluorescence detection and immunoaffinity column cleanup: methodology and Swiss data. *J Chromatogr B Biomed Appl*. 666(1):85-99.
- 22- Rosener H, Rohrmann B, and Peiker G., 2000. Ochratoxin A in serum. *Archiv fur Lehnsmineallrygiene*, 51,104-107.
- 23- Boudra H and Morgavi DP, 2006. . Development and validation of a HPLC method for the quantitation of ochratoxins in plasma and raw milk. *Journal of Chromatography B*. 843(2):295-301.

---

**24-** Lino CM, Baeta ML, Henri M, Dinis AM, Pena AS, Silveira MI., 2008. Levels of ochratoxin A in serum from urban and rural Portuguese populations and estimation of

exposure degree. Food Chem Toxicol. 46(3):879-85.

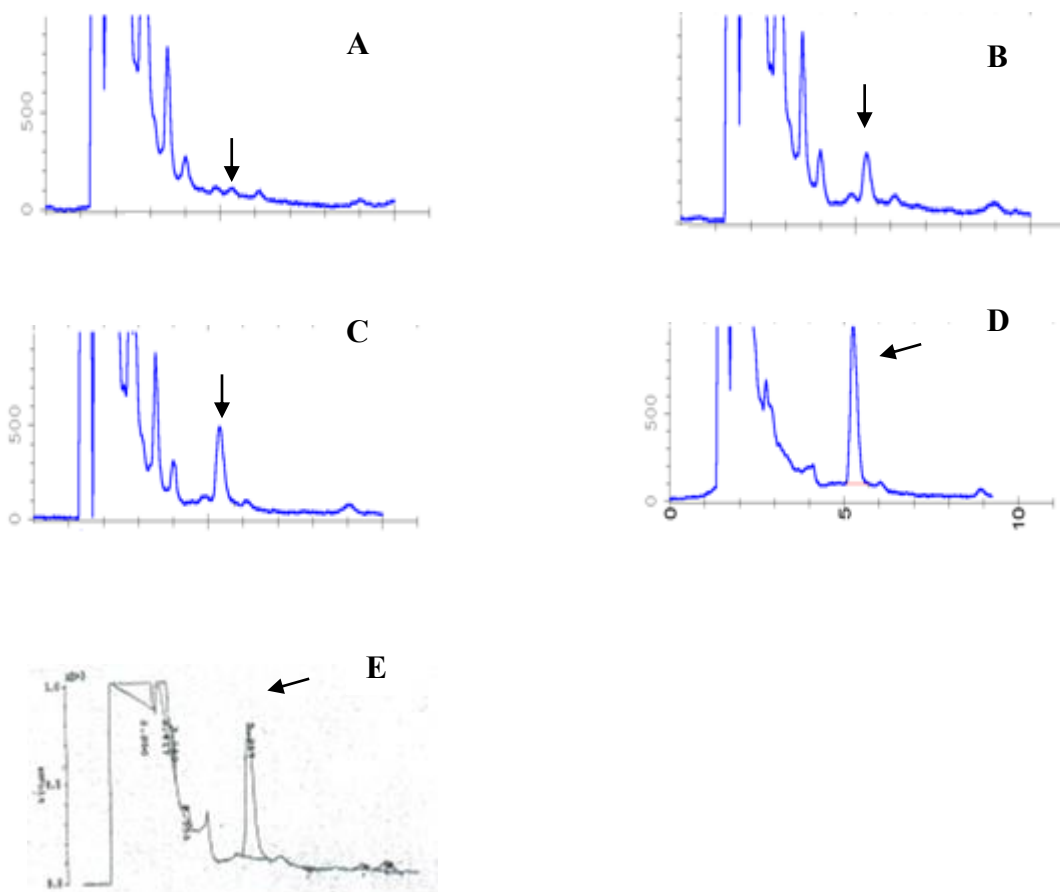
**Table 1:** Mean recovery of ochratoxin A from artificial contaminated human serum samples (n=5) for each concentration range.

<b>OTA concentration added to serum (ng/ml)</b>	<b>Mean OTA measured (ng/ml)</b>	<b>S.D</b>	<b>c.v (%)</b>	<b>Rec (%)</b>
0.3	0.29	0.04	13.8	96.67
1	0.97	0.09	9.28	97.0
2	2.02	0.04	1.98	101.0
4	3.89	0.10	2.57	97.25

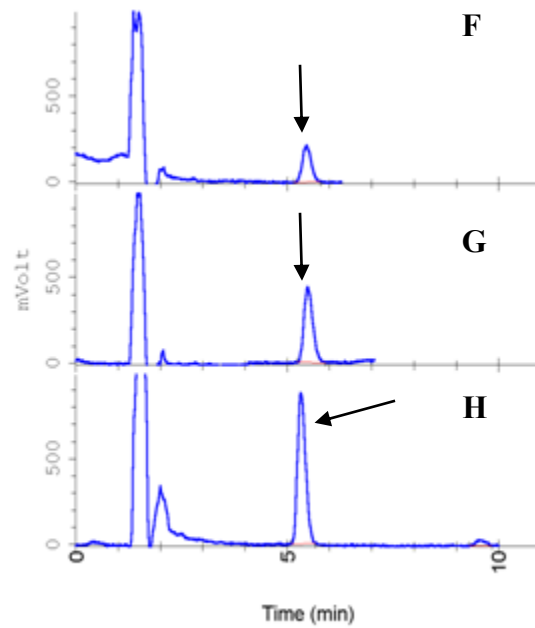
**Table 2:** OTA concentrations in human serum samples analyzed.

<b>Concentration of OTA (ng/ml)</b>	<b>No. of positive sample (%)</b>
0.1-0.19	26 (70.3%)
0.2-0.28	6 (16.2%)
0.34-0.37	3 (8.1%)
0.7	1 (2.7)
2.88	1 (2.7)





**Figure 1:** HPLC separation of ochratoxin A on ultrasphere C18 column with 0.08M  $\text{H}_3\text{PO}_4$ : acetonitrile: isopropanol (45:45:10) as mobile phase and fluorescence detection: excitation at 330 nm and emission at 460 nm (A) blank serum sample; (B) serum sample spiked with OTA (1ng/ml); (C) serum sample spiked with OTA (2ng/ml); (D) serum sample spiked with OTA (4ng/ml); (E) serum sample of individual naturally contaminated with 2.88ng/ml OTA.



**Figure 2:** HPLC separation of ochratoxin A on ultrasphere C18 column with 0.08M H<sub>3</sub>PO<sub>4</sub>: acetonitrile: isopropanol (45:45:10) as eluent and fluorescence detection: excitation at 330 nm and emission at 460 nm (F, G, H) 1, 2, and 4 ng/ml standard ochratoxin A, respectively.