Determination of Atrazine Metabolites in Human Urine: Development of a Biomarker of Exposure

Anne D. Lucas,[†] A. Daniel Jones,[‡] Marvin H. Goodrow,[†] Steven G. Saiz,[§] Craig Blewett,[§] James N. Seiber,[†,∥] and Bruce D. Hammock[*,†]

Departments of Entomology and Environmental Toxicology and Facility for Advanced Instrumentation, University of California, Davis, California 95616, and Department of Pesticide Regulation, California Environmental Protection Agency, Sacramento, California 94271-0001

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Enzyme-linked immunosorbent assays (ELISAs) are reported for the detection of atrazine and its principle metabolite in human urine. The ELISAs can be used with crude urine or following extraction and partial purification by methods described in this report. GC, MS, and HPLC techniques were used to confirm and complement the ELISA methods for qualitative and quantitative detection of urinary metabolites. A series of samples from workers applying this herbicide confirmed a mercapturic acid conjugate of atrazine as a major urinary metabolite. The mercapturate was found in concentrations at least 10 times that of any of the N-dealkylated products or the parent compound. Atrazine mercapturic acid was isolated from urine using affinity extraction based upon a polyclonal antibody for hydroxy-*s*-triazines and yielded products sufficiently pure for structure confirmation by MS/MS. In a pilot study monitoring applicators, a relationship between cumulative dermal and inhalation exposure and total amount of atrazine equivalents excreted over a 10-day period was observed. On the basis of these data, we propose that an ELISA for the mercapturate of atrazine could be developed as a useful marker of exposure.

Introduction

Atrazine is one of the most heavily used agricultural pesticides in North America (*1*) and has been identified as a major groundwater contaminant in the U.S. (*2, 3*). This herbicide is used to control weeds in a variety of crops, retard weed growth in management of wildlife habitats, and maintain rights of way (*1, 4*). The volume of atrazine use, the possibility of exposure during application, and its common detection in ground and surface water, coupled with its uncertain impact upon the environment and human health, point to the importance of the development of techniques to evaluate exposure rapidly and accurately.

Although there are a number of papers concerning human monitoring for atrazine exposure, attempts to quantify the parent compound indicate that only a small percentage of atrazine is excreted unchanged in the urine (*5, 6*). Urinary concentrations of N-dealkylated metabolites reflected the degree of external exposure (*7*), but the levels of these metabolites could not be extrapolated to determine an absorbed dose. Animal metabolism studies of the *s*-triazines have all demonstrated the excretion of N-dealkylated products (*7–13*). However, the individual forms and amounts of these metabolites found in the urine and feces vary among species, studies, and dose (*7–12*). Metabolic studies of atrazine metabolism in humans have identified trace amounts of free parent compound (*6*) in urine. To date, no conjugated metabolites of atrazine have been reported in human studies.

Here we report an enzyme-linked immunosorbent assay which can detect thioether conjugates of atrazine in human urine at the sub-ppb level. Studies involving persons exposed to this herbicide during application indicate GSH conjugation and mercapturic acid formation as major metabolic pathways. In addition, by using antibodies bound to enzyme-linked immunosorbent assay (ELISA)[1] plates for immunoaffinity extraction of human urine samples, selective extraction of the triazine urinary metabolites is accomplished. Following the rapid and selective isolation of the analyte(s) of interest, confirmation methods such as mass spectrometry can be performed readily.[2]

Materials and Methods

Chemicals. Alkaline phosphatase (AP), keyhole limpet hemocyanin (KLH), and *p*-nitrophenyl phosphate enzyme substrate tablets were obtained from the Sigma Chemical Co. (St. Louis, MO), and affinity-purified goat anti-mouse and goat anti-rabbit antibodies were purchased from Boehringer Mannheim (Indianapolis, IN). All solvents used, with the exception of those used in chemical synthesis, were of residue grade from J. T. Baker (Phillipsburg, NJ) or Fisher Scientific (Pittsburgh, PA). Solvents utilized in synthesis were of reagent grade from the same companies. A Sybron/Barnstead Nanopure II water system set at 16.7 MΩ·cm provided water for all aqueous solutions. The

* Address correspondence to this author at the Department of Entomology, University of California, Davis, CA 95616, (916) 752-7519.
† Departments of Entomology and Environmental Toxicology, University of California.
‡ Facility for Advanced Instrumentation, University of California.
§ California Environmental Protection Agency.
∥ Present address: Center for Environmental Science and Engineering, University of Nevada, Reno, NV 89557.

