Immunochemical Studies of Urinary Metabolites of Sterigmatocystin in Rats

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An indirect enzyme-linked immunosorbent assay (ELISA), using polyclonal antibodies against sterigmatocystin (St)–hemiacetal (HA)–bovine serum albumin (BSA), was developed for the detection of St metabolites in rat urine. The detection limits for St in buffer solution and St metabolites in diluted urine without cleanup were 30–64 pg/mL and 5 ng/mL, respectively. Among three cleanup methods tested for the recovery of St metabolites from rat urine, both the C-18 reversed-phase cartridge and the immunoaffinity column provided good recovery; XAD-2 gave the lowest recovery and had the most interference in the HPLC. Identification of the urinary metabolites was achieved by HPLC in combination with ELISA for the samples before and after enzymatic hydrolysis. Only one immunoreactive St metabolite was detected in the urine by HPLC–ELISA. After purification by an immunoaffinity column and hydrolysis with β-glucuronidase, the St metabolite was converted to St. Sulfatase hydrolysis produced no change. Semipreparative HPLC purification of the St urine metabolite followed by fast atom bombardment MS revealed a m/z of St. Exposing the St urine metabolite to UV light resulted in a decrease in immunoreactivity as determined by ELISA. The results suggest that the urine metabolite is a glucuronide conjugated on the phenolic position of St, and the metabolite was very unstable in solution.

INTRODUCTION

Sterigmatocystin (St) is a naturally occurring hepatotoxic and carcinogenic mycotoxin produced by fungi in the *Aspergillus*, *Monocillium*, *Bipolaris*, *Chaetomium* genera and *Penicillium luteum* (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 experimental animals. Contamination of St in foods and feed has been reported periodically; incidences of St exposure in livestock and humans have also been reported (Betina, 1989; CAST, 1989; Chu, 1991a). Thus, even though the prevalence of St is not as widespread as that of AFB, its carcinogenicity and incidence in foods are reason for concern (CAST, 1989; Chu, 1991a; Sun et al., 1988). Sterigmatocystin has been found to be metabolized by the cytochrome P450 system to an active epoxide, which is then free to react with nucleic acids resulting in St–DNA adducts (Essigmann et al., 1979). Very little is known about the metabolic pathways that lead to the elimination of St. By oral administration of ^{14}C-labeled St to vervet monkeys, a major urinary metabolite with a glucuronide conjugated at the phenolic group of St was isolated (Theil and Steyn, 1973). Fifty percent of the recovered radioactivity in the urine was due to this metabolite. Most recently, Raney et al. (1992) have demonstrated the formation of a St–glutathione adduct, which could be another way for the body to eliminate the toxin.

With advances in the development of immunochemical methods for monitoring of the environmental toxicants, enzyme-linked immunosorbent assays (ELISAs) have been shown to be effective in monitoring urinary metabolites of several mycotoxins including aflatoxin–DNA adducts, aflatoxin M_1 (AFM_1) (Chu, 1991b), and T-2 toxin (Lee et al., 1990). An ELISA method was used by Zhu et al. (1987) for monitoring the excretion of AFM_1 in human urine after exposure to AFB_1. A linear correlation of dietary intake of AFB_1 and excretion of AFM_1 in human urine was found by these investigators. Groopman et al. (1985) developed a monoclonal antibody–affinity column that could capture a wide variety of metabolites of AFB_1, including AFM_1, AFP_1, and AFB_1–N^7-guanosine, in the urine of experimental rats and in the urine of humans exposed to AFB_1.

In the present study, a competitive indirect ELISA was established to monitor St metabolites in the urine of rats that had been dosed with St. The ELISA was also used to identify immunoreactive metabolites after separation of the sample by HPLC or TLC and by enzymatic hydrolysis. Details of the ELISA and the use of various immunochemical approaches for the isolation, purification, and characterization of the urinary metabolites of St are presented in this paper.

MATERIALS AND METHODS

A. Materials. Sterigmatocystin was produced in rice inoculated with *Aspergillus versicolor* NRRL 15653 (16 days at 30 °C) and purified according to the method of Steyn and Rabie (1975). Briefly, the toxin was extracted with chloroform and then subjected to chromatography with a Kieselgel 60 (70–230 mesh, 7 × 50 cm) column equilibrated with hexane. Stepwise elution with 4 L each of hexane, methylene chloride, and 1, 1.5, 2, and 10% MeOH in methylene chloride followed. Sterigmatocystin was eluted in the 1.5–2% MeOH in methylene chloride fractions. Fractions containing St as the major component were pooled. The solution was concentrated under reduced pressure nearly to saturation. An equal volume of hexane was added dropwise. The solution was stored at 4 °C for several days and allowed to crystallize. The crystalline St was filtered and washed with cold hexane. Crystals were dissolved in a minimum amount of chloroform and recrystallized as described above. St–hemiacetal (St–HA) was prepared in a manner similar to the method used by Li and Chu (1984). Briefly, 5 mg of St in 10 mL of acetone and 50 μL of 10% sulfuric acid in water was refluxed at 60 °C for 2 h. After reaction, the mixture was evaporated to near dryness, and 10 mL of water was added. Hemiacetal of St

