Urinary Excretion of Sterigmatocystin and Retention of DNA Adducts in Liver of Rats Exposed to the Mycotoxin: An Immunochemical Analysis

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An indirect ELISA was used to monitor the excretion of sterigmatocystin (St) in urine and retention of St-DNA in the liver of male Fischer rats dosed with St. ELISA revealed that 3.1% of the administered dose (1, 4, 8, and 16 mg of St/kg, ip) was excreted within 24 h. At the 8 mg/kg dose, 86% of the total St urine metabolites was excreted within the first 24 h. In the St-DNA adduct retention study, a linear dose-response relationship (4, 8, 12, and 16 mg/kg) was found by both ELISA and HPLC methods for livers collected 24 h after dosing. Kinetics analysis for the rats dosed at 8 mg/kg revealed that St-DNA adducts were rapidly eliminated; 73% of the original liver St-DNA adducts was eliminated 12 h after exposure. While the total amount of St-DNA adducts in the liver decreased with time, the relative amount of St-formamidopyrimidine (FAPy) increased compared to the amount of St-Guan in the liver. Present results showed that ELISA data correlated well with the HPLC results and ELISA could be used for monitoring St exposure both in humans and in animals.

INTRODUCTION

Sterigmatocystin (St) is a naturally occurring hepatotoxic and carcinogenic mycotoxin produced by fungi in the *Aspergillus*, *Bipolaris*, and *Chaetomium* genera and by *Penicillium luteum* (Betina, 1989; CAST, 1989; Chu, 1991a; Cole and Cox, 1981; van der Watt, 1974). It is known to be a precursor of aflatoxin B_1 (AFB_1) biosynthesis and is 10–100 times less carcinogenic than AFB_1 (Betina, 1989; CAST, 1989; Chu, 1991a) in test animals. Like AFB_1, St has been found to be metabolized by the cytochrome P450 system to an active epoxide, which could then be reacted with nucleic acids to form a DNA-adduct (Essigmann et al., 1979, 1980, 1982) and with glutathione to form a St-glutathione conjugate (Raney et al., 1992). However, St-glucuronide conjugate was the only St metabolite excreted both in feces and in urine (Theil and Steyn, 1973; Olson and Chu, 1993b). Contamination of St in foods and feed as well as incidences of St exposure in livestock and humans have also been reported (CAST, 1989; Chu, 1991a, Sun et al., 1988). Thus, even though St is not as widespread as AFB_1, its carcinogenicity and incidence in foods are reasons for concern. Because of the lack of a sensitive and specific method for monitoring St in foods and body fluids and also the lack of a systematic epidemiological study, the role of St in human carcinogenesis is still not known.

In view of the recent wide use of immunochemical methods for monitoring of various environmental toxicants and carcinogens in body fluids (Chu, 1991b; Groopman et al., 1985), we have recently developed an enzyme-linked immunoassay (ELISA) using polyclonal antibodies against St-hemiacetal that could detect both St-glucuronide and St-DNA adducts (Olson and Chu, 1993a,b). To test if this method could be used for monitoring of St exposure in humans, we have tested the effectiveness of this approach for monitoring the kinetics of urinary excretion of St and the formation of St-DNA in the livers of rats dosed with varying amounts of St. The data obtained by ELISA were compared with HPLC analysis. Results obtained from various studies as compared with previous related work are presented in this paper.

MATERIALS AND METHODS

Materials. Nuclease P_1, fatty acid-free bovine serum albumin (BSA), *o*-phenylenediamine (OPD), Tween 20, keyhole limpet hemocyanin (KLH), and sodium dodecyl sulfate (SDS) were purchased from Sigma Chemical Co. (St. Louis, MO). Hydrogen peroxide, 3-chloroperoxybenzoic acid, and dimethyl sulfoxide (DMSO) were obtained from Aldrich Chemical Co. (Milwaukee, WI). Goat anti-rabbit IgG peroxidase secondary antibody, goat anti-rabbit IgG biotin, streptoavidin, and biotin peroxidase were procured from Boehringer Mannheim Biochemicals (Indianapolis, IN). Male Fischer rats (100–150 g) were obtained from Harlan Sprague-Dawley (Madison, WI). Sterigmatocystin was prepared from fungal cultures of *Aspergillus versicolor* as described by Olson and Chu (1993b). Antibodies against St-hemiacetal (HA) showing good cross-reaction with St, St-HA, and St-DNA adducts and other immunochemical reagents such as St-HA-KLH were prepared as previously described (Olson, 1991; Li and Chu, 1984). All inorganic chemicals and organic solvents were of reagent grade quality or better.