[1] Abbreviations: ELISA, enzyme-linked immunosorbent assay; TFAA, trifluoroacetic anhydride; SPE, solid-phase extraction; AP, alkaline phosphatase; KLH, keyhole limpet hemocyanin; FAB-MS, fast atom bombardment mass spectrometry; FAB-HRMS, fast atom bombardment high-resolution mass spectrometry; PBS, phosphate-buffered saline (0.1 M); PBSTA, phosphate-buffered saline (0.1 M) with Tween20 (0.05%) and sodium azide (0.02%) added; CDNB, 1-chloro-2,4-dinitrobenzene; CAD/MIKE, collisionally activated dissociation/mass-analyzed ion kinetic energy; NPD, nitrogen phosphorus detector; SIM, selective ion mode.

[2] Immunochemical reagents mentioned in this paper are available for research purposes or can be commercially licensed from the Office of Technology Licensing, University of California, Berkeley, (410) 643-7201.

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pentafluoropropionic anhydride (99%), 2,2,3,3,3-pentafluoro-1-propanol (97%), THF, and trifluoroacetic anhydride (TFAA) were purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI). Solid-phase extraction (SPE) columns (C_2, C_{18}, and phenyl; all 3-mL volume, 500-mg packing) were from Analytichem International, Inc. (Harbor City, CA). Microtiter plates were purchased from both Nunc (4-42404, Roskilde, Denmark) and Dynatech Laboratories, Inc. (001-012-9200, Chantilly, VA). Technical atrazine was donated by Shell Agriculture Chemical Co. (Modesto, CA); *N*-acetyl-L-cysteine was obtained from Calbiochem Corp. (San Diego, CA). The analytical *s*-triazine standards were a gift from the Ciba-Geigy Corp. (Greensboro, NC). The purity of the analytical standards used in this study were confirmed by observing a single UV-dense spot on a silica gel TLC plate (0.25-mm precoated silica gel 60 F254 plastic sheet from E. Merck, Darmstadt, Germany). Using a developing solvent of 2:13:35 THF–ethyl acetate–hexane (v/v) plus 2% acetic acid, atrazine had an R_f = 0.57, deethylatrazine an R_f = 0.43, and deisopropylatrazine an R_f = 0.36. With this solvent system the mercapturic acid conjugate of atrazine, hydroxyatrazine, and the didealkylated metabolite remained at the origin or streaked slightly between R_f = 0.0–0.21. When developing the plate with ethyl acetate, atrazine had an R_f = 0.74, deethylatrazine an R_f = 0.73, deisopropylatrazine an R_f = 0.69, and didealkylatedatrazine an R_f = 0.46. Under these conditions, hydroxyatrazine did not move and the mercapturic acid conjugate of atrazine generated a UV-dense streak from R_f = 0–0.22. When this ethyl acetate-developed plate was dried and redeveloped with 87:11:2 v/v acetonitrile–water–acetic acid halfway up the TLC plate (to just below the didealkylated UV spot), hydroxyatrazine still did not move and the mercapturic acid conjugate of atrazine formed a single UV-dense spot at R_f = 0.74 relative to the new solvent front. Additional evidence of standard purity was seen in observing a single peak for a given standard in the HPLC, GC, and GC/MS methods detailed in the instrument section. Details of the compounds synthesized for this study, either as inhibitors (**2a–d, 4a**) or for immunizing and ELISA haptens, are described in Goodrow et al. (*14*) or in the synthesis section (see Figure 1 and Table I for structures).

