ESTIMATION OF OCHRATOXIN A IN THE HUMAN BLOOD OF ROMANIAN POPULATION

Anca Vartolomei Zlavog⁴, Magdalena Cuciureanu², Elena Şchiriac³, Ionela Popa (Morariu)¹, Camelia Diaconu⁴, Rodica Cuciureanu¹

University of Medicine and Pharmacy “Grigore T. Popa” - Iaşi
Faculty of Pharmacy
1. Department of Environmental and Food Chemistry
Faculty of Medicine
2. Department of Pharmacology
3. Praxis Medica Laboratory, Iasi
University of Medicine and Pharmacy “Dunărea de Jos”- Galaţi
Faculty of Pharmacy
4. Department of Pharmaceutical Sciences

ESTIMATION OF OCHRATOXIN A IN THE HUMAN BLOOD OF ROMANIAN POPULATION (Abstract): Ochratoxin A (OTA) is a toxic secondary metabolite of fungi belonging to the Aspergillus and Penicillium genera, its presence in human blood being the primary indicator of exposure. Aim: In the present study we determined OTA in 38 blood samples collected from healthy Romanian subjects of both genders. Material and methods: The OTA was determined through the direct, competitive, solid-phase immunoenzymatic method; the minimum quantification limit for determining OTA in serum samples was 0.0289 ng/mL. Results: The positive sample percentage was 100%. OTA concentrations varied between < 0.04 ng/mL and 1 ng/mL. There were no significant differences between OTA concentrations in men versus women (0.24 ± 0.20 ng/mL versus 0.17 ± 0.15 ng/mL, p=0.3527). Conclusions: The analyzed blood samples exhibit a very high degree of exposure to OTA, but in only approximately 10% of the subjects exceeded 0.5 ng/mL, considered the threshold for OTA-induced renal pathology. Keywords: OCHRATOXIN A, BLOOD SAMPLES, IMMUNOENZYMATIC METHOD

Ochratoxins (A, B, C) are a group of compounds with related chemical structure, a isocoumarin derivate being linked to L-b-phenylalanine by an amide bond.

OTA is a hepatotoxic, nephrotoxic, carcinogenic, teratogenic, immunotoxic and neurotoxic compound that contaminates foods before harvesting or, more frequently, during storage (1). The mycotoxin is naturally produced by Penicillium verrucosum in temperate areas and by Aspergillus ochraceus and, more rarely, by Aspergillus carbonarius in tropical regions. The toxicity of OTA results from the inhibition of protein synthesis, disruption of calcium homeostasis, increased lipid peroxidation, mitochondrial dysfunction, and also through the effects at the level of transcription and transduction (2).

The purpose of this paper is to determine OTA in blood samples collected from Romanian healthy volunteers through a
solid-phase direct, competitive immuno-enzymatic method.

**MATERIAL AND METHODS**

*Reagents and equipment.* STAT FAX 303 Plus ELISA microplate readers, (Awareness Technology, USA).

All reagents were multianalyte, supplied in a compact kit:

1. Calibrators with OTA in the concentration range 0.0 – 0.40 ng/mL: each kit contains multianalyte calibrators with values attributed through a reference method that attests that the values of the analytes were established according to reference materials certified by WHO for 6 levels of concentration (0.0, 0.02, 0.05, 0.1, 0.2, 0.4 ng/mL), in liquid conditioning form, that can be used as such.

2. Reagent kit for the quantitative determination of OTA, code DE991OCH01MS (Demeditec Diagnostics GmbH, Germany) containing all components for the ELISA reaction necessary for the determination of OTA.

*Analyzed samples.* Blood samples were collected from 38 healthy volunteers from Iasi, Romania. This study was approved by the Ethics Committee of the “Grigore T. Popa” University of Medicine and Pharmacy and was carried out in accordance with standard operation procedures. Written informed consent was obtained from all subjects before initiation of the study.

