Assessment of dietary exposure to ochratoxin A in the UK using a duplicate diet approach and analysis of urine and plasma samples

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The approach to assess exposure to ochratoxin A from the diet by the analysis of human plasma and urine samples has been developed. Composite duplicate diet samples from 50 individuals and corresponding plasma and urine samples were obtained over 30 days. Samples were analysed using sensitive methods capable of measuring ochratoxin A at 0.001 ng g⁻¹ in food, 0.1 ng ml⁻¹ in plasma and 0.01 ng ml⁻¹ in urine. Analysis of the foods indicated ochratoxin A levels contributing to an average intake in the range 0.26–3.54 ng kg⁻¹ bw day⁻¹ over the 30 days. Ochratoxin A was found in all plasma samples and in 46 urine samples. The correlation between the plasma ochratoxin A levels and ochratoxin A consumption was not significant (95% confidence limit). However, a significant correlation was found between ochratoxin A consumption and the urine ochratoxin A concentration expressed as the total amount excreted. This new work offers the possibility of using ochratoxin A in urine as a simple and reliable biomarker to estimate exposure to this mycotoxin.

Keywords: ochratoxin A, biomarkers, duplicate diets, urine, plasma, intake

Introduction

Ochratoxin A is a mycotoxin produced by fungi such as Penicillium verrucosum and Aspergillus ochraceus. It is principally found to occur as a result of poor storage of commodities or poor agricultural practice during the drying of produce (Moss 1996). Unlike those mycotoxins that are field-contaminants, because ochratoxin A is a storage-related mycotoxin, its occurrence is sporadic, and it will be also be unevenly distributed in contaminated food products. Thus, sampling is a major concern in terms of establishing average levels of contamination of ochratoxin A in foods (Gilbert 1996). Levels of occurrence of ochratoxin A are low, being in the ng g⁻¹ or sub-ng g⁻¹ range (MAFF 1987, 1993, van Egmond and Speijers 1995, Jørgensen 1998).

Historically, ochratoxin A was thought to occur almost exclusively in cereals such as a wheat, rye, barley and oats that had not been adequately dried or had not been dried quickly enough before storage, or had been poorly stored and subsequently had become moisture damaged (Scudamore et al. 1999). By this route it was recognized that animal feed could be contaminated with ochratoxin A and could subsequently be transferred to animal products for human consumption such as pigs’ kidneys, blood sausages and milk (Ominski et al. 1996, Valanta and Goll 1996, Skaug 1999). It is only more recently as the sensitivity of analytical techniques have improved, that it has become apparent that ochratoxin A occurrence is more widespread than previously assumed. Various beans and pulses, coffee, cocoa and dried fruit such as currants and sultanas can also be contaminated (MacDonald et al. 1999). Additionally, as ochratoxin A can survive many food processing operations, products derived from these raw materials such as instant coffee, and cocoa products can be contaminated (Stegen et al. 1997, Leoni et al. 2000), as can derived products such as wine and beer albeit at much lower levels of contamination (Majerus and Otteneder 1996, Zimmerli and Dick 1996, Legarda and Burdaspal 1998).

As a general rule, estimating exposure to a contaminant from analysis of individual foods invariably...
involves significant uncertainty (Douglas and Tennant 1997). In the case of ochratoxin A, the wide range of food types, the sporadic occurrence and the sub-ng g\(^{-1}\) levels at which ochratoxin A is found make assessment of exposure through analysis of foods particularly problematic.