Synthesis of *N*-Acetyl-*S*-{4-(ethylamino)-6-[(1-methylethyl)amino]-1,3,5-triazin-2-yl}-L-cysteine (the Mercapturic Acid of Atrazine). A heterogeneous mixture of 0.80 g (3.7 mmol) of atrazine, 0.49 g (3.0 mmol) of *N*-acetyl-L-cysteine, 0.72 g (8.6 mmol) of sodium hydrogen carbonate, 3 mL of ethanol, and 1 mL of water was heated under reflux for 15 h in a nitrogen atmosphere. Reaction progress was followed by TLC developed with a THF–ethyl acetate–hexane (2:13:35 v/v) solution. Compounds were detected first by viewing under UV light (254 nm) and then by exposure to iodine vapor. Atrazine was observed at R_f = 0.60 while the mercapturic acid of atrazine remained at the origin (R_f = 0.00). Although some of the atrazine remained unreacted after this time period, the reaction mixture was filtered to remove the insoluble materials. The filtrate was evaporated to dryness, and the white solid residue was dissolved in 10 mL of water. This aqueous mixture was washed thoroughly with chloroform (3 × 10 mL), then filtered through Celite, and finally acidified with concentrated hydrochloric acid to pH 1 to produce a white solid. This mixture was cooled, and the white solid was collected, washed thoroughly with water, and dried under vacuum to obtain 0.35 g (34%) of the mercapturic acid of atrazine. Since a reproducible melting point could not be obtained, the sample was recrystallized from ethanol–water (1:1 v/v) to provide white crystals (0.27 g, 26%), but again no definitive melting and/or decomposition point was observed. The final product showed a single UV-dense TLC spot with an R_f = 0.47 using a mixture of acetonitrile, water, and acetic acid (88:11:2 v/v) and a single UV-dense peak on HPLC as outlined in the instrument section. IR (KBr) 3428 (w, amide NH), 3295 (m, NH), 1705 (m, acid C=O), 1665 (s, amide I C=O), 1556 (vs, C=N) cm^{-1}; ^{1}H NMR (Me_2SO-d_6) δ 12.7 (br, 1 H, OH), 8.24 (d, J = 7.9 Hz, 1 H, amide NH), 7.1 (m, 2 H, 2 NH), 4.5 (m, 1 H, $CHCO_2$), 4.0 (m, 1 H, CHN), 3.3 (m, 4 H, CH_2N and CH_2S), 1.84 (s, 3 H, CH_3CO), 1.10 (d, J = 6.6 Hz, 6 H, 2 CH_3), 1.06 (t, J = 6.6 Hz, 3 H, CH_3) (the 12.7, 8.24, and 7.1 ppm peaks disappeared with added D_2O); ^{13}C NMR (Me_2SO-d_6 at 80 °C) δ 177.3 (acid C=O, C_1), 171.1 (amide C=O), 169.1 (ArC_2), 163.9 ($ArC_{4\ or\ 6}$), 163.2 ($ArC_{4\ or\ 6}$), 52.1 (C_2), 41.4 (CHN), 34.6 (CH_2N), 30.5 (CH_2S, C_3), 22.2 ($2CH_3$ plus CH_3CO), 14.5 (CH_3); FAB-MS m/z 343 (M + H$^+$); FAB-HRMS m/z calculated for $C_{13}H_{23}N_6O_3S$ 343.1554, observed 343.1560.

Synthesis of Atrazine Glutathione Conjugate. Synthesis of this compound was performed enzymatically using purified mouse GST donated by Alan Buckpitt.[3] A 10 μM solution of atrazine in 0.1 M phosphate-buffered saline (PBS), pH 7.4, was placed with 1 unit of GST (the amount of GST necessary to catalyze the production of 1 μM of CDNB–GSH conjugate per minute) and 5 mM of GSH. The disappearance of the atrazine and appearance of the glutathione conjugate were followed using ELISAs described below. After 30 min, no quantifiable amount (<0.003 μM) of atrazine remained, and the reaction mixture was partitioned against chloroform to remove any traces of unreacted parent compound. ELISA analysis using antibody 357 (detailed below) which is very selective for atrazine indicated no atrazine remained unreacted. The purity of this conjugate was assessed in part by ELISA analysis of the aqueous layer. Hydrolysis was the only anticipated side reaction, but a hydroxy-*s*-triazine-selective assay indicated there was none of this side product.

Spectral Equipment. IR spectra were recorded on an IBM IR/32 FTIR spectrometer (Danbury, CT). The ^{1}H-NMR and ^{13}C-NMR spectra were obtained using a QE-300 spectrometer (General Electric NMR Instrument, Fremont, CA) operating at 300.1 and 75.5 MHz, respectively; chemical shifts are expressed in ppm downfield from tetramethylsilane. Fast atom bombardment low- and high-resolution mass spectra (FAB-MS, FAB-HRMS) and MS/MS were obtained on a ZAB-HS-2F mass spectrometer (VG Analytical, Wythenshawe, U.K.), using xenon (8 keV, 1 mA) for ionization and 3-nitrobenzyl alcohol as the matrix. Poly(ethylene glycol) 300 was added to the matrix as a mass calibrant for FAB-HRMS. CAD/MIKE (collisionally activated dissociation/mass-analyzed ion kinetic energy) daughter spectra were obtained using helium as the collision gas.

GC/NPD Conditions. A Hewlett Packard 5890 instrument (Avondale, PA), equipped with a nitrogen phosphorus detector (NPD) and in a splitless mode, was used in the analysis of atrazine and its three N-dealkylated products. In order to adequately separate these four compounds, a system consisting of a DB-225 5-m × 0.53-mm column connected to a DB-1 15-m × 0.25-mm column was used. All capillary columns were obtained from J&W Scientific (Folsom, CA). Gas flow rates were set at 2.9 mL/min for the helium carrier gas, 27.1 mL/min for the helium makeup gas, 3.7 mL/min for hydrogen, and 97 mL/min for air. A temperature program began at 200 °C, held for 11 min, followed by an increase of 15 °C/min to 220 °C. The final hold time was approximately 15 min, depending on the extraneous peaks present in the sample. Limits of quantitiation for 10-mL urine extracts were approximately 10 ng/mL for atrazine and both of its mono N-dealkylated products and 1 μg/mL for the didealkylated metabolite. Neither the mercapturate conjugate nor hydroxyatrazine chromatographed on this system.

