Cyclopiazonic Acid Residues in Milk and Eggs†

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The mycotoxin cyclopiazonic acid (CPA) was detected in the milk from ewes and the eggs from chickens that were given oral doses of the toxin. Three lactating ewes were given CPA for 2 days at a rate of 5 mg/kg of live weight. Thin-layer chromatographic analyses of dried, defatted milk samples showed that CPA was present in milk at an average concentration of 236 ng/g within 24 h of the first dose. The concentration of CPA reached a high of 568 ng/g, but none was detectable by the ninth day after the first dose. Laying hens were given oral doses of CPA in two separate studies: an acute study over 9 days with dose groups of 0.0, 2.5, 5.0, and 10.0 mg/kg of live weight and a chronic study over 4 weeks with dose groups of 0.0, 1.25, and 2.5 mg/kg of live weight. Eggs from birds in all dose groups throughout both studies contained CPA. The concentrations of CPA were much higher in egg whites than in egg yolks, averaging approximately 100 and 10 ng/g, respectively.

Keywords: *Mycotoxins, Aspergillus flavus, Penicillium, sheep, chickens*

INTRODUCTION

Cyclopiazonic acid (CPA) is a toxic indole tetramic acid that is produced by many species of the genera *Aspergillus* and *Penicillium* (Dorner et al., 1985). The toxin has been found in cheese (Le Bars, 1979), corn (Gallagher et al., 1978; Widiastuti et al., 1988), and peanuts (Lansden and Davidson, 1983; Urano et al., 1992), and it was implicated in a human intoxication involving contaminated kodo millet (Rao and Husain, 1985). These observations point out the possibility of human exposure to CPA through consumption of contaminated foodstuffs. In addition, Norred and co-workers (Norred et al., 1985, 1988) demonstrated the possibility of exposure as a result of consumption of contaminated animal tissues because significant accumulation of CPA in tissues of rats and chickens resulted from oral doses of the toxin.

Other potential sources for human exposure to CPA are eggs and milk from animals that have consumed CPA-contaminated feed. However, the risk from such exposure is unknown because there are no reports of CPA accumulation in eggs or milk. In this paper we report the results of studies designed to measure the accumulation of CPA in milk and eggs from lactating ewes and laying hens, respectively, that were given oral doses of the toxin.

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MATERIALS AND METHODS

Toxin. The CPA used in these studies for dosing and as thin-layer chromatography (TLC) standards was purified from cultures of *Penicillium griseofulvum* (NRRL 3523). The fungus was maintained on Czapek agar slants, and it was grown in Fernbach flasks containing the following medium: glucose, 60 g; $NaNO_3$, 4.5 g; K_2HPO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; KCl, 0.5 g; $FeSO_4 \cdot 7H_2O$, 10 mg; $ZnSO_4 \cdot 7H_2O$, 17.6 mg; $Na_2B_4O_7 \cdot 10H_2O$, 0.7 mg; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.5 mg; $CaSO_4 \cdot 5H_2O$, 0.3 mg; $MnSO_4 \cdot H_2O$, 0.11 mg; distilled H_2O to 1 L. The pH was adjusted to 5.5 with HCl (Neethling and McGrath, 1977). CPA was purified from chloroform extracts of 10-day-old cultures as previously described (Dorner et al., 1983). Purity of CPA was determined to be >98% on the basis of TLC analysis and its UV extinction coefficient at 284 nm.

Ewes. Three crossbred lactating ewes were administered CPA in gelatin capsules at a rate of 5 mg/kg of live weight per day for 2 days. Samples of milk were collected before the first dose was given (day 0) and then days 1, 2, 4, 9, and 20 after the first dose.

Chickens. *Acute Study.* Crossbred laying hens were allocated to four groups of five, housed individually in cages, and fed a commercial layer ration. Daily doses of 0, 2.5, 5.0, or 10 mg of CPA/kg of live weight were administered orally in gelatin capsules for 9 days. Eggs were collected daily, and yolks and whites from birds in each dose group were pooled separately for analyses.

