Ochratoxin A Content of Human Sera Determined by a Sensitive ELISA

László Solti1,2, Ferenc Salamon3, Illdikó Barna-Vetró1, Ágnes Gyöngyösi1, Erzsébet Szabó1, and Anna Wölfling1

1Agricultural Biotechnology Center, H-2101 Gödöllő, P.O. Box 411, Hungary, 2University of Veterinary Science, H-1400 Budapest, P.O. Box 2, Hungary, and 3Szent János Hospital II. Department of Internal Medicine, Budapest, Hungary

Abstract

A sensitive, monoclonal antibody-based ELISA test was developed and used for quantitative determination of ochratoxin A (OA) in human sera. The measuring range of this test (without sample dilution) was 0.2–2.0 ng/mL, and the detection limit was 0.2 ng/mL. The OA concentrations of 355 sera samples varied from <0.2 to 10 ng/mL OA, but 75% of the samples contained 0.2–1.0 ng/mL. This amount reflects a tolerable daily intake (TDI) value of toxin. However, in some cases (6.8%), more than 1.0 ng/mL OA was measured, which is probably a result of elevated intake of OA, which may even exceed the “virtually safe dose”. Our data indicate that, like in many other countries, OA is present in food or feed products available in Hungary, and in order to save the health of consumers, their regular control is desirable.

Introduction

Food laws must include specific legislation imposing limits or tolerance levels in foods for the concentrations of specific contaminants such as mycotoxins. Currently, about 60 countries have set up regulations for acceptable levels of mycotoxins in food and feed. Most of the existing worldwide regulations for mycotoxins refer to aflatoxins, but several countries regulate other mycotoxins as well (1). Among the nephrotoxic mycotoxins, ochratoxin A (OA) was the only one for which proposals or official limits were determined by at least 11 countries in 1990. The acceptable levels range from 1 to 50 µg/kg for food and from 100 to 1000 µg/kg for animal feed (2). Some studies have demonstrated mutagenic, teratogenic, and carcinogenic effects of OA. Based on a recent carcinogenicity study with OA in rats, a “tolerable daily intake” (TDI) in humans was also estimated and ranged from 0.2 to 4.2 ng/kg body weight (BW). The FAO/WHO Joint Expert Committee on Food Additives recently established a provisional “tolerable weekly intake” (TWI) level of 112 ng/kg BW corresponding to 16 ng/kg BW daily, which was calculated on the lowest damaging level in the kidneys of pigs, which are the most sensitive species (2). In view of its potential carcinogenicity, a daily OA intake in the order of 5 ng/kg BW may be a reasonable estimate for a “virtually safe dose” (VSD) (3).

The toxicokinetics of OA differ widely among the investigated animal species; the rhesus monkey showed the longest half-life value of 21 days (4). In human blood, these data vary between 20 and 50 days. As OA binds strongly to serum proteins and accumulates, its blood concentration may be higher than the daily toxin intake (5). In order to estimate the possible human health risk of OA (5), it is necessary to measure its blood concentration and calculate the daily toxin intake of the population. Several methods have been used for analysis of feeds, foods (6–8), and body fluids (3,4,9–14). For the determination of OA in body fluids (human and animal plasma and milk), different analytical methods, such as spectrofluorometry (12) and high-performance liquid chromatography (HPLC) (3,4,15), are commonly used. The detection limits of HPLC range from 5 to 10 ng/g.

Recently, some reports have been published about sensitive immunological methods (e.g., RIA, ELISA) using polyclonal or monoclonal antibodies for OA determination in porcine serum (6,15–17). ELISA is simple, reliable, requires relatively inexpensive equipment, and enables a large number of samples to be run daily. Despite these advantages, few papers have been published on the analysis of ochratoxin A in human plasma by this technique (10).

During a 5-year research program, a sensitive, monoclonal antibody-based ELISA has been developed for the determination of OA in cereals. In the present study, this test was modified and applied for quantitative measurement of OA in human serum.

Materials and Methods

Antibody

Monoclonal antibody against OA was developed as published elsewhere (18). Crossreactivity of this antibody with OA and ochratoxin B was 100 and 9.3%, respectively, and no crossreactivity was measured with ochratoxin α, coumarin, and L-β-phenylalanine.
Toxin label
The carboxylic group of OA was conjugated directly to horseradish peroxidase (RZ = 3.0) by a mixed anhydride method described by Märtlbauer et al. (7). The working dilution of this conjugate was determined in direct ELISA.

Serum samples
In a 3-month period between March and July 1995, a total of 355 serum samples from random internal medicine patients was collected at the Szent János Hospital. The samples were kept frozen before analysis.

