

Ochratoxin A in Pig Blood: Method of Analysis and Use as a Tool for Feed Studies

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A procedure is presented for screening the quality of feed in respect to ochratoxin A contamination based upon the analysis of ochratoxin A in pig blood. Representative samples from large feed lots may be obtained by using pigs as in vivo sample collectors which enrich the toxin and forms homogeneous samples in the blood. The spectrofluorometric procedure for ochratoxin A analysis (K. Hult and S. Gatenbeck, *J. Assoc. Off. Anal. Chem.* **59**:128-129, 1976) has been adapted to pig blood and has been simplified to involve only three extraction steps. A volume of 2.5 ml of blood or plasma is needed, and the detection limit is 2 ng of ochratoxin A per ml. The disappearance of ochratoxin A from pig blood as a function of time has been studied. A feeding experiment with ochratoxin A has been performed, and the time course of the concentration of ochratoxin A in blood has been followed during the experiment.

The increasing evidence indicating that ochratoxin A is occurring in pigs and their feed in Sweden (2, 5) and Denmark (3) has emphasized the importance of a broader knowledge of the general distribution of the toxin. A study with such a goal must be based on a great number of analyses, and therefore we have simplified the fluorometric method of ochratoxin A analysis (1) as much as possible. This method has been adapted to analysis of ochratoxin A in blood or plasma from pigs, because as this report will show, ochratoxin A is retained for a considerable time in the blood system of living pigs. The pig can be looked upon as an in vivo sample collector which forms an average, homogeneous blood sample reflecting the ochratoxin A content in its feed.

MATERIALS AND METHODS

Analysis of ochratoxin A in blood or plasma. To a 50-ml nylon centrifuge tube, 2.5 ml of blood (or plasma), 5.0 ml of chloroform, and 20 ml (for plasma, 10 ml) of a solution containing 0.05 M HCl and 0.1 M MgCl₂ are added. Extraction of ochratoxin A is carried out during 10 min in a tube-turning apparatus rotating 20 turns per min. The mixture is centrifuged for 10 min at 10,000 × *g*. From the bottom of the tube, 3.0 ml of the ochratoxin A-containing chloroform is transferred with a syringe into a 15-ml glass centrifuge tube. The chloroform is washed once with 1.5 ml of water on a tube shaker. The phases are separated by centrifugation with a table centrifuge (Wifug). It is important that no emulsion remains so that all water can

be discarded. A 3.0-ml portion of 0.04 M tris(hydroxymethyl)aminomethane buffer, adjusted with sulfuric acid to pH 7.5 (20°C), is added to the chloroform solution, and ochratoxin A is extracted into the buffer by using a tube shaker. The mixture is centrifuged to obtain a clear buffer solution. A 2.0-ml fraction of the buffer extract is transferred to a round borosilicate glass cuvette. A 100- μ l solution of carboxypeptidase A is added [100 U/ml in 0.04 M tris(hydroxymethyl)aminomethane sulfuric acid buffer, pH 7.5, 1 M sodium chloride]. The sample is cooled in an ice bath for 10 min. The fluorescence excitation spectrum is recorded from 320 to 400 nm, at 450-nm emission. The sample is incubated at 37°C for 2 h and cooled in an ice bath for 10 min. The fluorescence spectrum is recorded again. The loss of fluorescence at 380 nm is proportional to the concentration of ochratoxin A.

Experiment with intravenously administered ochratoxin A. Ochratoxin A was dissolved at a concentration of 1 mg/ml in 0.85% (wt/vol) aqueous sodium bicarbonate. The solution was sterilized by filtration through a membrane filter (Millipore Corp.) before use. Each of three pigs was injected intravenously (auricular vein) with one dose of ochratoxin A, calculated to correspond to 50 ng of toxin per g of pig. As a control, one pig was given only the buffer solution without ochratoxin A.

During a period of 23 days, 9 blood samples of approximately 5 ml each from each pig were taken from the anterior vena cava. Heparin was used as an anticoagulant, and the samples were either stored frozen or analyzed immediately. Single samples were used.

The animals were of Swedish Landrace, belonged to the same litter, and were about 14 weeks old at the

beginning of the experiment. The weights of the pigs during the experiment are shown in Table 1.

Pigs number 1 (control) and 2, and pigs 3 and 4 were kept and fed in pairs. The feed consisted of commercial, compounded pig feed.

