Enzymatic and Immunological Approaches for the Quantitation and Confirmation of Ochratoxin A in Swine Kidneys

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ABSTRACT

Several techniques for the quantitation and confirmation of ochratoxin A (OA) in swine kidneys were examined. Naturally and artificially contaminated swine kidneys were analyzed for OA by conventional high-performance liquid chromatography (HPLC) analysis. Samples were additionally tested by enzyme-linked immunosorbent assay (ELISA) or treated with carboxypeptidase A followed by HPLC analysis (enzymatic method). Correlations (r values) between the conventional HPLC procedure and the ELISA, using artificially contaminated samples, were 0.88 and 0.81 (P < 0.05) respectively, while the corresponding values between the conventional HPLC procedure and the enzymatic method were 0.89 and 0.98 (P < 0.05). The ELISA gave a more direct estimation of OA contamination than the enzymatic procedure. The enzymatic method also had a reproducible tendency to underestimate or overestimate the amounts of OA in kidney. This was found to be dependent on the source of contamination, as artificially and naturally contaminated kidney samples resulted in linear regression analysis slopes of 0.38 and 2.8, whereas the slopes for the ELISA method were 1.13 and 0.92, respectively. The results with the naturally contaminated kidneys suggest that other naturally occurring forms of OA also occurred in swine kidney. Regardless of this effect, the enzymatic method accurately confirms the presence of OA and related compounds in kidney. The techniques are simple and will complement conventional HPLC analysis for the detection, quantitation, and confirmation of OA in swine kidneys.

Key words: Ochratoxin A, ELISA, HPLC, swine kidneys, confirmation, carboxypeptidase A, occurrence

Ochratoxin A (OA) is a highly toxic and frequently occurring fungal metabolite. It is produced as a consequence of secondary metabolism in certain species of Penicillium and Aspergillus. Ochratoxin A is a known nephrotoxin and potent carcinogen which can accumulate in the kidneys of animals fed a tainted diet (13, 15, 18–22, 26–28). This mycotoxin has been implicated as a causal agent of porcine nephropathy and human Balkan endemic nephropathy (2, 3, 14–17). Several countries regulate its presence in agricultural commodities (29); thus it is essential that accurate detection and confirmatory techniques be developed to minimize the possibilities of false and potentially costly condemnation (29). Enzyme-linked immunosorbent assays (ELISA) rely on the unique and highly specific interaction between an antibody and its cognate antigen. These unique specificities have allowed the development of several quantitative assays for OA in swine kidney (29). The ability to quantitatively convert OA to ochratoxin alpha (Oα) by enzymatic hydrolysis followed by conventional high-performance liquid chromatography (HPLC) analysis would further complement the identification of OA. This approach, when performed in tandem with conventional HPLC should minimize the likelihood of false positives. This study investigated the quantitation and confirmation of OA in contaminated swine kidneys using immunological and enzymatic methods. Special attention was given to the performance of these techniques in artificially and naturally contaminated samples.

MATERIALS AND METHODS

Materials

Ochratoxin A and 4-hydroxy-ochratoxin A (4-OH-OA) were isolated from wheat contaminated with Aspergillus ochraceus NRRL 3174 (33). Ochratoxin α was prepared by acid hydrolysis of OA (6). Ochratoxin B (OB) was obtained by catalytic dechlorination of OA with palladium on activated carbon (4). Ochratoxin C (OC) was prepared by esterifying OA with ethanol (8). para-Nitrophenyl phosphate, diethanolamine, goat anti-rabbit immunoglobulin G (IgG) coupled to alkaline phosphatase, and carboxypeptidase A from bovine pancreas were obtained from Sigma Chemical Co., St. Louis, MO. Microtiter plates (Falcon 3911, Microtest III) were purchased from Becton Dickinson Labware, Oxnard, CA. All solvents and reagents used were of analytical grade. Fresh swine kidneys were purchased from Manitoban retailers in the fall and winter of 1994. The kidneys were visually examined and found free of macroscopic lesions.