Studies on the Excretion of St in Urine of Rats Dosed with St. For dose-response study, Fischer rats (Harlan Sprague-Dawley) were administered varying ip doses (1, 2, 4, 8, and 16 mg/kg) of St dissolved in DMSO. Four rats were dosed at each level; one rat for each dose served as the control. Twenty-four hours before dosing, urine was collected from all rats to serve as a blank. Rats were housed in metabolic cages (Nalgene, Rochester, NY) and fed ad libitum. Urine was collected into a light-protected bottle in a dry ice ethanol bath for a 24-h period and then stored frozen until the samples were analyzed. At the end of 24 h, the cages were washed with water followed by MeOH. All of the washes were pooled and kept frozen for later analysis. Indirect ELISA was performed on collected urine samples and cage washes after appropriate dilutions (100–10000-fold) in 0.01 M PBS buffer. Urine sample diluted to more than 100 times did not interfere with the ELISA.

For time course study, three rats were administered ip 8 mg of St in DMSO/kg of body weight. The urine was collected 0.5, 1, 2, 3, 4, 6, 9, 12, 18, 22, 28, 34, 42, 50, 59, 63, and 70 h after dosing as described above.

Formation of St-DNA Adducts in the Liver of Rats Dosed with St. In the study of St-DNA formation, rats (three per group) received an intraperitoneal injection of St at doses of 0

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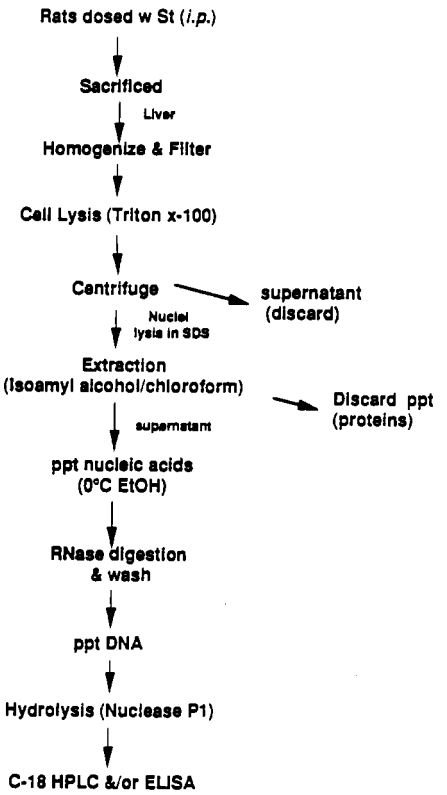

Figure 1. Schematic of protocol for the extraction of DNA from rat liver. Protocol is according to that of Croy and Wogan (1981).

(DMSO only), 1, 2, 4, 8, and 16 mg of St (in DMSO)/kg of body weight. The animals were sacrificed 24 h after dosing. The livers were excised and immediately rinsed with 0.05 M Tris-HCl buffer (pH 7.5), wrapped in foil, placed in plastic bags, and kept on ice. Extraction of DNA from the cut up and homogenized liver DNA was carried out according to the procedure of Croy and Wogan (1981) except that the concentration of RNase used was 10 μg/mg of DNA instead of the prescribed 100 μg of enzyme/mg of DNA. A schematic diagram of the protocol is shown in Figure 1. Detail of the protocol were described by Olson (1991).

For time course response of St–DNA adduct formation, rats were injected ip with 8 mg of St/kg of body weight. At 1, 2, 5, 12, 24, and 48 h after administration of St, two rats were sacrificed and the livers excised as described above. Two livers were combined, and the St–DNA adducts were purified. Purified St–DNA adducts were analyzed by avidin–biotin-enhanced ELISA and by reversed-phase HPLC.