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was extracted from the mixture with 5 mL of $CHCl_3$ three times. The chloroform extracts were passed through a Sep-Pak silica gel cartridge (Waters Associates, Milford, MA) and washed with 10 mL of chloroform. The hemiacetal of St was eluted from the Sep-Pak cartridge with 10 mL of 50% MeOH in chloroform with a final yield of recovery around 79%.

Antibodies against St–HA were produced according to the method of Li and Chu (1984). St–hemiacetal was conjugated to fatty acid-free BSA, keyhole limpet hemocyanin (KLH), or polylysine (PLL) by the reductive alkylation method (Li and Chu, 1984; Olson, 1991). The molar ratios between hapten and carrier protein were 21:1 and 25:1 for St–HA–BSA and St–HA–KLH, respectively. Bovine liver β-glucuronidase, limpet sulfatase, UDP-glucuronic acid, phenobarbital, fatty acid-free bovine serum albumin (BSA), *o*-phenylenediamine (OPD), and Tween 20 were purchased from Sigma Chemical Co. (St. Louis, MO). Dimethyl sulfoxide (DMSO) and hydrogen peroxide were obtained from Aldrich Chemical Co. (Milwaukee, WI). Goat anti-rabbit IgG peroxidase was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). Male Fischer rats (100–150 g) were obtained from Harlan Sprague-Dawley (Madison, WI). Microtiter plates were purchased from Nunc (Roskilde, Denmark). The Sep-Pak C-18 cartridge and C-18 reversed-phase column cartridge for Z-module were purchased from Waters Associates (Milford, MA). All other inorganic chemicals and organic solvents were of reagent grade quality or better.

B. Treatment of Rats. Fischer rats fed ad libitum were administered varying ip doses (4, 8, 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 permit later comparisons. Rats were housed in metabolic cages (Nalgene, Rochester, NY). Urine was collected into a light-protected bottle in a dry ice/ethanol bath for a 24-h period then stored frozen until the samples were analyzed. At the end of 24 h, the cages were washed with water and methanol. The cage washes were saved for later analysis. Indirect ELISA was performed on collected urine samples and cage washes. Samples analyzed by ELISA were diluted 10, 100, 1000, and 10 000-fold in 0.01 M PBS buffer.

C. Competitive Indirect ELISA. The indirect ELISA was performed according to the protocol described by Fan et al. (1984). Briefly, 100 μL of St–HA–KLH, prepared by a reductive alkylation method (Olson, 1991) in 0.05 M carbonate buffer (1.0 μg/mL), pH 9.6, was added to each well of the ELISA plate (Nunc) and incubated at 4 °C overnight. St–HA–polylysine and St–HA–BSA (both at 1.0 μg/mL) were also tested. Because St–HA–KLH was found to be more stable, this conjugate was used throughout the study. 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 diluted in PBS containing 0.1% BSA together with 50 μL of various concentrations of standard St solution or 50 μL of diluted sample solution 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 conjugate at 1:20 000 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, 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 on a Thermomax microplate reader (Molecular Devices, Menlo Park, CA).

D. Establishing Conditions for Cleanup of Urine Samples. *1. Effect of Control Urine Sample on Indirect ELISA.* The effect of control urine sample on the ELISA was determined as follows. Urine samples collected from DMSO vehicle control rats were diluted 10-, 100-, and 1000-fold in PBS buffer (0.01 M potassium phosphate buffer with 0.1 M NaCl, pH 7.4) and analyzed by indirect ELISA. Control urine was compared with the urine from rats exposed to St in the same rat exposure experiment.