**Toxicological assessment of ochratoxin A**

Ochratoxin A is nephrotoxic and has been found to be a potent renal carcinogen in the male rat (National Toxicology Programme 1989). The IARC has classified ochratoxin A as Group 2B, a possible human carcinogen. Between 1989 and 1995, various bodies such as the JECFA proposed tolerable daily intakes (TDI) for ochratoxin A ranging from 1.5 to 16 ng kg\(^{-1}\) bw day\(^{-1}\). The JECFA re-evaluated ochratoxin A data in February 2001 and maintained the previously proposed PTWI, additionally undertaking a quantitative risk assessment modelling the impact of possible regulatory levels of 5 and 20 ng g\(^{-1}\) in cereals and cereal products. Attempts to assess exposure to ochratoxin A from dietary calculations have indicated possible levels of 1.1–4.5 ng kg\(^{-1}\) bw day\(^{-1}\) in Canada ( Kuiper-Goodman and Scott 1989, Kuiper-Goodman et al. 1993), and levels from 0.7 to 4.6 ng kg\(^{-1}\) bw day\(^{-1}\) in the EU (EC 1997). At the European level, possible regulatory limits for ochratoxin A in cereals (5 and 3 ng g\(^{-1}\)), dried vine fruit (10 ng g\(^{-1}\)), roasted coffee (3 ng g\(^{-1}\)), wine (0.5 ng g\(^{-1}\)) and beer (0.2 ng g\(^{-1}\)) will be reviewed before the end of 2002, with the intended aim of controlling dietary exposure to ochratoxin A (EC 1998).

Most of the above risk assessments have been based on estimations of exposure based on measurements of known sources of ochratoxin A in the diet. This has meant that exposure estimates based on cereals and meat have not included contributions from more recently discovered sources such as coffee, dried fruit and wine (Kuiper-Goodman et al. 1993). The large variability in contamination levels and sampling difficulties means that large numbers of individual food samples need to be analysed. Estimates of exposure based on determining ochratoxin A in foods will thus inevitably have large sources of error. Additionally, the fact that estimated exposure levels using methods based on food intake/food analysis which are inherently inaccurate appear to indicate exposure close to the TDI makes it important to have a better approach to assessing ochratoxin A exposure.

**Alternative approaches to assessing ochratoxin A exposure**

It has been accepted for some time that determination of ochratoxin A in human blood can be used as a good indicator of human exposure to this mycotoxin from the diet (Breitholtz et al. 1991, Ruprich and Ostry 1993, Maaroufi et al. 1995, Zimmerli and Dick 1995). However, assessment has always been based on assumptions and extrapolations concerning ochratoxin A bioavailability and clearance rates based on animal data (Breitholtz et al. 1991, Schlatter et al. 1996). In an attempt to have reliable human exposure data to ochratoxin A, as well as corresponding plasma and urine samples, a 1-month duplicate diet study was carried out in the UK. A full description of the study protocol, the inclusion and exclusion criteria used to screen the volunteers, and other details will be published in MacDonald et al. (2001). Fifty volunteers took part in the study of which 11 were vegetarian and seven consumed an ethnic diet. Subjects kept a daily food diary and gave a blood sample at the start of the study (day –30). At the beginning of the second month (day 0), subjects commenced daily collection of duplicate portions of all food and drink they consumed, which were continued for the entire second month. They maintained their food diaries during this period. The sampling schedule is shown in table 1. All samples were stored immediately at \(-20^\circ\text{C}\). Blood samples were taken from each volunteer once per week for the duration of the study (days 0, 7, 14, 21 and 28). Urine was collected for a 24 h once per week during this time (taken on days –1, 6, 13, 20 and 27) an aliquot taken and stored at \(-20^\circ\text{C}\).

**Table 1. Sampling schedule for collection of duplicate food, urine and blood samples.**

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Days on which samples/information collected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duplicate food</td>
<td>0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28</td>
</tr>
<tr>
<td>Urine</td>
<td>0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28</td>
</tr>
<tr>
<td>Blood</td>
<td>0, 7, 14, 28</td>
</tr>
</tbody>
</table>
Foods samples were prepared on a weekly basis, i.e. four samples per subject. A monthly composite sample was prepared by taking a relative proportion from each weekly sample. The samples were blended together before a subsample was taken for analysis. The plasma sample taken at the beginning of the study (day −30) was analysed. This was used to determine if the subjects had measurable ochratoxin A in their plasma before they participated in the study. Each subject supplied five plasma samples (taken on days 0, 7, 14, 21 and 28 of the study). Composite plasma and urine samples were produced for each subject by combining subsamples (0.5 and 10 ml respectively) from each of the five samples, vortex mixing and storing.

The experimental details of analytical methods involving immuno-affinity column clean-up and HPLC determination with fluorescence detection are described in MacDonald and Brereton (in press).