*Method.* The quantitative determination of OTA in human serum samples was realized through a solid-phase, direct, competitive immunoenzymatic method. The standards (calibrators) or samples are added to the polystyrene microwells coated with high-affinity antibodies and, if present, OTA couples with the antibodies. Next, OTA conjugated to the enzyme horseradish peroxidase (HRP) is added, forming bonds with the antibodies that have not yet been occupied by the OTA from the standard or sample. After a reaction incubation interval, the content is removed, the microwells are washed, and the chromogenic (3,3',5,5'-tetramethylbenzidine) (TMB) substrate is added. TMB soluble substrates yield a blue color when detecting HRP. The intensity of the color is directly proportional with the quantity of conjugate and inversely proportional with the quantity of OTA present in the standard or in the sample. The reaction is stopped by adding an acid solution that changes the color from blue to yellow. The intensity of the color is spectrophotometrically determined at 450 nm with a 630 nm reference filter.

The above described method for OTA analysis was linear for the concentration range 0.0-0.5 ng/mL, with the regression coefficient (R) = 0.968058367. The minimum detection limit was 0.0133 ng/mL and the minimum quantification limit for OTA determination in serum samples was 0.0289 ng/mL.

*Sample preparation.* To determine OTA in human blood, the blood is collected in vacuumserum tubes; the blood is centrifuged, the serum is separated and exposed to ethanol extraction: 750 µl absolute methanol is added to 250 µl serum. The mixture is homogenized, left at room temperature, centrifuged at 3500 rpm for 5 minutes and only the supernatant is used for OTA determination.

*Procedure.* The successive work steps to determine OTA in blood samples are described in tab. I.

*Statistical analysis.* The statistical analysis was performed using one-way analysis of variance (ANOVA) and Pearson's r. Tukey test was used for multiple comparisons (software Stats Direct version 2.6). A value of p < 0.05 was considered significant.
TABLE I

<table>
<thead>
<tr>
<th>Procedure for the determination of OTA from serum</th>
<th>BLANK</th>
<th>S₁ (0.0 ng/mL)</th>
<th>S₂ (0.02 ng/mL)</th>
<th>S₃ (0.05 ng/mL)</th>
<th>S₄ (0.1 ng/mL)</th>
<th>S₅ (0.2 ng/mL)</th>
<th>S₆ (0.4 ng/mL)</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 µl of contents from each mixing well of microtiter plate was transferred to corresponding Anti OTA antibody coated well from reaction microplate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Incubation</td>
<td>30 minutes at ambient temperature (22°C).</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wash</td>
<td>Decant the contents of the wells. Wells were washed 3 times with washing buffer PBS-Tween (pause between washes was 60 seconds). After the last wash was absorbed excess moisture by blotting on filter paper.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OTA HRP-conjugate</td>
<td>-</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
</tr>
<tr>
<td>Incubation</td>
<td>30 minutes at ambient temperature (22°C).</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wash</td>
<td>Were carried out three successive washes with washing buffer PBS-Tween. After the last wash was absorbed excess moisture by blotting on filter paper.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Substrate solution TMB</td>
<td>-</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
</tr>
<tr>
<td>Incubation</td>
<td>10 minutes at ambient temperature (22°C).</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stopping the reaction with acidic solution</td>
<td>-</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
</tr>
<tr>
<td>Reaction contents were homogenized by 2-3 successive pipetting.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Optical density was read at λ = 450 nm with 630 nm reference filter.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

RESULTS

The study population comprised 9 males (23.68%) and 29 females (76.32%) with an average age of 38.63 ± 13.08 years, 38.24 ± 13.61 for women and 39.88 ± 11.87 for men.

The majority of the study population (28 subjects) was living in urban areas (74.69%) whereas 10 patients (26.31%) were from rural areas. All study participants were white Caucasians and had an average Body Mass Index (BMI) of 22.98 ± 1.83 kg/m² for women and 23.36 ± 1.96 kg/m² for men, with no statistical difference between sexes.