Chronic Study. Three groups of six laying hens kept singly in cages were administered gelatin capsules containing 0, 1.25, or 2.5 mg of CPA/kg of live weight per day for 28 days. Eggs were collected on days 6–10, 14–17, 20–24, and 27–28 after dosing began. Yolks and whites from birds in each dose group were pooled daily for analyses.

CPA Analysis. Extraction and cleanup of CPA from milk and eggs was carried out with methodology similar to that used by Lansden (1986) for peanuts and corn and by Norred et al. (1987) for poultry meat. These methods utilized a chloroform/methanol extraction and partial purification by partitioning the extract with base, acidification, and reextraction with chloroform or dichloromethane.

Specifically, milk and egg yolk samples were first defatted with petroleum ether by blending at high speed for 1 min in a Waring blender with a solvent volume (milliliters) equaling 10 times the sample weight (typical sample weights were 20–40 g). Extracts were vacuum-filtered through glass microfiber filter paper, dried, and reweighed. Defatted milk and egg yolks were transferred to a 500-mL Erlenmeyer flask and extracted with 8 mL/g of chloroform/methanol/5 N HCl (80:20:0.25 v/v/v) for 30

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min on a wrist-action shaker at moderate speed (5 oscillations/s). The extract was vacuum-filtered with rinsing through glass microfiber filter paper and evaporated to dryness on a rotary evaporator at 40 °C. The residue was redissolved in chloroform, adjusted to 25 mL, and transferred to a 125-mL centrifugal separatory funnel. Twenty-five milliliters of 0.1 N NaOH and 5 mL of saturated NaCl solution were added, and the solution was shaken carefully and centrifuged at 1000*g* for 25 min. The upper, aqueous layer was collected and acidified with about 1.5 mL of 5 N HCl. This was shaken with 25 mL of dichloromethane and centrifuged at 1000*g* for 10 min. The dichloromethane layer was collected and evaporated to near dryness on a rotary evaporator at 40 °C, and it was quantitatively transferred to a 4-mL vial. Evaporation of solvent was completed under a stream of nitrogen, and the residue was redissolved in 200 μL of chloroform for TLC analysis. Samples of egg whites were extracted and prepared in the same way except that defatting was not necessary.

Quantiation was achieved by applying 10 μL of the sample to a precoated silica gel 60 F-254 HPTLC plate (10 × 10 cm; EM Industries) along with four standards containing 5, 10, 20, and 50 ng of CPA per spot. Plates were developed in ethyl acetate/methanol/ammonium hydroxide (85:15:10 v/v/v) and sprayed with 1% *p*-(dimethylamino)benzaldehyde in ethanol followed by 50% ethanolic sulfuric acid. CPA appeared as a bluish purple spot at R_f 0.36. The amount of CPA in the sample spot was estimated by comparing it with the four standard spots. Highly contaminated samples were diluted as necessary. The amount of CPA in the original sample was then calculated using the relationship

$$\text{CPA(ng/g)} = CV/ZW$$

where C is the nanograms of CPA in the sample spot, V is the final volume (microliters), Z is the volume spotted on the plate (microliters), and W is the weight of the sample (grams).

The limit of quantitation with this system for a 20-g sample was 5 ng/g. Recovery of CPA from dry milk and freeze-dried egg whites spiked at 10 and 100 ng/g ranged from 68 to 83%, which was similar to results reported by Lansden (1986) and Norred et al. (1987). Results reported below are not corrected for recovery but are adjusted to account for the fat removed during defatting.

RESULTS AND DISCUSSION

Occurrence of CPA in Milk. The effect of the administered CPA on the ewes was marked and rapid. Milk production and feed intake dropped substantially within 24 h of the first dose. By 48 h milk production was only 20% of normal and animals had increased respiration rates and body temperatures. Because of these factors, dosing of CPA was discontinued after 2 days. By 7–10 days daily milk production had returned to nearly the levels prior to administration of CPA.