Results and discussion

Duplicate diets

Although there have been significant advances in analytical methodology for the determination of ochratoxin A (Valenta 1998), none of the methods reported have achieved limits of detection below 0.05–0.1 ng g⁻¹ with good method performance characteristics. However, for the work undertaken in the present study, we have developed a method with a detection limit of 0.001 ng g⁻¹ in food (duplicate diet) samples. This improvement in the detection limit was achieved by using a multistage approach, taking a large sample for analysis, which was extracted into chloroform and concentrated into an aqueous solution. A further concentration was achieved using an immuno-affinity column whereby an equivalent of 56 g of sample was loaded onto the column. Finally, the use of large volume injections for HPLC (up to 2 ml) and post-column pH shift to enhance the fluorescence of ochratoxin A further improved the sensitivity of the analysis. An in-house reference sample with a mean level of 19.2 ng kg⁻¹, was used to establish recoveries which ranged from 70 to 90%, with a between batch CV of 8.8%.

Ochratoxin A was detected in all the composite diet samples, with reported results being corrected for recovery. The levels ranged from 10 to 115 ng ochratoxin A kg⁻¹ diet (mean 30.5 ng kg⁻¹, median 23.7 ng kg⁻¹). The amounts of food consumed by the subjects varied from 35.6 to 99.7 kg. By multiplying the concentrations of ochratoxin A in the individual diets by the total amount of food consumed during the study (weights recorded during sample preparation) the amount of ochratoxin A consumed by each individual were estimated to range from 577 to 7974 ng (mean 1949 ng, median 1383 ng).

Plasma results

The detection limit for ochratoxin A in plasma was established as 0.1 ng ml⁻¹ at a signal-to-noise ratio of 3:1. Ochratoxin A was detected in all the control samples (day −30) and all plasma samples composited for the duration of the study. Ochratoxin A levels found in the day −30 plasma samples ranged from 0.15 to 2.17 ng ml⁻¹, whereas levels determined in the composite plasma samples ranged from 0.4 to 3.11 ng ml⁻¹. These results are similar to other studies carried out on comparable population groups (Hald 1991). For example, in a study of 144 Canadian subjects, ochratoxin A plasma levels of 0.29–2.37 ng ml⁻¹ were found (Scott et al. 1998). The identity of ochratoxin A was confirmed in selected samples by the formation of the methyl ester.

Urine results

The method used for plasma was also applied to urine, with a limit of quantification of ochratoxin A of 0.01 ng ml⁻¹. Some samples contained ochratoxin A at less than the limit of quantification. Wherever possible, this level was ‘quantified’ to make the statistical evaluation easier (to avoid using a series of less than values). Ochratoxin A could not be detected in four samples. The corrected urinary ochratoxin A levels ranged from < 0.01 to 0.058 ng ml⁻¹. The urinary ochratoxin A levels found in this study are very similar to those reported for healthy individuals in a previous study (Castegnaro et al. 1991).
Assessment of biomarkers: statistical evaluation of the results

In figure 1 the results are plotted for total ochratoxin A exposure during the 30-day study against the ochratoxin A plasma levels, from the composite plasma samples. Similarly, the results for urine ochratoxin A levels are plotted in figure 2.

There was no good correlation (0.29) between plasma levels and ochratoxin A consumption. In contrast, there was a more significant statistical correlation (0.52) between the urine concentration of ochratoxin A and ochratoxin A consumption. Nevertheless, the observed correlation, whilst being statistically significant, because of wide confidence limits, would not be of immediate practical use. A non-parametric Kruskal–Wallis test was applied to the additional (demographic) data on subjects to test for any differences. There was a significant difference between the sexes in total ochratoxin A consumption that was not entirely due to differences in the amount of food eaten. All the volunteers who participated in the study lived in the same geographical area, so it is not clear if the differences noted between the sexes would apply to other regions of the UK or elsewhere.