All 38 plasma samples exhibited the presence of OTA in concentrations ranging between 0.04 ng/mL and 1 ng/mL. The average OTA concentration in the examined plasma samples was 0.22 ± 0.19 ng/mL. Seven subjects (18.42%) exhibited OTA concentrations less than 0.1 ng/mL, 27 subjects (71.05%) between 0.1 - 0.49 ng/mL, 3 subjects (7.89%) between 0.5 - 0.99 ng/mL, and 1 subject (2.63%) of 1 ng/mL (fig. 1). There were no significant differences in OTA concentrations between men and women (0.24 ± 0.20 ng/mL versus 0.17 ± 0.15 ng/mL, p=0.3527). Our results indicated no correlations between OTA concentrations and age or BMI.

![Fig. 1. The distribution of OTA concentration in blood samples from healthy Romanian subjects](image-url)

DISCUSSION

OTA can be present as a natural contaminant of food and, as a consequence, it can be traced in human blood, breast milk and
animal-based products. In European diet, cereals (barley, wheat, corn and oat) and their derivatives are viewed as the major source of OTA ingestion, representing approximately 50% of the total input (1).

The importance of determining OTA plasma concentrations is related to the fact that OTA levels higher than 0.5 ng/mL are linked to the development of renal diseases (3).

The nephrotoxic action of OTA in humans was demonstrated by the occurrence of Balkan endemic nephropathy (BEN) and is associated with renal and urethral carcinoma, and, less frequently, with carcinoma of the bladder (1, 4).

Still, there are insufficient epidemiological and clinical data to demonstrate the carcinogenic potential of OTA in humans and it is considered that other nephrotoxic agents like aristolohic acid could be involved in BEN (5).

Similar studies using a detection limit of 0.025 ng/mL identified OTA in 324 of the 341 analyzed blood samples. 62% of the samples contained OTA concentrations lower than 0.2 ng/mL, while 17 samples (5.2%) presented concentrations higher than 0.50 ng/mL, indicating a low exposure of Italian population (6).

European studies reported high percentages (97%) of OTA positive blood samples at comparable detection limits, data that are in agreement with our results (7, 8). In similar studies conducted in African or Near East countries, these percentages were lower; thus, in Morocco, using a detection limit of 0.08 ng/mL the percentage of positive samples was 60%, while in Lebanon, using a detection limit of 0.60 ng/mL the percentage dropped to 33% (9, 10).

As to the difference in plasma OTA concentrations between men and women, the results are heterogeneous. Several authors indicated that plasma OTA concentration is higher in men than in women, whereas other studies revealed no significant differences between sexes (11, 12). Giuseppe et al. (2012) signaled a significant age-sensitive difference between these values in men and women, differences that were not found in our study (6).

**CONCLUSIONS**

In conclusion, our results indicate an increased level of exposure to OTA (100% of the blood samples), but only approximately 10% of the subjects exceeded 0.5 ng/mL considered the threshold for OTA-induced renal pathology.

**REFERENCES**

Estimation of ochratoxin a in the human blood of romanian population


ANEMIA- RISK OF DEMENTIA IN THE ELDERLY

According WHO criteria’s, anemia is defined by a level of hemoglobin concentration less than 13 g/dL for men and 12 g/dL for women. This illness affects a quarter of adults over the age of 65. Another condition common in the elderly pathology is dementia. A study published in online issue of Neurology®, the medical journal of the American Academy of Neurology, show that anemia is associated with increased risk of dementia. The study group which includes 2552 elderly adults was investigated for anemia and cognitive disorders and memory for 11 years period. At baseline, 393 participants had anemia. At the end of the study, 23% of the patients with anemia have associated dementia. Only 17% of people without anemia have developed dementia. The increased risk of developing dementia for older adults with anemia has remained significant after adjusting for age, race, sex and education. These results suggest that anemia is a target for preventing cognitive disorders (Hong CH, Falvey C, Harris TB et al. Anemia and risk of dementia in older adults. Findings from the Health ABC study. Neurology, 2013 DOI: 10.1212/WNL.0b013e31829e701d).

Cătălina Luncă