2. Evaluation of Cleanup Procedures for HPLC Analysis of St Urinary Metabolites. The effectiveness of three methods was evaluated. The urine sample was first filtered through a Whatman No. 2 filter paper (Whatman, Hillsboro, OR) to eliminate the particulate matter. All samples were then subjected to cleanup with a XAD-2 resin column, C-18 Sep-Pak cartridge or with an anti-St–hemiacetal antibody affinity column. The optimum conditions for each method are presented below.

a. Cleanup with XAD-2 Column. The XAD-2 resin was activated by extensive washing with acetone, water, methanol, and water cycle. The urine samples were diluted 3-fold with water before being loaded onto the column. Two sizes of columns were used. For low urine volumes (2 mL or less), a 1 × 5 cm column was packed with activated Amberlite XAD-2 resin (Sigma). The column was washed with 50 mL of distilled water. The sample was allowed to sit on the column for 15 min. The column was then washed with 50 mL of water at a flow rate of 1.5 mL/min and eluted with 20 mL of methanol. The eluate was air-dried, resuspended in 50% MeOH in water, and then analyzed by competitive indirect ELISA. Urine collected from both exposed and control rats were compared according to the above method. For batch urine cleanup (20–30 mL of urine sample), a 2 × 100 cm column was prepared. The column was washed with 1 L of distilled water before using. After the sample was loaded and incubated for 15 min, the column was washed with 1 L of water, followed by elution with 500 mL of MeOH. The methanolic eluate was then concentrated by rotovap without heat. The concentrated eluate was analyzed by TLC using acetic acid/methylene chloride/acetone/MeOH (1/4/45/50) as the developing solvent.

b. Cleanup with C-18 Sep-Pak. A C-18 Sep-pak cartridge was rinsed with 5 mL of MeOH followed by 5 mL of water. One milliliter of urine sample was diluted 3-fold in water and then loaded onto the Sep-Pak. The Sep-Pak was washed with 20 mL of 10% MeOH in water followed by elution with 20 mL of 60% MeOH in water. The Sep-Pak was rinsed with 100% MeOH to elute any St from the cartridge. The wash and eluate were air-dried to reduce the volume and analyzed by ELISA.

c. Immunoaffinity Column Cleanup. Antiserum was conjugated to Carbolink coupling agarose gel according to the manufacturer's specification (Pierce, Rockford, IL). Briefly, 5 mg of antibody in 1 mL of 0.1 M potassium phoshate buffer (PB) was oxidized with 5 mg of sodium metaperiodate in 6 mL of buffer in an amber vial for 30 min at room temperature. The reaction was terminated by passing the reaction mixture through a desalting column (Pierce). Two milliliters of the oxidized protein sample (1.5 mg) was added to a 4-mL column that was packed with Carbolink agarose gel and equilibrated with 10 mL of 0.1 M PB, pH 7.0. The column, with contents, was capped and mixed by inversion at room temperature for 6 h. The coupling efficiency was approximately 78%.

For immunoaffinity chromatography purification, the affinity column (4 mL of gel) was equilibrated with 8 mL of 10 mM PBS at pH 7.5. One milliliter of undiluted urine was loaded onto the column, incubated at room temperature for 15 min, and then washed with 26 mL of PBS buffer followed by 8 mL of water. The bound ligands were eluted with 12 mL of 60% acetonitrile in water. The column was regenerated with 12 mL of 10 mM PBS, pH 7.5.

E. HPLC Determination of Immunoreactive St Metabolites in Urine. A combination of HPLC and ELISA was used for identification of immunoreactive St metabolites using the same approach as described by Chu and Lee (1989) for trichothecene mycotoxins. Samples after cleanup were separated by HPLC and monitored by a UV detector. High-performance liquid chromatography was carried out on a Beckman Model 100A HPLC with a 421A controller (Beckman Instruments, Irvine, CA) and a Model 440 detector (Waters Associates). Various metabolites of St-exposed urine after cleanup were separated on a Beckman ODS 0.5 × 25 cm C-18 reversed-phase column using the following step gradient at a flow rate of 1 mL/min: 5 min 100% 20 mM PB at pH 3.8, 1 min 10% MeOH in PB, 11 min 24% MeOH in PB, 8 min 26% MeOH in PB, 3 min 30% MeOH

in PB, 13 min 50% MeOH in water, 1 min 65% MeOH in water, 4 min 98% MeOH in water, 10 min 100% MeOH. Fractions (1 mL/fraction) were air-dried and resuspended in 0.25 mL of 10% MeOH in water for ELISA. A portion of the original injected material was saved and also assayed by ELISA. As soon as the retention times for the immunoreactive peaks were known, 0.5-mL fractions were collected at this point in the HPLC run. Standards of St and St-hemiacetal were run on the basis of the HPLC conditions described above to compare their retention times with that of the metabolite. HPLC was conducted on filtered urine from both St-exposed rats and control rats after both had been treated with an XAD-2 column.