Direct competitive ELISA
Coating. Microplate wells (Immunoplate F-8 Maxisorp, Nunc, Denmark) were coated with 150 μL rabbit Ig anti-mouse Ig (10 μg/mL) for 18 h at room temperature, and then washed three times with 0.05% Tween 20 in distilled water. Next, 120 μL of anti-OA (1:500) ascites fluid was pipetted into each well and incubated for 18 h at room temperature. After washing with distilled water, the coated plates were dried and stored at 4°C in a sealed foil bag for up to several months.

Immune reaction. Fifty microliters of toxin standards or extracted samples (for extraction procedure, see below) were pipetted into the precoated wells and incubated simultaneously with 50 μL of OA-peroxidase conjugate (1:4000) diluted in PBS for 1 h at room temperature.

Enzyme reaction. After the four washing steps, the wells were incubated with 150 μL of tetramethylbenzidine (TMB)/H2O2 substrate per well for 15 min. The color reaction was terminated by adding 50 μL of 6N sulfuric acid, and the optical density at 450 nm (OD450nm) was measured.

Evaluation. The standard curve of OA was obtained by plotting log10 concentration (x axis) against B/B0 (y axis), where

\[
B/B_0 = \frac{(\text{OD of standard or sample})}{(\text{OD of blank [no toxin added]})}
\]

OA concentrations in serum sample extracts were calculated by using a calibration curve and expressed in nanograms per milliliter. The nanogram-per-milliliter value was multiplied by 2 (when sample extract was used directly), which gave a measuring range of 0.2-4 ng/mL. If the OA value was more than 3.5 ng/mL, the extracts had to be further diluted and the concentration calculated using the appropriate multiplication factor. The slope of the standard curve is the color change per concentration unit.

Extraction and cleanup
Two milliliters of human serum, 2.5 mL 1M citric acid, and 4 mL dichloromethane were added to a centrifuge tube, vortex mixed for 5 min, sealed with parafilm, shaken for 1 h on a horizontal shaker at room temperature, and finally centrifuged for 20 min at 3000 × g. Three phases, aqueous (upper), white ring (middle), and dichloromethane (lower), were obtained after centrifugation. Two milliliters from the lower (dichloromethane) phase was transferred to another conical tube, and 2 mL of 1% sodium bicarbonate buffer (pH = 8.8) was added. The tube was sealed with parafilm and shaken for 30 min, during which OA went into the aqueous solution. The mixture was centrifuged (20 min at 3000 × g) in order to obtain a clear buffer solution. An aliquot (490 μL) of the upper buffer solution was pipetted to a test tube, and 10 μL of 1N HCl was added. The solution was mixed thoroughly, and 50 μL of this solution was used directly in the ELISA. If the expected OA concentration was higher than 3.5 ng/mL, the final sample solution was further diluted with 1% sodium bicarbonate buffer (pH = 8.0–8.5).

Recovery of OA from artificially infected human sera
To 2 mL of low (0.26 ng/mL) concentration human serum pool, 0.1, 0.25, 0.5, and 1.0 ng/mL OA were added, mixed, extracted, and assayed as shown previously.

Recovery of OA from mixed human serum
Sera of different OA concentration were prepared by mixing low (a = 0.26 ng/mL) and high (b = 2.73 ng/mL) OA concentrations of human serum pools in different rates (75% a + 25% b, 50% a + 50% b and 25% a + 75% b). The sera were extracted as a sample, and the OA contents were determined. The correlations between the expected and measured OA values of sera were calculated.

**Table 1. Intra- and Interassay Variations of Human Serum Pools**

<table>
<thead>
<tr>
<th>Pooled human serum</th>
<th>Intra-assay CV (%)</th>
<th>Interassay CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OA (ng/mL)</td>
<td>9.53</td>
<td>14.8</td>
</tr>
<tr>
<td>0.24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.2</td>
<td>7.87</td>
<td>10.2</td>
</tr>
</tbody>
</table>
Results

ELISA test

The sensitivity of any immunoassay is based mainly on the reaction of analyte with its specific antibody. The quality of this antibody (affinity, avidity, crossreactivity) is a crucial factor for development of sensitive assays. A monoclonal antibody raised earlier against OA (18) was used for developing a direct, competitive ELISA. This monoclonal antibody was coated indirectly to the microplate using anti-mouse IgG immunoglobulin raised in rabbits as the capture antibody. Figure 1 shows a typical calibration curve of OA under optimized conditions in a range of 0.1-2 ng/mL with a detection limit of 0.042 ng/mL in buffer solution (0 ± 2 SD). The most accurate part of the curve is the middle range; the ID_{50} value was 0.4 ng/mL OA. The within-assay and interassay coefficients of variation of the standard points of the calibration curve were each <10%.