Analyses. *1. Sample Treatment.* For the analysis of urinary metabolite, after appropriate dilution, the urine samples were directly subjected to indirect ELISA.

For the analysis of DNA adducts, samples after nuclease P_1 hydrolysis were subjected to a cleanup treatment with C_{18} Sep-Pak according to the method of Olson and Chu (1993a). Briefly, hydrolyzed sample was loaded onto a prewashed Sep-Pak C_{18} cartridge, washed with 20 mL of 10% MeOH in water, and eluted with 20 mL of 80% MeOH in water. The eluted samples were dried in N_2 gas and resuspended in 1.0 mL of 10% MeOH in water before being subjected to HPLC or the avidin–biotin-enhanced ELISA. The overall average recovery of St–Guan (114–1140 ng added) after C_{18} Sep-Pak cleanup was 91.2 ● 16.5% (ranged from 79 ± 9% to 106 ± 6%; n = 10) (Olson, 1991). Each DNA sample was analyzed by ELISA in triplicate.

2. HPLC Analysis. A Beckman Model 100A pump with a 421A controller equipped with a Beckman System Gold detector Model 167 (Beckman Instruments Co., Irvine, CA) and a μ-Bondapack C_{18} reversed-phase column (Waters Associates, Milford, MA) were used to determine St–DNA adducts. The column was equilibrated with a solvent system of 10% ethanol in 10 mM potassium acetate (pH 5.0). After injection of the samples and running in this solvent system for 10 min, a linear gradient ranging from 30% to 75% ethanol was then applied over a 30-min duration. The flow rate was maintained at 1 mL/min throughout the run. The elution was monitored at both 254 and 330 nm. The amount of adducts was determined by peak height as it corresponded to a St–Guan standard curve, which was established by performing HPLC on two St–Guan standards through a range of 18–180 ng.

3. Competitive Indirect ELISA. The indirect ELISA was performed according to the protocol described by Olson and Chu (1993a). Briefly, 100 μL of St–HA–KLH in 0.05 M carbonate buffer (pH 9.6, 1 μg/mL) was added to each well of the ELISA plate (Nunc, Roskilde, Denmark) and incubated at 4 °C overnight. After the solution was removed, the wells were washed three times with 0.1 mL of PBS–Tween solution (0.1 M sodium phosphate buffered saline containing 0.1% Tween 20, pH 7.4), twice with 0.2 mL of PBS–Tween, and three times with 0.32 mL of PBS–Tween with a semiautomatic Dynatech miniwasher (Dynatech, Alexandria, VA), and incubated with 0.1% gelatin in PBS (0.22 mL/well) at 37 °C for 40 min. The plate was then washed three times with 0.32 mL of PBS–Tween. Fifty microliters of antiserum (anti-St–HA–BSA) diluted in PBS containing 0.1% BSA together with 50 μL of various concentrations of standard St or 50 μL of diluted urine was added to each well, and the plate was incubated at 37 °C for 40 min. The plate was again washed with PBS–Tween as described above, followed by the addition of 100 μL of goat anti-rabbit IgG peroxidase (HRP) conjugate at 1:20000 dilution in PBS containing 0.1% BSA in each well. After incubation at 37 °C for 40 min, the plate was washed again, and 0.1 mL of peroxidase substrate containing 50 mM citrate/100 mM sodium phosphate buffer (pH 5.0), 3.7 mM *o*-phenylenediamine (OPD), and 1.2 mM hydrogen peroxide was added. Fifteen minutes after incubation at room temperature, the reaction was terminated by adding 0.1 mL of 1 N hydrochloric acid. The absorbance at 490 nm was determined from a Thermomax Microplate reader (Molecular Devices, Menlo Park, CA).

4. Avidin–Biotin-Enhanced ELISA. An avidin–biotin-amplified ELISA system was used for the determination of St–DNA adducts under conditions similar to the protocols described above with the following modifications. Instead of using goat anti-rabbit IgG–HRP conjugate as the marker, goat anti-rabbit IgG–biotin was added, incubated at room temperature for 45 min and followed by addition of 100 μL of streptoavidin–biotin–peroxidase complex, which contained 40 μg of streptoavidin, 3.5 units of biotin–peroxidase in 10 mM Tris-HCl with 0.1 M sodium chloride, and 0.1% BSA, prepared a minimum of 30 min before use. The plate was incubated at 37 °C for 15 min, washed as usual, and incubated with 100 μL of OPD at room temperature for 15 min to develop the color.