F. Semipreparative Purification of St Metabolite Urine and Mass Spectra Analysis. Purification of a larger amount of St metabolites was carried out on two (1 × 25 cm) semipreparative Beckman ODS C-18 reversed-phase columns connected in tandem using a linear gradient at a flow rate of 1.5 mL/min. Urine samples were subjected to XAD-2 cleaned-up before injection onto the column. After the sample was loaded, the column was eluted with 100% water for 15 min followed by a MeOH/water 0–100% linear gradient for a duration of 125 min. The eluate was monitored by a UV detector set at 254 nm. Standard St and St-hemiacetal were chromatographed to enable comparison of their retention times with that of the metabolite. Two-minute (3 mL) fractions were collected, and a portion was diluted 10-fold in 10 mM PBS and analyzed by competitive indirect St-hemiacetal ELISA. The pertinent fractions were pooled from three runs. Pooled fractions were dried by reduced pressure to remove the MeOH, frozen, and then lyophilized. Standards of St and St-hemiacetal were run according to the HPLC method described above to enable comparison of their retention times with that of the metabolite. The lyophilized material was resuspended in 10% MeOH in water and rechromatographed by semipreparative HPLC as before. The immunoreactive fractions were lyophilized and resuspended in MeOH/water as before and analyzed by fast atom bombardment mass spectra (MS 50TC high-resolution spectrophotometer fitted with an Iontec fast atom bombardment gun; Kratos Inc., Manchester, England).

G. Sensitivity of St Metabolite to UV Light. To study the stability of the metabolite under long-wave UV light (366 nm), the St urinary metabolite was partially purified by C-18 Sep-Pak cleanup as described above. The fraction eluted in 50% MeOH was extracted with $CHCl_3$, and the MeOH/water fraction was dried and resuspended in 10% MeOH and water. ELISA revealed that St Metabolite was in the aqueous fraction and not in the $CHCl_3$ fraction. A total of 325 ng of St units of St metabolite in 0.5 mL of 10% MeOH/PB was irradiated at 366 nm in a UV lightbox (Ultra-Violet Product Inc., San Gabriel, CA). Fifty-microliter aliquots were taken out 30 s and 1, 5, 10, and 20 min after UV exposure, diluted 10- and 100-fold in 10% MeOH in water, and analyzed by ELISA.

H. Determination of the Group Conjugated to the St Metabolite by Enzymatic Hydrolysis. For enzymatic hydrolysis, the immunoaffinity-purified St metabolite (free of St) was digested with either β-glucuronidase or sulfatase. The enzymatic activity was determined by hydrolysis of phenolphthalein glucuronide and *p*-nitrophenol sulfate for β-glucuronidase or sulfatase, respectively (Sigma Bulletin G-0251 and S-1629). In general, 200 ng of affinity-purified St metabolite in 200 μL of 10% MeOH in water in an amber vial was digested in an enzyme solution containing either 500 units (25 μL) of bovine liver β-glucuronidase in 0.8 mL of 0.05 M potassium acetate buffer (pH 5.0) or 5 units of limpet sulfatase (25 μL) in 0.8 mL of 0.05 M Tris-HCl buffer (pH 7.1). After digestion at 37 °C for 20 h, the reaction was stopped by adding 2 mL of acetonitrile. The solution was concentrated to the approximate original volume with N_2 gas for subsequent HPLC and ELISA analyses. Control experiments, with no enzymes added, were conducted in two different buffer solutions.

For HPLC analysis, a Waters 10-μm spherogel radiopak C-18 reversed-phase column in a Z-module column containment apparatus (Waters Associates) was used in this study. The following step gradient was used: 100% 20 mM potassium phosphate buffer (pH 3.8) for 5 min, 40% MeOH in buffer for 15 min, 50% MeOH in water for 5 min. The step gradient was continued with a 10% incremental MeOH increase every 5 min until 100% MeOH mobile phase was reached and maintained for 10 min.

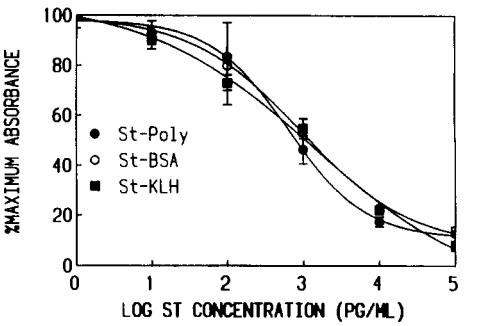

Figure 1. Standard curves of indirect ELISA of St using three different protein carriers as the immobile phase. The microtiter plate was coated with 100 μL of St-HA-protein carrier at a concentration of 1 μg/mL. Anti-St-HA-BSA antibody was used at a concentration of 1 μg/mL.