The occurrence of CPA in the milk of ewes is shown in Figure 1. The CPA concentration averaged 236 ng/g the day after the first dose was given. The concentration rose to 568 ng/g the day following the second dose. Although no CPA was given after day 1, CPA was still present in milk at an average concentration of 262 ng/g on day 4. By day 9 the ewes apparently had fully recovered and no CPA was detectable in the milk.

Occurrence of CPA in Eggs. *Acute Study.* All hens in the 10 mg/kg dose group and four of five hens in the 5 mg/kg group died during the 9-day study. Eggs were available in the 10 mg/kg group only on day 1 after dosing began and for 4 days in the 5 mg/kg group. All birds survived in the control and 2.5 mg/kg dose groups, and eggs were available throughout the trial for these groups.

The occurrence of CPA in the pooled egg whites and yolks is shown in Figure 2. Most striking was the fact that CPA accumulated almost exclusively in the whites (Figure 2A), and it appeared within 24 h after dosing began. On

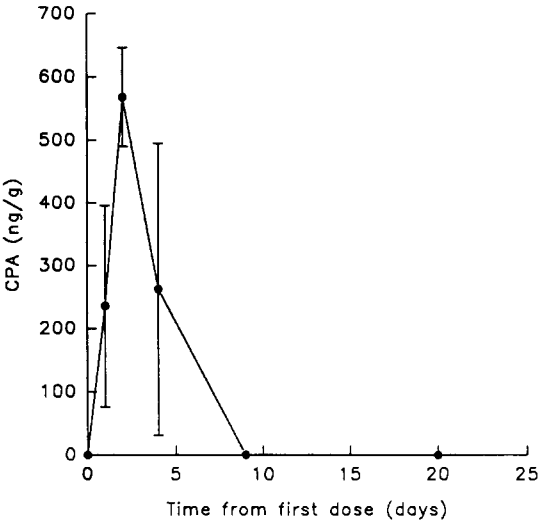

Figure 1. Concentrations of CPA in milk from ewes given oral doses of 5.0 mg of CPA/kg of live weight on days 0 and 1. Each point is the mean value for three ewes with standard deviations shown as vertical bars.

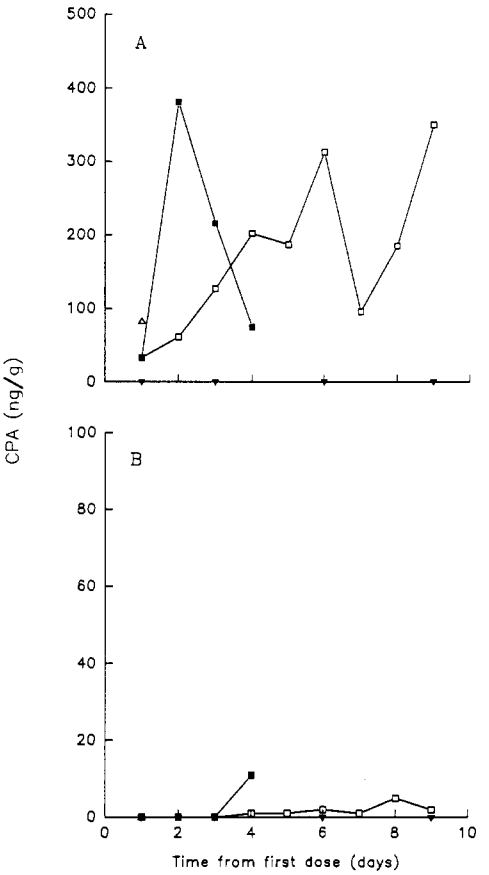

Figure 2. Concentrations of CPA in egg whites (A) and yolks (B) from chickens given daily oral doses of 0 (▼), 2.5 (□), 5.0 (■), or 10.0 (△) mg of CPA/kg of live weight for 9 days. Dosing began on day 0. Plotted points represent the values for pooled samples.

day 1, the concentration of CPA in egg whites was highest (82 ng/g) in the 10 mg/kg group, but that was the only day eggs were available from that group. In the 5 mg/kg group, CPA reached a high concentration of 381 ng/g in egg whites on day 2 and then declined as feed intake, live weight, and egg production declined.