RESULTS

Dose–Response Relationship between ip Administration of St in Rats and St Metabolite Excreted in the Urine. Results for the dose-related excretion of St are shown in Figure 2; there was a linear relationship between St exposure and the amount of St metabolites excreted in the urine samples as analyzed by indirect ELISA. A 16-fold increase of dose resulted in a 15-fold increase in metabolites in the urine. The values for the St metabolites excreted were based on a St standard and therefore expressed in terms of St immunoequivalents and not in terms of the absolute concentration of the St metabolite. By a linear regression analysis of the data we obtained the following values: slope = 30.9 ± 1.0; r^2 = 0.9969; and standard deviation of the residuals, 12.8. From the slope of this regression, it is apparent that 3.1% of the dosed St was found in the urine.

Kinetics of Excretion of St Metabolites in Urine after a Single Exposure to St. The kinetics of the elimination of the St urine metabolite after a single St exposure is shown in Figure 3. It is apparent that the elimination was rapid. Within 24 h approximately 86% of the total urine St metabolite was excreted. At 63 h, an average level of St metabolites in the urine was around 45

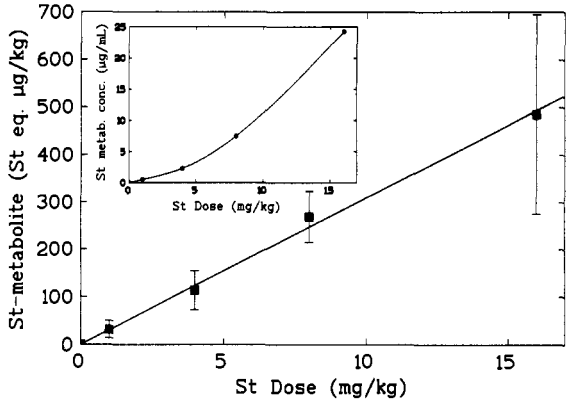

Figure 2. Dose-response curve for the excretion of St in urine of rats as measured by ELISA. Four groups of four rats received a dose of 1, 4, 8, or 16 mg of St/kg of rat weight. The St metabolite in the urine (y axis) is expressed in St equivalent units based on a St standard in the ELISA per kilogram of body weight of rat. Urine was collected for a 24-h period after dosing. The inset shows the concentration of St metabolite (micrograms per milliliter) in the urine collected for 24 h as a function of dose.

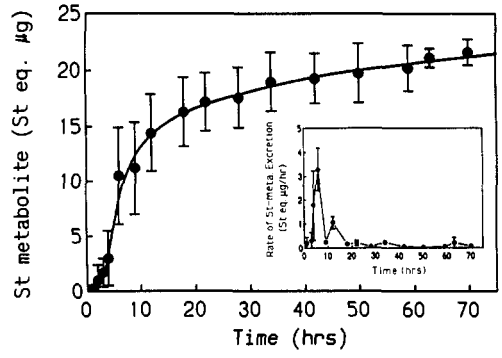

Figure 3. Excretion of St metabolites as a function of time as measured by an indirect ELISA. A group of three rats was ip injected at a level of 8 mg/kg of rat weight. Urine samples were collected at appropriate time intervals, and the St metabolitie was assayed by an indirect ELISA using St as the standard. St metabolite on the y axis is the additive excreted St metabolite and is expressed in St equivalent units. The inset shows the rate of St metabolite excretion, expressed as the amount of St metabolites excreted in urine per hour for each time interval, using data from the same experiment.

ppb. At the time of termination of the study, the rate of excretion was 59 ng of St/h. The kinetics demonstrates an exponential relationship with a slight lag phase during the first 2 h after exposure. When the rate of St metabolite excretion was plotted as a function of time (inset of Figure 3), several peaks were observed. These data indicate that enterohepatic recycling had occurred.