RESULTS

A. Competitive Indirect ELISA of Urine Samples Obtained from Control Rats and Rats Dosed with St. Initially, three different protein carriers were compared as the immobile phase in the competitive ELISA using anti-St-HA-BSA antiserum. Results shown in Figure 1 indicate that the displacement curves for the ELISA using St-HA-polylysine, St-HA-BSA, or St-HA-KLH are relatively the same. Using St-HA-BSA or St-HA-KLH as the immobile phase, the St concentration causing 50% displacement from maximum absorbance was 1.6 ng/mL; using St-polylysine, the 50% displacement value was 1 ng of St/mL. However, because St-HA-polylysine was unstable over time, and also because St-HA-BSA was the conjugate used in the immunization, St-HA-KHL was used in solid phase for all of the ELISAs in the present study. Assuming the concentration of St causing a decrease of 10–20% in maximum absorbance as the detection limits for the ELISA, as low as 30–64 pg of St/mL could be measured (Figure 1).

To determine the nonspecific interference, ELISAs were performed for the urine samples collected from rats over a period of 24 h after dosing with 4, 8, and 16 mg of St/kg and also for the controls. No interference was observed when all of the samples were diluted 100 times before ELISA. However, nonspecific interference was observed when the samples were diluted 10 times before the assay. At this dilution, the nonspecific interference was only about 1.9% of the value of the urine collected for a 24-h period for the rats dosed with the least amount of St (4 mg/kg). The relative percent of nonspecific interference due to St metabolite as compared to the actual signal was highest for the lowest dose. For the doses used in the present study (4–16 mg/kg), nonspecific interference was not a problem because the urine samples had to be diluted more than 100 times for the ELISA. On the basis of the limit of detection of the ELISA and diluting the urine sample 100-fold, the lowest amount of St metabolite that could be detected would be around 5 ng of St equivalent/mL or 0.25 ng/assay.

B. Efficacy of Three Cleanup Procedures for Urine Samples for HPLC. *1. XAD-2 Column.* Using a small XAD-2 column (2-mL sample with a capacity of 1.5 μg of St), all of the St metabolite was recovered in the MeOH eluate and not in the wash. The total recovery was approximately 60% and did not increase when less than 2 mL of urine was used. TLC analysis of the MeOH eluate from XAD-2 cleanup of a urine from rats dosed at 8 mg

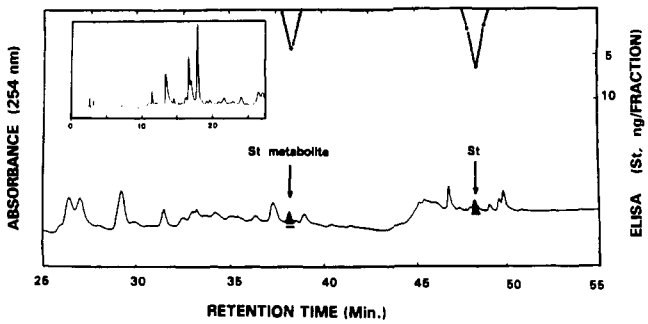

Figure 2. HPLC–ELISA chromatogram of XAD-2 cleaned up urine from a rat exposed to 8 mg of St/kg of body weight. Analysis was carried out under the conditions described in the text. Peaks were monitored at 254 nm, and 1-min fractions were collected and analyzed by indirect ELISA. The immunoreactive peaks are shown at the top (negative peaks). The insert shows the elution pattern of the first 25 min. Immunoreactive peaks were not detected in those fractions.

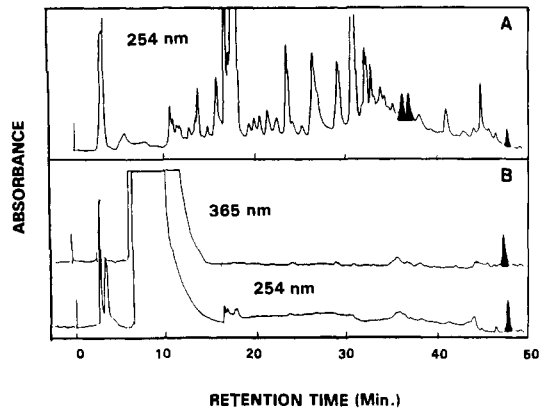

Figure 3. Rechromatograph of an immunoreactive fraction from a previous HPLC run of St-exposed rat urine after XAD-2 cleanup. A urine sample from a rat dosed at 8 mg/kg was subjected to XAD-2 cleanup and then was separated by C-18 HPLC using a Beckman ODS column (25 × 0.5 mm). Fractions were collected and analyzed by indirect St–hemiacetal ELISA (top). Fractions 72–74 of (A) were immunoreactive. Fraction 74 was rechromatographed (B) under the same conditions. The St metabolite peak shifted to the retention time for St and was still immunoreactive.

of St/kg revealed a blue fluorescent spot that was distinct from spots seen in the control urine when visualized under long-wave UV light (366 nm). In addition, an orange-red fluorescent spot was seen with the same R_f value as St.