Ochratoxin A feeding experiment. For feeding experiments, nine pigs were used: one control pig and four groups with two animals in each, which per os were given daily doses of 10, 50, 300, and 1,750 μg of ochratoxin A per pig, respectively. The ochratoxin A was given in gelatin capsules. Gelatin capsules (no. 3; Parke Davis & Co.) were filled to half the volume with lactose, and the ochratoxin A, dissolved in chloroform, was pipetted on top of the lactose. After 2 h, when the chloroform had evaporated, the capsules were closed.

Ochratoxin A was fed daily, 2 h after the morning feed had been given to the animals. Blood samples were taken just before the capsules were given. Heparin was used as an anticoagulant, and plasma was separated by centrifugation. The plasma samples were either analyzed immediately or stored frozen. All analyses were performed in duplicates.

The different groups of animals were kept and fed separately, except for the control pig, which was kept together with the pigs receiving the lowest dose of ochratoxin A. The feed consisted of commercial compounded pig feed. Portions of 1.5 kg of feed were given per pig per day.

Ochratoxin A was given during 14 days, and the increase of ochratoxin A concentration in the plasma was followed. After this period, the disappearance of ochratoxin A from the plasma was followed during 16 days. Weights of the pigs during the experiment are shown in Table 2.

Standards. The changes in fluorescence of standard solutions of ochratoxin A in buffer were measured as described above. Diluted standard solutions of ochratoxin A in buffer were prepared from a stock solution (10^{-4} M ochratoxin A) in buffer, which was stored frozen. The concentration of the stock solution was determined spectrophotometrically at 380 nm, using a value of $5,680 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for the extinction coefficient.

Apparatus. A Turner 430 spectrofluorometer with scanning attachment was used. The spectrofluorometer was equipped with a polarizer in the emission pathway, which is necessary when measuring low amounts of ochratoxin.

Carboxypeptidase A. Carboxypeptidase A was

TABLE 1. *Weight and increase in weight of pigs during the experiment with intravenously administered ochratoxin A^a*

Pig no.	Wt (kg)		Increase in wt (kg/day)
	Day 0	Day 21	
1	32.0	40.0	0.38
2	35.0	43.0	0.38
3	34.0	44.0	0.48
4	27.0	32.5	0.26

^a Dose was 50 ng of the drug per g of body weight. Pig no. 1 is a control, without any ochratoxin A injected.

TABLE 2. *Weight and increase in weight of pigs, and doses given during the feeding experiment*

Pig no.	Dose (μg /pig per day)	Wt (kg)		Increase in wt (kg/day)
		Day 0	Day 18	
5	0	21.0	25.5	0.25
6	10	28.5	31.5	0.17
7	10	28.5	31.5	0.17
8	50	32.0	37.0	0.28
9	50	29.5	36.0	0.36
10	300	26.5	32.5	0.33
11	300	31.0	36.0	0.28
12	1,750	34.0	39.0	0.28
13	1,750	31.0	34.5	0.19

obtained from Sigma Chemical Co., St. Louis, Mo. The preparation used was C 6510 carboxypeptidase A (EC 3.4.12.1). This quality of the enzyme has been treated with diisopropylfluorophosphate to eliminate trypsin and chymotrypsin activities.

RESULTS AND DISCUSSION

Analysis of ochratoxin in blood and plasma. The method described here had an average fluorescence recovery for ochratoxin A of 67% for blood samples (in the concentration range of 2 to 200 ng/ml), as compared with standards prepared in pure buffer. The loss of fluorescence intensity was composed of at least two different components, nonquantitative extraction and quenching of the fluorescence in the extract. By introducing known amounts of ochratoxin A into buffer extracts, the quenching contribution to the loss was measured to be 10% of the fluorescence of the standards. The remaining 23% of the loss was mainly the result of a nonquantitative extraction of ochratoxin A from the chloroform phase into the buffer.

The recovery of ochratoxin A from plasma was higher than that from blood. In the concentration range of 2 to 200 ng/ml, the recovery was 78%.

The linearity of the method from 2 to 200 ng/ml had a correlation coefficient of 0.9994. Above 200 ng/ml, the method was not linear, and the recovery of ochratoxin A from plasma decreased to 68% at 1,600 ng/ml.