Dose-Response Relationship of St Administration and St-DNA Adducts Retained in the Rat Liver. Results for dose-related formation of St-DNA adducts in the rat liver 24 h after ip administration of St are shown in Figure 4; a linear relationship was seen. The regression statistics for the HPLC data are as follows: slope = 28.5 ± 4.7; x intercept = 1.9; r^2 = 0.9492; standard deviation of the residuals from the line, 41.7; P value = 0.0257. The regression statistics for the ELISA data are as follows: slope = 25.4 ± 5.0; x intercept = 1.97; r^2 = 0.9267; standard deviation of the residuals from the line, 45.13; P value = 0.373. There was little difference in the ELISA vs the HPLC results. Data obtained from both methods were in good agreement when the standard deviation was taken into account for the three rats at each dose. Therefore, analysis of St-DNA adducts by ELISA is a reliable method that is confirmed by HPLC. On the basis of these data,

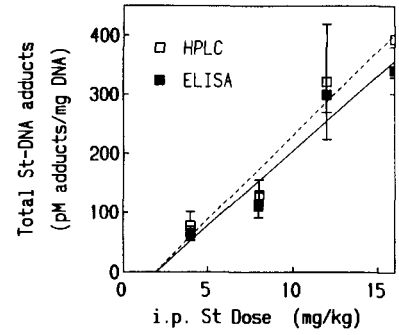

Figure 4. Dose-response relationship for the retention of St-DNA adducts in the rat liver 24 h after exposure of the mycotoxin. For each dose of St, three rats were ip injected with various doses of St. The rats were sacrificed 24 h later, and the DNA was extracted from the livers. The hydrolyzed St-DNA adducts after cleanup were analyzed by an avidin-biotin-amplified indirect ELISA and by HPLC. Values obtained by ELISA are based on standard St-Guan. Values obtained by HPLC are based on measured peak height, at 254 and 330 nm, compared to a St-Guan standard curve. The dotted line was generated for the HPLC data by linear regression. The solid line was generated for ELISA data by linear regression. The concentrations of adducts are expressed as picomoles per milligram of DNA in the y axis.

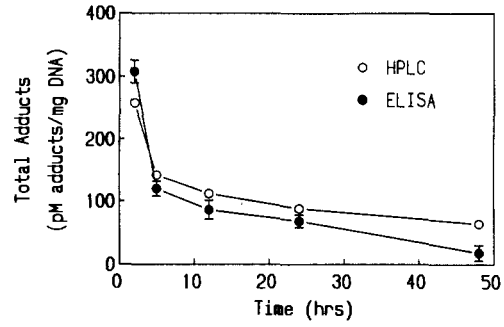

Figure 5. Time-response relationship for the retention of St-DNA in rat liver after the animal received the mycotoxin. Five groups of two rats were given an ip injection of 8 mg of St/kg. Each group was sacrificed, and the liver DNA was extracted at one of the following designated times after St exposure (1, 5, 12, 24, 48 h). The hydrolyzed St-DNA adducts after cleanup were analyzed by avidin-biotin amplified indirect ELISA and by HPLC. The concentrations of adducts are expressed as picomoles per milligram of DNA in the y axis.

the levels of St adducts in DNA were estimated as 2.1 adducts/10^5 nucleotides for the 4 mg/kg dose level and 10.5 adducts/10^5 nucleotides for the 16 mg/kg dose. A 4-fold increase in dose resulted in a 5-fold increase in St-DNA adducts.

Time-Response Relationship of St Administration and St Adducts Retained in the Rat Liver. The kinetics of retention of St-DNA adducts retained in the liver DNA is shown in Figure 5. On the basis of the ELISA data, the St-DNA adducts were quickly eliminated from the liver DNA; approximately 73% of the adducts was eliminated within 12 h. The ELISA results also correlate the elimination trend shown by HPLC analysis. The relative compositions of St-Guan and St-formamidopyrimidine (FAPy) in the St-DNA adducts retained in the liver were determined by HPLC, and the results are shown in Figure 6. A rapid decrease in St-Guan corresponded to a rapid increase in St-FAPy for the first 12 h following St exposure. The decrease in relative percent of St-Guan leveled off after 12 h.