When the XAD-2 cleanup fraction was separated by analytical reversed-phase HPLC and the fraction analyzed by ELISA, two immunoreactive peaks with retention times of 38.4 and 48.2 min were observed; one peak corresponded to the retention time for St (Figure 2). These immunoreactive peaks corresponded to HPLC peaks absorbing at 254 and 330 nm. A HPLC chromatogram of control urine did not reveal peaks at the same retention times as the immunoreactive peaks.

From an analytical HPLC run of a cleaned-up urine sample, the more polar immunoreactive peak (fraction 74 of Figure 3A) was collected and rechromatographed by HPLC. Only a large St peak was seen in the chromatogram (shaded peak in Figure 3B). These results indicate that the urine metabolite may be unstable and revert to the parent compound. Only one St urinary metabolite was isolated from the urine of St-exposed rats.

2. C-18 Sep-Pak. The C-18 reversed-phase Sep-Pak provided a quick, effective means of cleaning up urine samples without the problem of metabolite loss experienced with the XAD-2 column cleanup. This method was

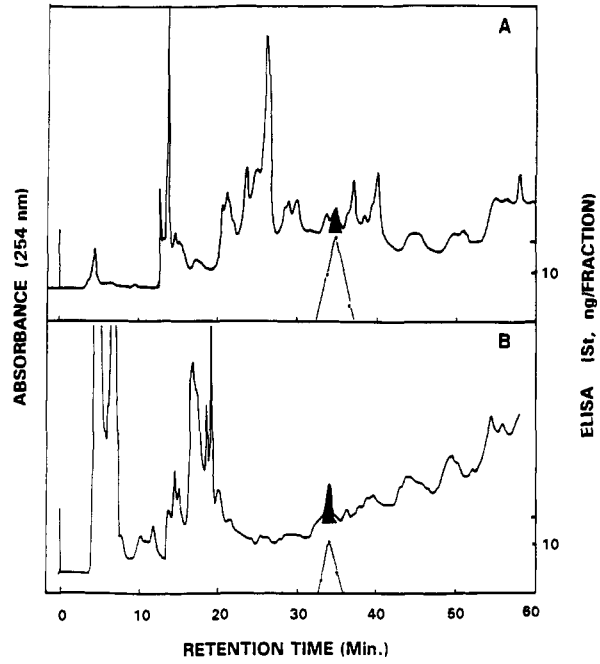

Figure 4. HPLC–ELISA chromatograms of urine from a rat exposed to St after cleanup by C-18 Sep-Pak (A) and immunoaffinity column (B). Urine from a rat exposed to a 4 mg/kg dose of St was subjected to cleanup by either C-18 Sep-Pak (top) or immunoaffinity column and then chromatographed on a Waters C-18 cartridge for Z-module radial compression system. The eluate was monitored at 254 nm at a 0.05 attenuation. The gradient conditions are the same as described in the text for the enzymatic digestion experiment. Indirect ELISA was used to measure the St metabolite in 1-min fractions eluted from the run.

able to clean up about 1 mL of urine with almost 100% recovery of St at levels between 1 and 1.5 μg/column. Larger volumes of urine resulted in sample loss. A HPLC chromatogram of a urine sample after the Sep-Pak treatment is shown in Figure 4A. An immunoreactive peak with a retention time of 34 min was observed. Under the same conditions the retention times for St and St–hemiacetal were 48.5 and 18 min, respectively. The samples after cleanup did not reveal the parent St compound. It is still very difficult to quantify St by UV absorption methods.