GC/MS Conditions. A Hewlett Packard 5890 instrument with a Model 5970 mass-selective detector (MSD) was used to confirm atrazine and all N-dealkylated compound identities by retention times and detection of characteristic ions in their mass spectra. The column was a 40-m × 0.178-mm DB-5 with 0.40 μM film, with helium gas flow at 0.4 mL/min in a splitless mode. The temperature of the injector port was set at 250 °C. The temperature program commenced at 50 °C with 0.7-min hold followed by programming up to 160 °C at 50 °C/min. From 160 °C to the final temperature of 320 °C, the rate was 5 °C/min with a final hold of 5 min. Data acquisition was conducted in the selective ion mode (SIM) with a dwell of 20 ms for each ion. Atrazine (retention time 20.8 min) was detected by monitoring

[3] Department of Veterinary Pharmacology and Toxicology, University of California, Davis, CA 95616.

m/z 215, 200, and 173 from 19.5 to 22.0 min, deethylatrazine (18.9 min) for *m/z* 188, 172, and 145 from 18.8 to 19.5 min, deisopropylatrazine (18.7 min) for *m/z* 173, 158, and 145 from 17.5 to 18.8 min, and the didealkylated atrazine (16.7 min) for *m/z* 145, 110, and 68 from 15.0 to 17.5 min. Limits of quantitation were approximately 10 ng/mL for atrazine, deethylatrazine, and deisopropylatrazine and 1 μg/mL for the dideakylated metabolite. Again, neither the mercapturate conjugate nor hydroxyatrazine chromatographed.

HPLC Conditions. An Altex HPLC system, consisting of two Model 110A pumps, a SpectoMonitor 1 UV detector (λ = 254 nm), and a C_{18} 3-μm packing, 10-cm column (Rainin, Woburn, MA) were used to fractionate the urine samples. A mobile phase of 75% methanol and 25% water with 0.05% TFA at a flow rate of 0.5 mL/min provided adequate separation of the water-soluble metabolites of atrazine. Limits of detection using this HPLC system were 10 μg/mL for atrazine, deethylatrazine, deisopropylatrazine, and the mercapturate conjugate of atrazine and 100 μg/mL for hydroxyatrazine and the didealkylated metabolite.

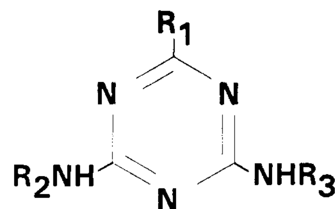
ELISA Reagents and Procedure. All ELISA optical density readings were performed with a V_{max} microplate reader (Molecular Devices, Menlo Park, CA) and calculated by the method of Rodbard (*15*). The monoclonal antibody used to quantitate the atrazine mercapturate conjugate was developed by Karu et al. (*16*) and has been well characterized by Lucas et al. (*17*) and Schneider and Hammock (*18*). The polyclonal antibody (2266) based ELISA used to extract the urine samples for the atrazine mercapturate conjugate and to quantitate hydroxyatrazine used the same format and enzyme tracer as previously detailed (*18*),[4] while an ELISA selective for atrazine, antibody 357 (*19*), was also employed.

Solid-Phase Extraction. Atrazine and its three N-dealkylated products were extracted from urine using a C_2 SPE column. The column was prewashed with ethyl acetate, acetone, and water at pH 10 before sample addition. Ten milliliters of urine was brought to pH 10, 1 g Na_2SO_4 was added slowly while stirring, and the mixture was filtered and passed through a C_2 SPE column using a vacuum. After the urine had passed through the column, the column was dried under vacuum for 10–15 min. Elution with 2 mL of ethyl acetate yielded an extract suitable for GC/NPD and GC/MS analysis. Less than 3% of the mercapturate conjugate or 1% of hydroxyatrazine were present in the ethyl acetate extract under these conditions because neither were retained by the C_2 column.

An acid extraction system using a phenyl SPE column was also developed. Prewashing the column with acetone and water acidified with HCl (pH 2.7) preceded the addition of 10 mL of urine acidified to pH 2.5–3. The column was washed with acidified water and dried under house vacuum for approximately 10 min, and the metabolites were removed from the SPE column with 2 mL of acetone. This acetone extract was then reduced to dryness under a gentle stream of nitrogen and was redissolved in PBSTA [phosphate-buffered saline (0.1 M) with Tween20 (0.05%) and sodium azide (0.02%) added] for ELISA analysis or mobile phase for HPLC analysis. Under these conditions, 99% of atrazine, 70% of either of the mono N-dealkylated products, 50% of hydroxyatrazine, and no measurable didealkylatedatrazine were retained using this SPE system.