The best extraction of ochratoxin A from blood was obtained at a pH between 2 and 3. The magnesium chloride in the hydrochloric acid prevented the extraction of colored substances from the blood. These substances diminished the fluorescence of ochratoxin A by quenching if they were allowed to enter the buffer phase.

The original procedure for enzymic hydrolysis of ochratoxin A (1) was modified by selecting a different buffer and a different enzyme quality. The pH of the buffer was adjusted with sulfuric

acid instead of hydrochloric acid. A more purified quality of carboxypeptidase A was used as specified in Materials and Methods. These changes were necessary to avoid the formation of an opaque precipitation during the incubation with the enzyme.

The method could be applied on plasma, serum, or whole blood with or without heparin. The higher relative concentration of ochratoxin A in plasma and serum compared with that of whole blood (5) made those samples more convenient when analyzing low concentrations of the toxin.

Disappearance of ochratoxin A from pig blood after intravenous administration. The concentration of ochratoxin A in the blood as a function of time is shown in Fig. 1. No ochratoxin A was observed at any time in the control pig (number 1). The results are adjusted for the dilution of the toxin by increases in the weight of the pigs. To make the best fit of the blood concentration as a function of time, the following equation has been applied

$$A_t = (A_0^{-0.54} + 0.0133t)^{-1/0.54}$$

where A_0 and A_t represent nanograms of ochratoxin A per milliliter of blood at time zero and time t , respectively, and t is the time measured in days.

The disappearance of ochratoxin A from the blood was slightly slower than that published previously for kidney, liver, muscles, and fat (4).

Ochratoxin A feeding experiment. The ochratoxin A feeding experiment consisted of two parts, the ochratoxin A feeding part and the part observing the elimination of ochratoxin A from the pigs. During the first part, the ochra-

toxin A concentration in plasma was continuously increasing until a steady-state concentration was reached. At this concentration, the amount of ochratoxin A taken up into the plasma and the amount that disappeared from the plasma during 1 day were equal. The results, showing the concentrations of ochratoxin A in plasma as a function of time, are given in Fig. 2.

It can be seen from Fig. 2 that in all pigs the concentrations of ochratoxin A in plasma were approaching a steady state before the end of the ochratoxin A feeding period. If the means of the steady-state concentrations were compared with the doses given, calculated as nanograms of ochratoxin A per gram of feed, the following relationship was obtained: plasma concentration of ochratoxin A (nanograms per milliliter) = $1.5 \times$ concentration of ochratoxin A in feed (nanograms per gram).

The important conclusion from this relationship was that ochratoxin A in a contaminated feed could be expected to appear in a more concentrated form in the plasma than in the consumed feed.

The decreases in the concentrations of ochratoxin A in the plasma of the pigs started at the end of the ochratoxin A feeding period. Only one group, given the lowest dose of ochratoxin A (Fig. 2D), showed an increase instead of a decrease in the concentration of ochratoxin A. This was explained by a contamination of ochratoxin A in the feed, because the control pig at the same time contained some ochratoxin A. If the amount of ochratoxin A in the control was subtracted from the toxin concentrations of the pigs in this group, the values obtained agreed with the steady-state concentrations. The other groups were not influenced by this small amount of ochratoxin A.

The broken lines in Fig. 2 indicate the calculated disappearances of ochratoxin A starting with the means of the steady-state concentrations for each group of animals; the disappearances were calculated by the equation derived in the experiment with intravenously administered ochratoxin A. The parameters given in the equation were valid only for concentrations in plasma. The plasma values had to be converted into the corresponding concentrations in whole blood by the factor 0.43 before use. The agreement between calculated and observed disappearances of ochratoxin A from the plasma was good. Only in the case of the highest dose given (1,750 μg per pig per day; Fig. 2A) could a pronounced discrepancy be seen. The reason for this is not understood for the moment. The conclusions from this feeding experiment were that the ochratoxin A was concentrated in the plasma as compared with the feed and that the

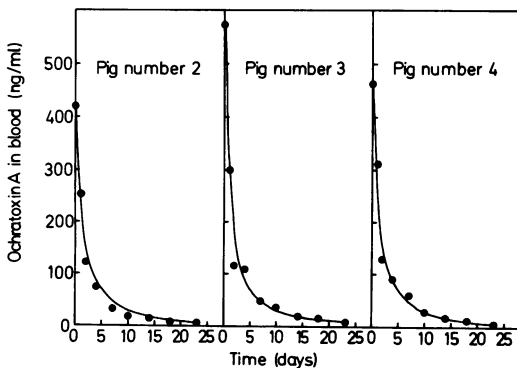


Fig. 1. Disappearance of ochratoxin A from pig blood. Each pig was injected with 50 ng of ochratoxin A per g of body weight at time zero, and the concentration of ochratoxin A in the blood was followed during 23 days. The points represent the experimental results, and the curves are calculated from the equation given in the text.