Concentrations of CPA in egg whites from birds dosed with 2.5 mg/kg of live weight generally increased over the first 6 days of the trial to a concentration of 313 ng/g. The

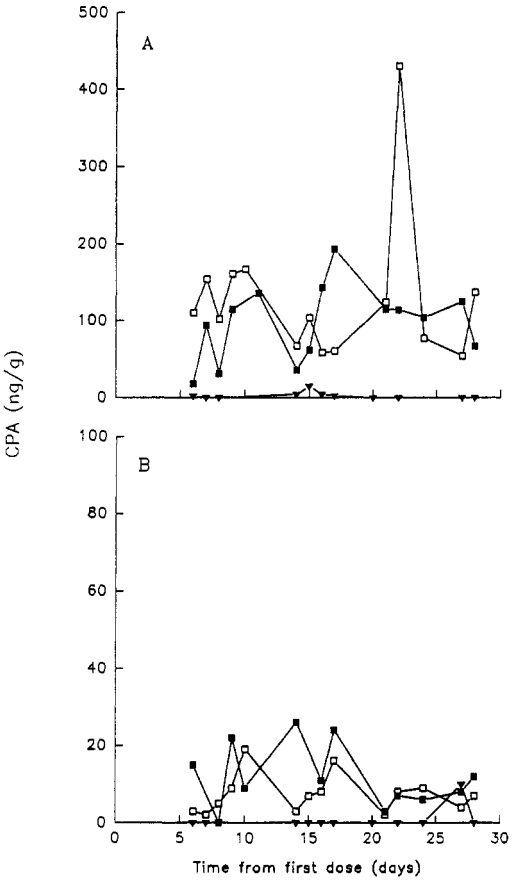

Figure 3. Concentrations of CPA in egg whites (A) and yolks (B) from chickens given daily oral doses of 0 (▼), 1.25 (□), or 2.5 (■) mg of CPA/kg of live weight for 28 days. Dosing began on day 0. Plotted points represent the values for pooled samples.

concentration dropped to 96 ng/g on day 7 and then increased to 350 ng/g by day 9.

Essentially no CPA was found in egg yolks (Figure 2B). In the eggs from hens dosed with 2.5 mg/kg of live weight, CPA was not found in yolks until day 4 at 1 ng/g. The highest concentration in yolks from that group was 5 ng/g on day 8. CPA was found in yolks from the 5.0 mg/kg group only on day 4 at 11 ng/g.

Chronic Study. All hens survived the 28-day trial with the most noticeable response to CPA being a reduction in egg production and egg shell quality. Eggs were first collected for analyses on the seventh day after dosing began, and CPA occurrence in the egg whites and yolks is shown in Figure 3. As in the acute study, most of the CPA was detected in the whites. Egg whites from birds that received 1.25 mg/kg CPA contained a high concentration of CPA of 430 ng/g on day 22. However, the concentration was usually in the range 60–160 ng/g, averaging 105 ng/g for all values excluding day 22. The concentration of CPA in whites from the 2.5 mg/kg group fluctuated in the range 18–193 ng/g, averaging 97 ng/g.

Slightly higher concentrations of CPA were found in yolks from the chronic study than from the acute study. In the 1.25 mg/kg group, the concentration of CPA in yolks ranged from 2 to 19 ng/g and averaged 7 ng/g. In the 2.5 mg/kg group, the concentration in yolks ranged from 0 to 26 ng/g and averaged 12 ng/g.

The occurrence of CPA in milk and eggs is potentially a cause for concern among consumers. This is particularly true in areas where animals may be exposed to feed containing significant concentrations of the toxin. Surveys of CPA in agricultural commodities have been extremely limited, but given the number of species of *Aspergillus* and *Penicillium* known to produce the toxin, the potential for natural CPA contamination would seem to be high. It is clear from the results of this study that potential sources of human exposure to CPA include milk and eggs, thus establishing a need for further studies on the effects of CPA and the scope of its occurrence.

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