3. Immunoaffinity Column. The capacity of the immunoaffinity column for St, as determined by loading 5 mL of standard solution at a concentration of 500 ng of St/mL, was found to be 0.72 ± 0.21 μg/mL of affinity gel with a recovery of 88%. After 20 runs, the column was still able to purify the St metabolite from urine. Results of HPLC–ELISA analysis of a urine sample containing St metabolites after immunoaffinity column cleanup are shown in Figure 4B. Samples cleaned up by this method showed several elution peaks (254 nm) at the beginning of the HPLC run and up to 20 min into the run. After 22 min, there was only one elution peak distinct from a blank HPLC run (Figure 4B). The blank run (no cleanup) showed many elution peaks 7 min after the start of the HPLC, and it was impossible to observe the elution peak for St metabolites. From a comparison of the chromatograms shown in Figure 3A and 4A,B, it is apparent that immunoaffinity column chromatography is the only approach that could be used for the sample cleanup for the detection of St metabolites by the HPLC method.

C. Mass Spectra of Urinary St Metabolite Purified by Immunoaffinity Chromatography and HPLC. Mass spectral analysis of the materials collected from the immunoreactive peak of HPLC by fast atom bombardment

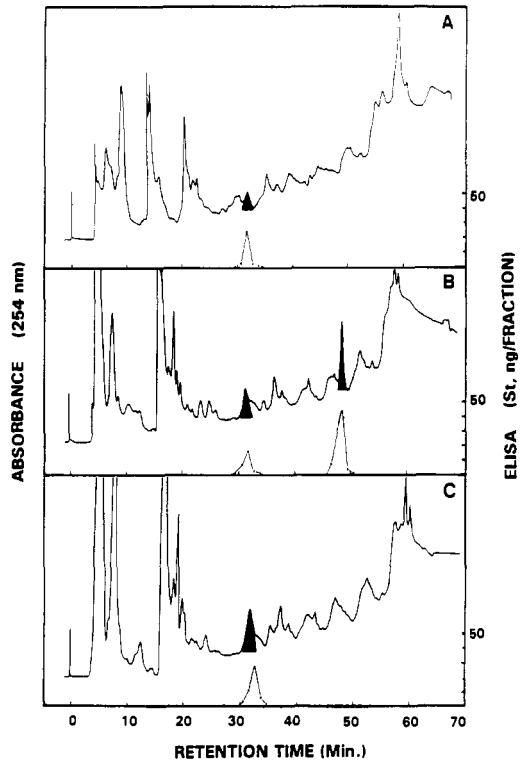

Figure 5. HPLC–ELISA chromatogram of St metabolite in control buffer (A) and after hydrolysis with β-glucuronidase (B) and sulfatase (C). St urine metabolite from St-exposed rats purified by immunoaffinity chromatography was incubated at 37 °C in a control buffer. (A) of 0.05 M potassium acetate, pH 5.0, or in the presence of β-glucuronidase (B) or sulfatase (C). After 20 h, the reaction was stopped with acetonitrile and the contents were analyzed by reversed-phase HPLC monitored at 254 nm and by indirect ELISA of the eluate fractions.

revealed the same mass peak as St (325 m/z). Since retention time of the peak collected was that of the St metabolite, the MS data suggest that the St metabolite was unstable. It might be converted to St after the sample was collected, or it might be completely fragmented during the FAB/MS spectral analysis. Because all of the sample was used for the analysis, no other physicochemical method was used for further characterization.

D. Enzymatic Digestion of Urinary St Metabolite. The results from the enzymatic digestion of the St metabolite are shown in Figure 5. The HPLC immunograms indicated that only one immunoreactive peak was in the control (Figure 5A) and there was no metabolite breakdown in the buffer alone. After digestion with β-glucuronidase, this peak is partially converted to a peak with the same retention time as St (Figure 5B). Digestion by sulfatase (Figure 5C) did not show any change from the control in the HPLC immunochromatography pattern. The enzymatic digestion of urinary St metabolite was only successful in urine samples that had been subjected to immunoaffinity purification. No hydrolysis by β-glucuronidase was observed for samples prepared by filtration or by cleanup with the XAD-2 column or by C-18 Sep-Pak.

E. Sensitivity of St Metabolite to UV Light. The stability of St metabolite to the methanolic PBS solution under UV light was studied by the indirect ELISA. The results showed a sharp decline of St immunoreactive units (from 400 to 230 ng) during the first 2.5 min of UV irradiation, followed by a gradual leveling off (to 120 ng) and no further decrease after 10 min. These results suggest that the St metabolite may be photosensitive and that caution is warranted when handling St-exposed urine samples. These observations are similar to those for AFB_1, which is also sensitive to light in MeOH and water. Such inactivation is due to the light-catalyzed interaction of these solvents with the double bond of the dihydrofuran in the AFB_1 molecule (Wei and Chu, 1973). The apparent decrease in immunoreactive units of St metabolites after exposure to UV light indicates that the St metabolite still has a dihydrofuran ring available for such interaction. The ELISA sensitivity for St hemiacetal, i.e., hydroxy group on the furan position, is less than for St.