Swine kidney sample preparation

The OA extraction and spiking procedures were similar to those previously described (5). In brief, OA standards in methanol at appropriate concentrations were added to the kidney tissue and
allowed to dry at room temperature. The control kidney samples used for spiking had been previously tested by HPLC and ELISA and were found negative for free OA and Ou (data not shown). For extraction, 5 ml of double-distilled water was added to 5 g of thawed swine kidney cortex in a capped Nalgene centrifuge tube. The mixture of kidney and water was shaken and then homogenized (Janke and Kunkel, Ultra-Turrax T25, Homogenizer) for 30 s. Ethyl acetate (10 ml) was added to the kidney slurry followed by homogenization for 30 s. An additional 10 ml of ethyl acetate and 250 µl of concentrated H₂PO₄ were added. The mixture was shaken and rehomogenized. The extract was centrifuged for 30 min at 10,000 × g. Two 8-ml aliquots of the supernatant were removed and dried under nitrogen. Prior to the analysis, the two evaporated supernatants were reconstituted either with 4 ml of methanol for the ELISA and conventional HPLC analysis or with 1.2 ml of Tris buffer (0.04 M, pH 8.5) for enzymatic treatment. For all analysis, not spiked and spiked samples were prepared in triplicate and analyzed in triplicate.

**Conventional HPLC analysis**

Analytes were separated on a Waters Novapak ODS reversed-phase C18 (25 cm by 4.6 mm) analytical column using a gradient mobile phase which consisted of double-distilled water adjusted to pH 2.1 with H₂PO₄ (solvent A) and HPLC-grade methanol containing 10% isopropanol (solvent B) at a flow rate of 1.5 ml/min and a temperature of 40°C. The amount of solvent B in the mobile phase was increased linearly from 30 to 48% in the first 10 min of the run, kept constant at 48% for 6 min and increased linearly to 70% over 9 min. During this time period all detectable compounds were eluted. The column was washed with 90% of solvent B for 6 min and further equilibrated with 30% of solvent B for 10 min. The HPLC system was similar to that previously described (5). Fifty microliters of the centrifuged kidney extract supernatant was injected onto the column. Ochratoxin peaks were detected with a Shimadzu Model RF535 fluorescence detector set at 333 nm for excitation and at 450 nm for emission. Pure ochratoxin A was obtained from Sigma Chemical Co., St. Louis, MO and used as a standard (0.25 ng/µl).

**ELISA method for quantitation and confirmation**

The procedures for the preparation of the rabbit antisera and ovalbumin-OA (OV-OA) and the ELISA were similar to those described previously (5). In brief, the microtiter plates were coated with 2.5 µg per well of the OV-OA dissolved in 0.1 M phosphate-buffered saline (pH 7.2), and incubated overnight at 4°C. The plates were washed twice with pH 7.2 phosphate-buffered saline containing 0.01% Tween-20 (pH 7.2; PBS-T), and then air dried at 25°C. Fixed amounts of kidney extract (10 µl) or OA standards diluted in methanol and rabbit anti-OA antisera (140 µl) diluted with pH 7.2 PBS-T were added to each appropriate well in the OV-OA-coated microtiter plate. The microtiter plates were incubated at 37°C for 60 min, the contents of the plate were emptied, and the individual wells washed three times with pH 7.2 PBS-T. Goat anti-rabbit IgG alkaline phosphatase conjugate (150 µl) diluted 1:5,000 in pH 7.2 PBS-T was added to each well and the microtiter plates were incubated for 60 min at 37°C. The plates were washed six times with pH 7.2 PBS-T and dried thoroughly. Alkaline phosphatase substrate solution (1 mg of p-nitrophenyl phosphate per ml of diethanolamine buffer, pH 9.8) was added to each well (150 µl) and the microtiter plates incubated for 30 min at 37°C. Absorbency was determined at 405 nm using a Model 450 microtiter plate reader (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada). The concentration of OA in the samples was determined from curves relating absorbency to the concentration of the OA standard. The relative cross-reactivities of the rabbit antisera used in this study had been previously determined to be 100, 80, 3.33, 10, 1.4, 0, and 0.04% for ochratoxins OA, OC, OB, Ou, 4-OH-OA, 1-phenyllalanine, and citrinin, respectively.

**Enzymatic method for quantitation and confirmation**

Dried kidney extracts were prepared as described above. Each replica sample containing 1.2 ml of Tris buffer (0.04 M, pH 8.5) was divided into two equal fractions of 600 µl. Carboxypeptidase A (200 µl) was added to one fraction and 200 µl of Tris buffer was added to the other fraction. Carboxypeptidase A was prepared by diluting one volume of a commercial carboxypeptidase A stock solution (1.296 units per ml) with three volumes of the Tris buffer. The samples were incubated for 3 h at 37°C and then overnight at 4°C. Following overnight incubation, samples were immersed in boiling water for 5 min, sonicated (Metert Electronic Ultrasonic Cleaner) for 30 s and then centrifuged at 10,000 × g for 15 min. Supernatants were then analyzed directly by HPLC as described above. The extent of conversion of OA to Ou was assessed by comparing enzyme-treated samples with non-enzyme-treated swine kidney controls and the OA standards. The presence of trace quantities of Ou or Ou-like compounds in the original and naturally contaminated samples were accounted for by the subtraction of the peak areas of the unhydrolyzed samples from the peak areas of the hydrolyzed samples (Figure 1).