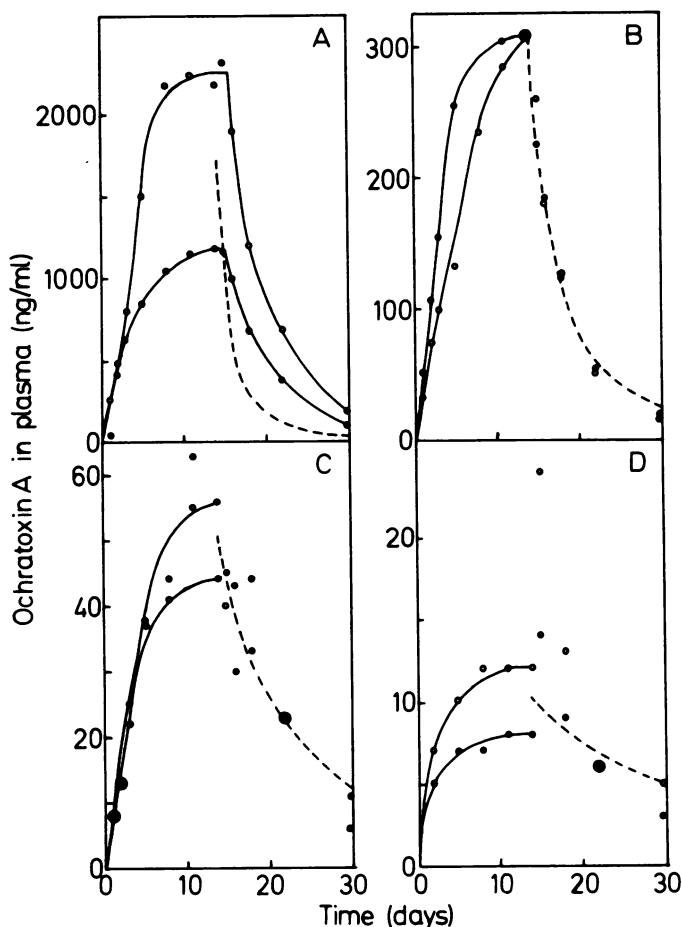


FIG. 2. Concentration of ochratoxin A in pig blood during ochratoxin A feeding experiment. Two animals in each group (A, B, C, and D) were, per os, given daily doses of 10, 50, 300, and 1,750 μg of ochratoxin A per pig, respectively. The last ochratoxin A doses were given on day 13. Symbols: \circ , \bullet , experimental points from the two animals in each group, respectively; (---) the calculated disappearance of ochratoxin A according to the equation given in the text.

disappearance of ochratoxin A was fast at high concentrations in the plasma but low concentrations remained for a very long time.

Conclusions. One of the main problems when dealing with mycotoxin contamination of feed is the sampling. The aim of the sampling should be to get a representative sample of the feed given to the animals. In the case of ochratoxin A, the problem could be solved by using the pigs as samplers. Ochratoxin A will be retained in the pigs, and the concentration of the toxin in the blood will be a good measure of the amount of toxin that the pigs have been exposed to. The heterogeneous distribution of ochratoxin A often observed in feed will appear as a homogeneous and average sample in the blood of the pigs. The blood is easily collected in the slaughterhouse or can be taken from animals in vivo.

We showed earlier that pigs from two small herds fed with spontaneously occurring ochratoxin A-contaminated feed had, within each herd, almost the same concentration of ochratoxin A in kidneys or blood (5). From herds that are fed with the same feed it should therefore be sufficient to analyze only one pig from each herd to get a good knowledge of the occurrence of ochratoxin A in the feed given to the herd. The described analytical procedure will allow screening for ochratoxin A in large areas and performance of comprehensive epidemiological investigations, including living individuals, without an unreasonable amount of analytical work.

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