DISCUSSION

In the present study, the conditions needed for monitoring St metabolites in urine from St-exposed rats were established. For high levels of exposure, urine samples could be used directly in the ELISA after dilution to more than 10-fold. Sample cleanup is necessary for low levels of exposure, and either C-18 Sep-Pak or immunoaffinity chromatograph could be used for this purpose. Concentration and cleanup by XAD-2 resin is not recommended due to the instability of the St metabolites after this treatment. Various HPLC–ELISA analyses of the urine samples also revealed that only one immunoreactive metabolite was excreted. The metabolite was more hydrophilic than St and unstable after UV exposure and XAD-2 cleanup treatment. It fluoresced blue at 366 nm and has a m/z of 325. Sterigmatocystin was formed after hydrolysis of the metabolite with β-glucuronidase. These data suggest that the metabolite is St–glucuronide, which was formed as result of conjugation of glucuronic acid with St at the phenolic position. The result is consistent with the study by Theil and Steyn (1973); they found that St–glucuronide was the only metabolite in the urine sample after monkeys were exposed to St.

Since St and AFB_1 have the same difuran end group that may undergo epoxidation, it is reasonable to expect that similar metabolites might be formed in both toxins via the epoxide (Essigmann et al., 1979, 1982; Wong and Hsieh, 1980; Raney et al., 1992). Similar to the properties of anti-aflatoxin–HA (Pestka et al., 1982), the antisera used for the ELISA in the present study have been shown to cross-react with a number of metabolites including St modified at the furan position (i.e., dihydro-St, St–hemiacetal, St–DNA adducts) and with St modified at the phenolic position, o-carboxy-St (Olson, 1991); thus, it is reasonable to expect that if these metabolites are present in the urine sample, they should be readily detectable by the ELISA. However, we have not found any metabolites derived from St modified at the furan ring. Because UDP-glucuronide transferase in the endoplasmic reticulum is a high capacity and readily accessible enzyme, much of the St may be metabolized by this enzyme before catalyzing by cytochrome P450 to form the reactive epoxide. In contrast, AFB_1 must first be hydroxylated by the P450 system before elimination (Neal et al., 1981). For example, the rate-limiting step for the formation of AFP_1–S-glucuronide metabolite is the demethylation of AFB_1 to AFP_1; once the hydroxyl group is formed, the hydroxyl is glucuronidated rapidly (Holeski et al., 1987). In an in vitro study, Raney et al. (1992) have recently demonstrated that St was readily conjugated to glutathione after activation; the contribution of this type of conjugation in the overall in vivo metabolism of St is not known.

The reason for the failure to demonstrate St–sulfate in the urine remains obscure. It was expected that the sulfate–St conjugate would be found in the urine from St-exposed rats. Glucuronide and sulfate conjugates are routinely excreted in urine from rats administered phenolic

compounds. For example, Fischer rats exposed to phenol excreted 45% of the metabolized compound as the sulfate conjugate and 40% as the glucuronide conjugate (Mulder and Pilon, 1975). Rats exposed to the phenolic compound harmol excreted 75% of this compound as the sulfate conjugate and 25% as the glucuronide conjugate (Mulder and Pilon, 1975). In the present study, we have found that the phenol glucuronide conjugate was unstable under XAD-2 cleanup; the phenolic link of the *O*-carboxy-St compound was also unstable (Olson, 1991). These observations suggest that a sulfate conjugate may also be unstable and revert to St.

Using ^{14}C-labeled St, Walkow et al. (1985) found that most of the metabolized St is eliminated in the feces and assaying fecal material may reveal other St metabolites. When harmol (a multicyclic phenol) was administered by ip injection in rats, over 50% of the dose was recovered in the feces as the phenol glucuronide or phenol sulfate (Mulder and Pilon, 1975). The major aflatoxin metabolites AFP_1–glucuronide and AFB_1–*S*-glutathione are excreted in the feces (Holeski et al., 1987). However, handling and extraction of fecal samples can be difficult, resulting in greater variability than metabolites analyzed in urine (Muecke, 1985).

In conclusion, results obtained from the present study show that the ELISA could provide a means of routine analysis of urine from suspected St-exposed populations. That the St–glucuronide was the only metabolite isolated in the urine of St-exposed rats and monkeys suggests that this metabolite may be found in many mammalian species and be a good parameter for assessing St exposure. However, in view of the instability of the metabolite, care should be taken during sample collection and analysis; one should also avoid using an XAD-2 column and plastic tubes as well as exposure of the samples to light.

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