Chloroform Extraction. Urine samples were also extracted using an equal volume of chloroform. One milliliter of chloroform was added to 1 mL of urine and shaken vigorously for 1 min and then allowed to stand until the two phases completely separated. The organic layer was removed and evaporated to dryness under a gentle stream of nitrogen. This chloroform residue was reconstituted using 1 mL of PBSTA prior to analysis by ELISA and quantitated concurrently with the remaining aqueous layer.

Affinity Extraction. Affinity extraction of urine to isolate the immunoreactive metabolites was accomplished by using the monoclonal antibody described above or the polyclonal antibody raised against a hydroxytriazine antigen (Figure 1), but having some cross reactivity for the mercapturate (see Table I for the cross reactivity of these antibodies). For extraction of the mercapturate of atrazine, the anti-hydroxytriazine polyclonal antibody was used. As the maximum tolerance of this assay for raw urine was approximately 3% urine in PBSTA, a 3% solution of urine was made and 100–200 μL/well of the sample placed on a goat anti-rabbit/anti-hydroxyatrazine antibody coated plate. Following 1-h incubation time, the plate was thoroughly washed, and 100 μL of water (pH 2.75) was added to each well. Three hours proved sufficient to extract the bound mercapturate from the polyclonal antibody. The acidic extraction mixture was then removed with a multichannel pipet, and the wells were washed with a small aliquot of the acidified water. The combined acidic extract was passed through the phenyl SPE scheme as described above. In order to desalt the resulting solution, the acetone extracts from the phenyl columns were dried completely under a stream of nitrogen, and then 20 μL of pentafluoropropionic anhydride and 100 μL of pentafluoropropanol were added to esterify the carboxyl group. After 1 h, the derivatized atrazine mercapturate was again evaporated to dryness under a stream of nitrogen, 2 mL of water was added, and the solution was then passed through a C_{18} SPE column, dried under vacuum, and finally eluted with 2 mL of acetone. This procedure yielded an extract of the atrazine mercapturate suitable for FAB MS/MS confirmation. Aliquots of atrazine mercapturate standard and of urine samples from pesticide applicators were monitored after each step to determine the recovery of the highly immunoreactive material using the monoclonal antibody AM7B2.1. Mercapturate concentrations over 0.108 ng/mL in urine exceeded the binding capacity of the polyclonal antibody 2266. However, no significant loss (>25 ng/mL) was observed for this SPE extraction and derivitization procedure.



Reagent	R_1	R_2	R_3
Alkaline Phosphatase Enzyme Tracer	Cl	Et	$(CH_2)_2C(O)$-NH-AP
Monoclonal Antibody AM7B2.1 Immunizing Hapten	$S(CH_2)_2C(O)$-NH-KLH	Et	iPr
Polyclonal Antibody 2266 Immunizing Hapten	OH	Et	$(CH_2)_2C(O)$-NH-KLH
Polyclonal Antibody 357 Immunizing Hapten	Cl	iPr	$(CH_2)_5C(O)$-NH-KLH

Figure 1. Structures of the haptens coupled to keyhole limpet hemocyanin (KLH) for immunizing and for attaching to alkaline phosphatase (AP) used as an enzyme tracer in all ELISAs. The structures indicated in the cross reactivity data from Table I conform to the generic *s*-triazine structure depicted here.

Human Sample Acquisition. For recovery studies from human urine, 5 individuals who do not work with or use this herbicide donated the samples. No GC interference was observed from those who had consumed coffee, cigarettes, or alcohol. However, a large number of late eluting peaks were seen on the GC/NPD for those who had ingested large quantities of carbonated beverages.

Urine samples from atrazine applicators were collected by the California Environmental Protection Agency, Worker Health and Safety Branch. Dermal and inhalation exposure of a single mixer/loader/applicator was compared to the urinary levels of atrazine metabolites in urine over a 10-day work period. The worker made four applications to preemergent sudan grass during this work period and was monitored by Worker Health and Safety personnel for atrazine exposure using dermal dosimetry and air sampling. Dermal dosimetry consisted of a 100% cotton long-

[4] Unpublished data.