**RESULTS AND DISCUSSION**

Ochratoxin A is routinely detected in swine kidneys in most northern and central European countries. Denmark, Germany, and the Netherlands have found OA in 20 to 100, 14 to 21 and 7 to 100% of all pig kidneys tested, in amounts ranging from 0.5 to 19.5, 0.1 to 16.4, and 0.2 to 240 ng/g, respectively. Several other countries have been identified as having OA in swine products including Hungary, Sweden, Poland, Belgium, and Czechoslovakia with incidences and concentrations ranging from 1.6 to 79.2% and 0.2 to 104 ng/g, respectively (19, 30). The high incidence of OA in the blood of Manitoba swine also strongly suggests that the kidneys of these animals contain OA (21, 24), since blood is frequently used as an indicator of tissue and diet contamination (15). There are unfortunately few studies on the natural occurrence of OA in swine kidneys in Canada (18). This oversight may have resulted as a consequence of the difficulty in the analysis of this mycotoxin in swine kidney; therefore, we have attempted to simplify the analysis and confirmation of this toxin in swine kidney.

High-performance liquid chromatography is by far the most common method for the analysis of OA in swine tissues (29). This technique is sensitive and accurate; however, its performance depends greatly on the extent of sample clean-up (12, 29). Co-elution of contaminants may interfere with the accurate estimation of the concentration of OA in tissue samples. The presence and concentration of OA in kidney should therefore be confirmed using an independent procedure. Chemical derivatization of OA such as esterification of its carboxyl group to form the methyl ester (23) has enabled analysts to determine OA in grain commodities by conventional HPLC. Quantitative esterification was however found to be unacceptable in animal tissues (23). In addition, the samples required extensive sample clean-up...
FIGURE 1. HPLC separation profiles for ochratoxin standards (A), naturally contaminated swine kidney extract (B), and naturally contaminated swine kidney extract following enzyme treatment (C). The solvent gradient consisted of two components, distilled water adjusted to pH 2.1 with $\text{H}_3\text{PO}_4$ (solvent A) and 90% methanol with 10% isopropanol (solvent B).

FIGURE 2. Relationship between the concentration of ochratoxin A in spiked (- - - , line A), or naturally contaminated (- - - - , line B) kidney samples determined by HPLC and ELISA. Prior to chemical derivitization (23). Mass-spectrum analysis is possible, especially when coupled with HPLC; however, this approach requires extensive sample preparation and expensive instrumentation (1). Immunochemical methods, in contrast, are similar in sensitivity, but have a much lowered requirement for sample clean-up (5). Also, the unique specificities of the antibodies for OA provided an extra degree of confidence in the analysis. The antiserum selected for this study has previously been shown to be highly specific. This unique specificity and high sensitivity allowed the direct analysis of OA in artificially and naturally contaminated swine kidneys. The high correlations of the ELISA results with those of conventional HPLC ($r = 0.88$) and a linear regression slope of 1.13 using the artificially contaminated kidney samples indicated that the assay was potentially quantitative (Figure 2). The corresponding coefficient of correlation ($r$) using the naturally contaminated samples was 0.81 with the slope of the line being 0.92 (Figure 2). The slightly reduced correlation ($r = 0.81$) observed with the naturally contaminated samples suggests that variable amounts of naturally occurring compounds associated with OA contamination may interfere with the assay signal.

Enzymatic cleavage of the OA-amide bond using carboxypeptidase A has allowed several authors to confirm the presence of OA (11); however, this technique was generally considered to be only qualitative. The possibility of the quantitative conversion of OA in swine kidneys to OA was therefore examined and results were compared with conventional HPLC analysis. The correlation ($r$) between the amount of OA in artificially contaminated kidneys and the concentration of OA in the hydrolyzed samples was 0.89, indicating that the results of the two methods were closely related (Figure 3). The low slope of the regression line (slope = 0.378; $r = 0.89$; with $P < 0.05$) compared to the theoretical value of 0.635 suggested that there was a reproducible tendency to underestimate the amount of OA.
The relationship between the concentrations of OA in spiked (-■-) or naturally contaminated (-△-) kidney samples determined by HPLC analysis for OA and HPLC analysis for Oα following enzymatic hydrolysis of the samples. The expected slope of the line would be 0.635 (i.e., the ratio of molecular weight of Oα, 256, to that of OA, 403). The actual slopes of the lines were 0.38 ng Oα/ng OA for the spiked kidney (B) and 2.78 ng Oα/ng OA for the naturally contaminated kidney (A).