Plasma concentrations, although variable, appeared to depend on age, with higher levels at the age group 30–44 (figure 3). The relationship between plasma concentration and age was significant for day –30 (screening sample), but just short of the 5% significance level for plasma concentration during the study. The relationship did not appear to be due to differences in the amount of ochratoxin A consumed as this showed no significant relationship with age. There was also an indication of a relationship between the urine ochratoxin A concentration and the age of the subject, although it was not significant at the 5% level. This was due to lower urine levels in the over 45s. Despite the overall positive correlation between urine and plasma levels, they showed different trends with age. One possible cause for this could be that the efficiency of ochratoxin A removal from the body decreases with age, leading to higher plasma levels.

There were no significant differences associated with the ethnic diet of the subjects. Vegetarians had higher OTA consumption on average, although their plasma or urine levels were not significantly higher.
Ochratoxin A exposure estimated from plasma ochratoxin A levels

There have been two approaches to estimating ochratoxin A levels from plasma data. Renal clearance of ochratoxin A has been calculated to be 0.048 ml min⁻¹, corresponding to 0.99 ml kg⁻¹ bw day⁻¹ for a 70 kg person, and bioavailability assumed to be 50% (Schlatter et al. 1996). The second approach assumes the rate of renal filtration of ochratoxin A in humans is 0.033 ml min⁻¹ corresponding to 0.67 ml kg⁻¹ bw day⁻¹ (based on the clearance rate of inulin):

\[ k_0 = 0.99 \times C_p / 0.5 = 1.97 \times C_p, \]

(Schlatter et al. 1996)

\[ k_0 = 0.67 \times C_p / 0.5 = 1.34 \times C_p, \]

(Hagelberg et al. 1989),

where \( k_0 \) = continuous dietary intake (ng kg⁻¹ bw day⁻¹), 0.99 or 0.67 = plasma clearance (ml kg⁻¹ bw day⁻¹), 0.5 = bioavailability (fraction of toxin taken up) and \( C_p \) = plasma concentration (ng ml⁻¹).

The estimated daily intake ranged from 0.79 to 6.13 ng kg⁻¹ bw day⁻¹ (mean 2.15 ng kg⁻¹ bw day⁻¹) using the approach of Schlatter et al. (1996), and ranged from 0.54 to 4.17 ng kg⁻¹ bw day⁻¹ (mean 1.46 ng kg⁻¹ bw day⁻¹) using Hagelberg et al. (1989). Neither of these equations will be completely accurate, as one is based on a single human experiment (Schlatter et al. 1996) and the other on the plasma clearance of inulin not ochratoxin A (Hagelberg et al. 1989). The actual intakes calculated from the duplicate diet samples and subjects weights in this study were in the range of 0.26–3.54 ng kg⁻¹ bw day⁻¹ (mean 0.94, median 0.76 ng kg⁻¹ bw day⁻¹). This shows that both equations for estimation of exposure from plasma levels produce estimates of intake that are wide ranging and not particularly close to the known true levels assuming no exposure from other sources, e.g. dust. These estimates compared with known levels are illustrated in figure 4.

Conclusions

The determination of ochratoxin A levels in plasma and urine samples has been demonstrated to be a viable approach to assessing dietary exposure. Some significant relationships between urine and plasma ochratoxin A levels and dietary intake were found, but at the moment these are too weak to be used in a predictive manner. The correlation between urinary concentration and dietary intake appears to be stronger than the corresponding relationship between plasma levels and intake. Previous studies have used plasma levels to estimate intake, but it would appear from this study that this may not be the most suitable matrix. Analysis of urine could be a very useful route to monitor ochratoxin A exposure. However, the relationship between consumption and excretion needs to be defined further before it can be used to estimate actual intake levels. In fact, the correlation might be stronger if comparisons were made with urine excretion and previous day intake. Further research is needed to refine the relationship and gain a further understanding of the reasons why some subjects appear to deviate from the general trend. Temporal changes in urine levels need to be investigated to discover whether urine concentration reflects short-term changes in ochratoxin A consumption or is a stable long-term indicator. The total amount of ochratoxin A excreted in the urine by each individual must be accurately calculated to reduce the uncertainty in this assessment by using a composite sample. Analysis of the weekly samples of diet and urine will provide more information on both these questions.

References

Dietary exposure to ochratoxin A in the UK