Table I. Cross Reactivities of Antibodies Used in This Study for a Number of *s*-Triazines[a]

inhibitor	structure			cross reactivity		
	R_1	R_2	R_3	2266	AM7B2.1	357
atrazine	Cl	Et	*i*Pr	2	30	100
atrazine mercapturate	*N*-acetyl-L-cysteine	Et	*i*Pr	1.6	100	1.8
hydroxyatrazine	OH	Et	*i*Pr	38.4	2	0.8
simazine	Cl	Et	Et	2.4	9	7.5
hydroxysimazine	OH	Et	Et	100	<0.1	0.1
ametryne	SCH_3	Et	*i*Pr	<0.1	4	0.7
simetryne	SCH_3	Et	Et	1	1	0.1
propazine	Cl	*i*Pr	*i*Pr	<0.1	59	537
prometryne	SCH_3	*i*Pr	*i*Pr	<0.1	9	1.1
prometon	OCH_3	*i*Pr	*i*Pr	3.4	2	3.7
terbutryne	SCH_3	Et	$C(CH_3)_3$	<0.1	6	1.1
cyanazine	Cl	Et	$CCN(CH_3)_2$	<0.1	32	0.7
cyanazine acid	Cl	Et	$CC(O)OH(CH_3)_2$	<0.1	<0.1	<0.1
deethylatrazine	Cl	H	*i*Pr	<0.1	<0.1	0.8
deisopropylatrazine	Cl	Et	H	<0.1	<0.1	0.5
didealkylatedatrazine	Cl	H	H	<0.1	<0.1	1.0
melamine	H	H	H	<0.1	<0.1	1.9
2a	Cl	Et	$CH_2C(O)OH$	7	<0.1	0.3
2b	Cl	Et	$(CH_2)_2C(O)OH$	2	1	0.4
2c	Cl	Et	$(CH_2)_3C(O)OH$	1	2	1.9
2d	Cl	Et	$(CH_2)_4C(O)OH$	10	5	1.4
2e	Cl	Et	$(CH_2)_5C(O)OH$	96	20	4.9
4a	$SCH_2CH_2C(O)OH$	Et	*i*Pr	<0.1	91	0.2
glutathione conjugate	Glu-Cys-Gly	Et	*i*Pr	<0.1	85	1.5

[a] The values are presented relative to hydroxysimazine for the ELISA using the polyclonal antibody 2266, atrazine mercapturate for the monoclonal AM7B2.1, and atrazine for the polyclonal 357 with these particular analytes assigned a cross reactivity of 100% for their respective antibodies. Cross reactivities are determined by expressing the ratio of the IC_{50} of the chemical assigned to be 100% to the IC_{50} of the other compounds and expressed as a percent.

sleeved T-shirt and 100% cotton gloves worn under the normal work clothing and protective equipment. The worker wore cloth coveralls, leather boots, heavy rubber gloves, and sunglasses while pouring the liquid formulation atrazine (EPA #100-497) into a semiclosed mixing system. The worker removed his gloves whenever he drove the spray application vehicle. The spray vehicle was a large three-wheeled, 1200-gal tanker truck fitted with a 50-ft spray boom with an enclosed cab. Air samples were collected form this worker's breathing zone by drawing air through a glass fiber filter attached to an air pump with a preset flow rate of 1 L/min. Inhalation exposure was calculated by multiplying the air filter atrazine level by a breathing flow rate of 29 mL/min and dividing this value by the pump air flow rate. The sum of the atrazine from the T-shirt, gloves, and inhalation exposure is a rough estimate of the total exposure.

Biological monitoring consisted of daily urine collection, with all samples stored in an ice chest with dry ice. A preexposure sample was collected 1 day before the first application. Volumes, beginning times, and ending times for each sample were recorded.

Results and Discussion

Isolation of Immunoreactive Material from Urine. This project was started, at the request of the California Environmental Protection Agency, to compare the results from ELISA to those of GC for monitoring atrazine in urine. Although there is evidence that monitoring free atrazine in urine can provide exposure information (*6*), the levels are extremely low compared to the amount of metabolic products. It was postulated that the ability of the ELISA to bind various metabolites, in addition to the parent, would provide a stronger response and prove a useful tool in monitoring for exposure. Our initial results demonstrated high levels of immunoreactive material appearing in the urine of humans shortly after exposure to atrazine. The level of immunoreactive material that we detected by ELISA as 500–1000 times higher than those by GC based upon the parent compound. On the basis of the structure of the hapten used to raise the monoclonal antibody AM7B2.1 (Figure 1), we hypothesized that the immunoreactive material providing the enhanced response could be a mercapturic acid derivative. Multiple techniques were used to test this hypothesis.