Comparison of HPLC and ELISA Analysis of St-DNA Adducts from Rat Liver. The data from the time-response and the dose-response experiments for the

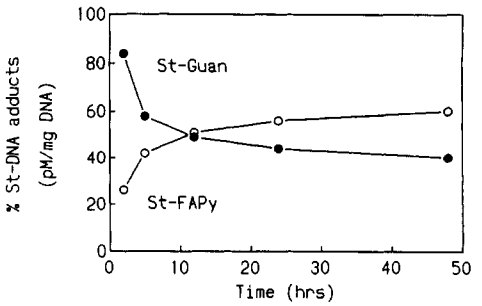

Figure 6. Percent of ST-Guan and ST-FAPy adducts as a function of time after an acute exposure to St in rats. Results are based on the same data as in Figure 5. The values of St-Guan and St-FAPy are based on peak height generated by HPLC analysis using a St-Guan standard curve. The concentrations of adducts are expressed as picomoles per milligram of DNA in the *y* axis.

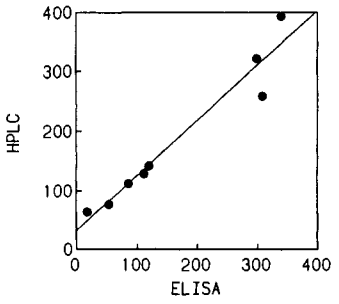

Figure 7. Comparison of values obtained for liver St-DNA adducts as determined by ELISA vs HPLC. Values were from the dose-response and time-response experiments of the rat exposure studies.

analysis of liver St-DNA adducts by ELISA and HPLC are compared in Figure 7; it is clear that results from both methods are linearly correlated. Data from HPLC were consistently higher than the results by ELISA (Figures 4 and 5). Near zero, HPLC results extrapolate to a false positive reading. This may reflect that the lower values were close to the detection limit of the HPLC method (20–25 ng/injection). The detection limit of the avidin-biotin-amplified ELISA was 40–60 pg/assay or a concentration of 0.8 ng/mL, lower than the levels encountered in this experiment.

DISCUSSION

In the present study, a linear dose-response relationship for the elimination of St for the rats injected with 1–16 mg of St/kg of body weight was observed. Approximately 3% of the total St administered was eliminated in the first 24 h. This value is considerably lower than the data obtained by Walkow et al. (1985) when ^{14}C-labeled St was used; they found as much as 7.5% of the total radioactivity in the first 24-h urine sample. This inconsistency might be due to the fact that St was used as a standard in the ELISA. Because St-glucuronide was the only urinary metabolite that had been demonstrated in rats (Olson and Chu, 1993b) and monkeys (Theil and Styen, 1973) and also because the antibodies used in the present ELISA had a higher cross-reactivity with St than with other St metabolites, the actual level of St metabolite would be higher if St-glucuronide is available for use as the standard in the ELISA. At the highest dose (16 mg/kg), there was a large standard deviation for the amount of St metabolite excreted in this group of rats (485 ± 210 μg of St metabolite/kg, Figure 3). Signs of kidney dysfunction, e.g., reduced volume of urine output of about 36–50% of that of control rats, were also evident at this dose. Because this dose is about one-fourth of St LD_{50} (Cole and Cox, 1981), metabolism and/or elimination of St may reach a saturation point and thus cause kidney dysfunction.

A rapid excretion of St-glucuronide was observed in the present study. This observation is in good agreement with those of Walkow et al. (1985). While we found that 86% of the St metabolite was eliminated within 24 h, Walkow et al. demonstrated a 78% elimination. In both studies, 95% of the total urine metabolite was excreted within 50 h. We found that the rate of excretion of St metabolite peaked at 6 h and decreased rapidly thereafter. The cyclic nature of the excretion rate indicated that enterohepatic recycling of the toxin may have happened. Because St is cleared very quickly, monitoring the urine metabolites of exposed individuals would require urine to be collected soon after exposure. For individuals chronically exposed to St, timing of urine collection would be less of a concern. Nevertheless, we had no problem measuring the St metabolites by ELISA even at 70 h after exposure at a dose of 8 mg/kg or at 24 h at 1 mg/kg.