Table II. Recovery of Ochratoxin A Added to Human Sera

<table>
<thead>
<tr>
<th>OA added (ng/mL)</th>
<th>Expected OA (ng/mL)</th>
<th>Detected OA* (ng/mL)</th>
<th>CV (%)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>1.26</td>
<td>0.84 ± 0.1</td>
<td>12.5</td>
<td>67</td>
</tr>
<tr>
<td>0.5</td>
<td>0.76</td>
<td>0.40 ± 0.02</td>
<td>8.5</td>
<td>53</td>
</tr>
<tr>
<td>0.25</td>
<td>0.51</td>
<td>0.30 ± 0.06</td>
<td>18</td>
<td>75</td>
</tr>
<tr>
<td>0.1</td>
<td>0.36</td>
<td>0.33 ± 0.06</td>
<td>18</td>
<td>91</td>
</tr>
</tbody>
</table>

* OA content of pooled human serum = 0.26 ng/mL.

Table III. OA Concentrations in Human Sera

<table>
<thead>
<tr>
<th>Number of samples</th>
<th>Range (ng/mL)</th>
<th>Relative frequency of occurrence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>355</td>
<td>&lt; 0.2-10.0</td>
<td>&lt; 0.2 ng/mL 0.2-1.0 ng/mL &gt; 1.0 ng/mL</td>
</tr>
<tr>
<td></td>
<td>18.2</td>
<td>75 6.8</td>
</tr>
</tbody>
</table>

Discussion

Among the naturally occurring mycotoxins, OA (synthesized by several Aspergillus and Penicillium strains) is quite common because these fungi grow well on cereals in different
climatic conditions. OA residues are known to occur in cereal crops as well as in edible pig tissues and porcine blood at concentrations that may be of health concern. Consumption of pork products from animals exposed to moldy feed is the most common source of OA. Natural occurrence of OA in pig products has been demonstrated in several countries, and the mean toxin level was found to vary from 4.4 to 13.8 ng/mL. In Denmark, carcasses with residue levels of OA in the kidney over 10 ng/g are considered contaminated. Another source is coffee, the beans of which frequently contain OA, which is not diminished by roasting. As feed and foodstuffs or their raw material can be contaminated with molds at any time, human exposure to OA is always a health hazard.

Some reports have been published about the development of immunoassays for OA in cereals and porcine and human serum samples (7,8,10). These tests used polyclonal or monoclonal antibodies and a complicated extraction procedure, but only few of them possessed acceptable detection limits (0.1 or 0.2 ng/mL serum), which are essential for OA determination in human sera (10) because the toxin is present in biological fluids at the parts-per-billion level.

Our monoclonal antibody-based ELISA with its detection limit of 0.2 ng/mL of OA in serum matrix fulfills this requirement. An additional advantage is the fast and easy sample cleanup with a mild (citric) acid for toxin purification.

As OA is known to be harmful, causing many serious disorders in the human, its level in human blood was investigated in several countries as summarized in a report by Zimmerli and Dick (3). According to this paper, most of the human sera in European countries contained OA in a concentration of <0.1 or 0.1–1.0 ng/mL. In the present study, 75% of the human sera contained 0.2–1.0 ng/mL of OA, which is in good agreement with the values found in Switzerland.

In Bulgaria, a significantly greater proportion of human serum samples from patients with endemic nephropathy or urinary system tumors or both contained more than 2 ng/mL serum compared with samples from nonendemic areas.

In a very recent publication (15), 52 of 100 human blood samples (52%) and 38 of 92 human colostrum samples (41%) analyzed by HPLC were reported to contain OA. In the present experiment, the prevalence of serum samples containing a detectable amount of OA was even higher (81.8%). However, only 6.8% of the 355 samples with OA concentrations above 1 ng/mL were regarded as positive, which reflected an elevated toxin intake. The increased number of positive samples assayed by highly sensitive analytical methods, along with the lack of a firm link between clinical diseases and serum OA concentration, indicates that a well-defined limit would be more informative than sample positivity alone when monitoring human exposure to OA.

Hungarian food law has recently set up new acceptable limits or modified the existing ones for aflatoxins, OA, deoxynivalenol (DON), zearalenone, fusarim T-2 toxin, (HT-2) fusarim HT-2 toxin, (DAS) diacetoxyscirpenol, nivalenol, and patulin in different food matrices. According to these regulations, the tolerance level of OA in roasted coffee or foods of plant origin is 10 ng/g.

There is an empirical formula by which the daily intake of OA can be estimated (3,4). According to this estimation, a daily OA intake of 0.7 ng/kg BW corresponds to a mean serum concentration of 0.25 ng/mL, whereas 5 ng/kg BW (which is regarded as the VSD) would result in a serum concentration of approximately 2 ng/mL. In our case, 24 blood samples (6.8%) contained over 1 ng/mL OA, and two of them contained considerably more than 2 ng/mL.

Conclusion

From our present survey, a direct conclusion regarding a later nephropathy or liver tumor cannot be drawn, but the data should call the attention of control organizations of each country to a thorough food and feed inspection, especially in connection with OA.

References


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