Initial support for this hypothesis was obtained by partitioning the urine with chloroform. Under conditions where the vast majority of parent compound spiked in blank urine partitioned into the organic phase, only traces of the immunoreactive material partitioned into the organic phase from urine collected from atrazine-exposed workers, as indicated by three ELISAs with differing selectivities for atrazine and its metabolites. The hydroxyatrazine and mercapturic acid conjugate of atrazine spiked in blank urine remained largely in the aqueous layer (Table II). Antibodies selective for atrazine (357) and for atrazine and its mercapturic acid conjugate (AM7B2.1), used to analyze both the chloroform and aqueous layers of urine from exposed workers, demonstrated that most of the immunoreactive response was contained in the aqueous layer and that this immunoreactive material was not atrazine (Table III). Analysis by the hydroxy-*s*-triazine-selective antibody 2266 indicated that no hydroxy-*s*-triazine was detected in the urine of atrazine applicators above 1 μg/mL. The pH of the chloroform-extracted urine (from 2.7 to 10.2) did not markedly change the partitioning behavior of these analytes when spiked into blank urine (see Table II). These data are consistent with the immunoreactive material being, at least in part, a water-soluble conjugate such as a mercapturic acid derivative.

By removing the water-soluble material from urine, the immunoassay based on the monoclonal antibody AM7B2.1 or polyclonal antibody 357 can be used for highly selective assays for parent triazines based upon the cross reactivity data shown in Table I. However, because no applicator urine samples contained a significant portion of immunoreactive material in the chloroform layer (Table III), efforts were concentrated on the aqueous phase. The assay

Table II. Partioning Behavior of Atrazine, Hydroxyatrazine, and the Mercapturate Conjugate of Atrazine between Urine and Chloroform at Various Spiking Levels and pHs[a]

compound	spike level (μM)	pH	average % IRM in $CHCl_3$ layer	CV %
atrazine	0.5	7		
	2.0	7	90	5
	10	7		
	0.5	2.7	84	6
	0.5	10	78	1
hydroxyatrazine	0.5	7		
	2.0	7	3	1
	10	7		
	0.5	2.7	6	9
	0.5	10	3	5
mercapturate conjugate of atrazine	0.5	7		
	2.0	7	1	0
	100	7		
	0.5	2.7	14	0.3
	0.5	10	0.4	0.7

[a] The distribution of immunoreactive material (IRM) was determined by an ELISA selective for the target analyte: antibody 357 for atrazine, 2266 for hydroxyatrazine, and AM7B2.1 for the mercapturate conjugate of atrazine.

Table III. Analytical Results for the Confirmed Urinary Metabolites of Atrazine Found in Field Workers[a]

sample	ppb atrazine mercapturate	ppb deethyl-atrazine	ppb deisopropyl-atrazine	% IRM in aqueous layer
CB24-W3-U1	48	ND	ND	83.7
CB22-W2-U2	8	ND	ND	
CB25-W5-U6	270	tr	tr	99.4
CB25-W3-U7	153	tr	tr	98.9
CB22-W3-U2	257	ND	ND	81.7
CB23-W3-U1	10	ND	ND	77.6
CB22-W3-U1	59	ND	ND	95.4
CB23-W2-U1	46	ND	ND	90.7
CB22-W3-U3	11	ND	ND	91.2
CB25-W5-U5	1756	44.6	45	99.0
AT01-1012	ND	ND	ND	
AT01-1014	32	10	10	90.2
AT01-1017	267	10	10	81.7
AT01-1018	9	ND	ND	
AT01-1019	49	49	49	83.6
AT01-1020	5	ND	ND	
AT01-1021A	25	ND	ND	87.6
AT01-1021B	tr	ND	ND	
AT01-1022	tr	ND	ND	

[a] The limit of detection for the ELISA-quantified mercapturate and the GC-quantified dealkylatrazine was 0.1 and 1 ng/mL, respectively, below which is reported as ND. Trace (tr) amounts of analyte indicate levels which are above ND but below the limit of quantitation, which is approximately 10 times that of the limit of detection. The distribution of the immunoreactive material (IRM) between chloroform and urine is expressed as a percent, having been evaluated by the monoclonal assay using antibody AM7B2.1 both before and after partitioning with chloroform. With the exception of sample CB25-W5-U5, the partition values using the atrazine-selective antibody 357 and the hydroxyatrazine antibody 2266 were all below the limit of detection of the assays. The percent IRM with antibody 357 indicated 90% of the IRM resided in the aqueous phase, indicating that most of the IRM is not atrazine. No samples yielded a response above the limit of detection using the antibody 2266.

reported here, using antibody AM7B2.1, can detect the mercapturic acid conjugate of atrazine down to 0.5 ppb in crude urine diluted to 25% with PBSTA buffer (Figure 2). Although Figure 2 indicates that this particular assay can be performed without any dilution or pretreatment of the urine, this is not always true. In examining samples