A linear dose-response relationship was observed for St-DNA adducts retained in the liver 24 h postadministration. A 4-fold increase in dose resulted in a 4.8-fold increase in the amount of DNA adducts. Elimination of St-DNA adducts in the liver was rapid; the level of St-DNA adducts in the liver decreased dramatically in the first 24 h postexposure and leveled off with time. This observation is in agreement with the ^{32}P postlabeling DNA experiment; Reddy et al. (1985) found a 2.4-fold increase in rat liver St-DNA adducts with a 3-fold increase in St dose. They also found a one-log decrease in St adducts retained in the liver 24 h postexposure. In our study, the relative amount of St-Guan in liver DNA decreased while the relative amount of St-FAPy increased (Figure 6); this observation is similar to those for AFB_1-Guan-DNA in which a rapid depurination of AFB_1-Guan with a concurrent conversion of the unstable bound guanosine adducts to stable formamidopyrimidine adducts was found (Wang and Cerutti, 1980).

It has been well documented that sterigmatocystin is less toxic and also less carcinogenic than AFB_1 (Engelbricht and Altenkirk, 1972; Krivobok et al., 1987; Stich and Laishes, 1975; Ueno and Kubota, 1976). Aflatoxin B_1 has been shown to be 10 times more potent than St in inducing tumors in Fischer rats (Lillehoj and Ciegler, 1968). Our data on the amount of DNA adducts formed at certain doses are consistent with the carcinogenic potential of both mycotoxins. For example, Fischer rats administered 0.6 mg of AFB_1/kg had the same adduct levels as rats given 6 mg of St/kg (Figure 4). Thus, rats need a St exposure 10-fold that of AFB_1 to form the same amount of DNA adduct. Since St-glucuronide was found to be the only urine metabolite, the UDP-glucuronide transferase may play a major role in metabolizing St and detoxification. Sterigmatocystin has a phenolic group for direct conjugation; in contrast, AFB_1 must be first hydroxylated before conjugation. Thus, St may be more efficiently conjugated and eliminated than AFB_1 and prevent much of the St from forming an active epoxide. However, the role of formation of St-DNA adducts for the overall toxicity and carcinogenicity of St is overwhelming. By chemical synthesis of DNA adducts via an epoxide generated by peroxidative activation, St produced about 10-fold more adducts than AFB_1, suggesting that St readily forms the epoxide and is just as reactive if not more so than the AFB_1 epoxide (Olson, 1991). Since the reactivity of the St epoxide is not less than that of AFB_1 epoxide, other factors must account for the greater toxicity of AFB_1. The

active formation of St–DNA in the liver after dosing with St also reiterates that glucuronidation of St would not give complete protection from DNA adduct formation.

Although we have not found any immunoreactive peaks in HPLC/ELISA other than the St–glucuronide for the urine samples, a small amount of St–Guan may still be present in the sample. During the first 8 h, the greatest concentration of St–glucuronide excreted was 700 nM; in contrast, the greatest concentration of St–DNA adducts eliminated from the liver was 10 nM. Assuming all of the eliminated St–DNA/St–Guan appeared in urine, the amount of St–glucuronide was 70 times more than the St–Guan. Even though the St–DNA is present in the urine, it could easily be overwhelmed by the large concentration of St–glucuronide. Thus, the appearance of St–glucuronide in the urine would suggest that St–DNA adducts were also formed.

Although consistently lower, the ELISA results correlated well with the HPLC findings in both the time– and dose–response experiments. The deviation of HPLC and ELISA results at lower adduct concentrations may be accounted for by the values being close to the limit of detection for HPLC. The limit of detection for HPLC was 25 ng/injection; the St–DNA adduct levels were considerably above the limit of detection (50 pg/assay) for ELISA.

In conclusion, the present results clearly demonstrate that the indirect competitive ELISA method gives a positive correlation between St exposure and the urine metabolite excreted. This has potential for monitoring populations with suspected St exposure. The ELISA method also was able to measure St–DNA adducts, providing a means of determining adduct levels from tissues for assessing St exposure and cancer risk. The ELISA provided a quick, easy, and relatively inexpensive technique that would be beneficial for monitoring St exposure in populations.

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