The reason for these 60% [(0.378/0.635) x 100] lower recoveries is unknown, but it may be related to the kinetics of the enzyme-substrate reaction and the ability of OA to inhibit the action of the enzyme carboxypeptidase A (4, 25). In contrast, a linear regression slope (2.78; r = 0.98; P < 0.05) for Oα was obtained for the naturally contaminated samples. These high values were unexpected and suggest that Oα was also derived from sources other than OA. The tendency to overestimate the amount of OA on the basis of its content of Oα after hydrolysis suggested that compounds similar to OA were present in the contaminated kidney. These naturally occurring contaminants presumably form Oα or a chromatographically similar compound when treated with carboxypeptidase A. It is conceivable that other forms of ochratoxin may have also been present in the sample, as OA is only one member of a large group of ochratoxins (30). It is also possible that OA is present in the form of protein adducts: recent work has demonstrated that OA is bound covalently to protein under natural conditions (32). We suspect carboxypeptidase A cleaves these bound OA forms from the protein adducts to free Oα. This also suggests that the direct measurements of free OA in kidneys may lead to systematic underestimation of the true levels of OA. It is unknown at this time if antibodies specific for free OA can recognize these conjugated forms, but it might be advisable to develop antibodies to these forms or to modify the standard assay procedure so as to obtain an estimation of total OA concentration. Notwithstanding, the enzymatic hydrolysis approach is an excellent confirmatory technique and with the use of a mathematical correction factor will facilitate the routine analysis and confirmation of OA in swine kidneys.

These results demonstrate the presence of OA in swine kidneys in Manitoba as determined by conventional HPLC, ELISA, and enzymatic analysis. The likelihood of all three techniques being incorrect is low. Although the sample size was small (n = 24) it can be seen that the range of OA concentration was relatively broad. Four samples were higher than 12 ppb, 14 were less than 10 ppb and the remaining were too low to be detected. Nine of the highest OA-containing samples were collected in October, while the remaining and lower values were from samples collected in December. The notable differences in contamination levels are consistent with seasonal differences in temperature and humidity. It has previously been reported that the time of sample collection can influence the incidences of OA contamination in swine blood (7, 21, 24). Higher concentrations of OA contamination were normally observed during harvest times when temperatures and humidity were more conducive to mold growth and lowest during the winter months (21). The extent of this contamination can be assessed and the presence of OA can be confirmed utilizing an approach similar to that used in the current study. It should be noted that in Denmark any level of OA leads to condemnation of the swine kidney. If concentrations of OA are greater than 10 ppb in the kidney all visceral organs are condemned, and if the concentrations are greater than 25 ppb the whole carcass is destroyed (13, 30). Under such regulations, 4 of 24 or approximately 16% of the viscera should have been condemned in Manitoba. Of particular concern is that the total amount of ochratoxin-like compound present in kidney may be greater than that detected as OA. As indicated above, OA may exist with different amino acid side chains (10), as an open lactone ring (32), or as conjugates (9, 31, 32) that may be hydrolyzed to Oα during digestion (31). The very high amounts of Oα produced upon carboxypeptidase digestion of kidney extracts relative to the amount of OA detected (sixfold more) would suggest that the concentration of other forms of OA in the kidney may be substantially greater than that of OA. The potential problem of OA contamination in meat products could therefore be considerably more serious than that indicated by HPLC analysis for OA only. This problem would be of particular concern if the different forms of OA that occur in kidneys have a toxicity similar to that of OA alone. Further research should be carried out to confirm these observations and, if correct, the potential health hazards of OA in Canadian pork products should be further assessed. This is particularly important in view of the high degree of OA contamination in pig blood in Western Canada, especially in the summer and fall months (7, 22, 24) and the corresponding occurrence of OA in human blood (7). It may be advisable to have seasonally dependant tolerance limits when OA concentrations are known to be highest.

ACKNOWLEDGMENTS

We thank Helena Stelovsky for excellent technical assistance. Financial support was from Natural Sciences and Engineering Research Council of Canada, University of Manitoba, and Manitoba Department of Agriculture.
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