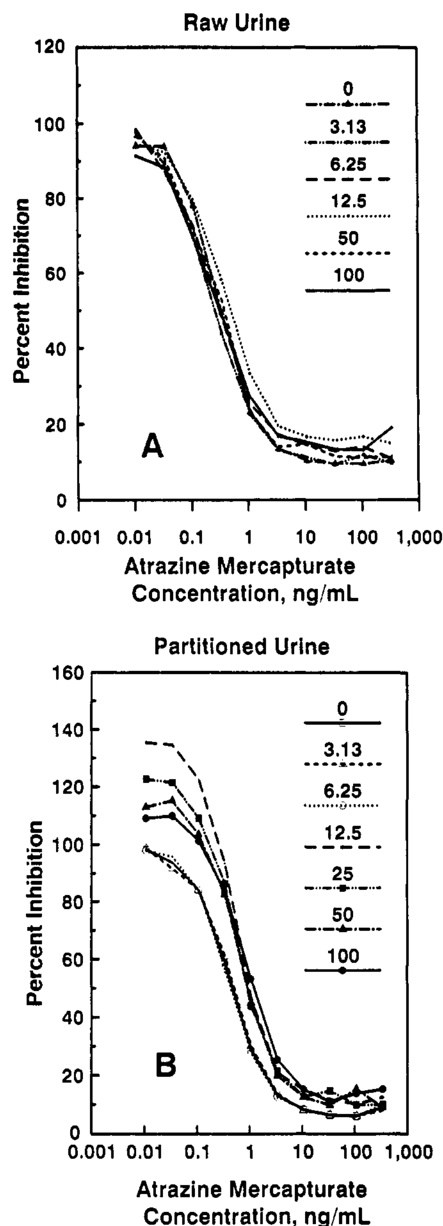


Figure 2. The average of quadruplicate standard curves of the atrazine mercapturate prepared in various dilutions of (A) raw urine in PBSTA and (B) the aqueous layer of urine following partitioning with $CHCl_3$. Concentrations of $CHCl_3$ follow extraction, evaporation, and reconstitution at 10, 5, 2.5, 1.25, and 0.613 times the initial volume of $CHCl_3$ extract. These studies were conducted to assess conditions for direct ELISA analysis using antibody AM7B2.1.

from a series of individuals not exposed to atrazine, the worst case indicated a dilution of urine to 25% in PBSTA buffer was necessary to avoid matrix effects. Because human urine may contain any number of components which may affect assay performance, a sample dilution of 25% was always performed.

A chloroform partition of raw urine exerted a mild perturbation on the performance of the assays. As seen in Figure 2, there is a loss in sensitivity of approximately half an order of magnitude when dilutions of partitioned urine were above 12.5% of the sample volume. However, the limit of detection using a dilution factor of 16 for the chloroform-saturated urine (the 6.25% value in Figure 2) to avoid this particular solvent/matrix effect was still quite reasonable at 3.2 ng/mL.

To further characterize this immunoreactive material, an acidic SPE system and a basic SPE system were

Table IV. Recovery from Spiked Blank Urine of (A) Atrazine and Its N-Dealkylated Products Using a C_2 Cartridge under Basic pH and (B) the Mercapturic Acid Conjugate of Atrazine Using a Phenyl SPE Cartridge with the pH at 2.75[a]

(A) Recovery of Atrazine and Its Deethyl- and Deisopropyl- Forms from Urine at pH 10 Using a C_2 SPE Cartridge[b]

spike level	atrazine	deethylatrazine	deisopropylatrazine
5–10 μg/mL	104	92	90
1–2 μg/mL	89	85	103
0.1–0.2 μg/mL	97	100	118
10–20 ng/mL	99	69	71

(B) Recovery of the Mercapturic Acid Conjugate of Atrazine from Acid Urine Using Phenyl SPE Columns[c]

spike level (ng/mL)	atrazine mercapturate	spike level (ng/mL)	atrazine mercapturate
342	77	17.1	108
34.2	83	3.42	110

[a] The values represent a composite recovery consisting of a minimum of three different blank urine sources and triplicate extractions per spiking level. [b] Less than 1% of hydroxyatrazine and 3% of the *N*-acetyl-L-cysteine form of atrazine was retained using the C_2 column. [c] In addition, 99% of atrazine was also retained and coeluted with the atrazine mercapturate under this system, as well as 70% of both deethyl and deisopropylatrazine and 50% of the hydroxyatrazine. No didealkylated product was detected.

developed to separate the postulated mercapturic acid conjugate (using the acidic SPE extraction) from parent or N-dealkylated metabolites (with the basic SPE column). In addition, these SPE extracts provided sample preparation prior to HPLC or GC analysis.

The SPE column used for acidic (phenyl) and basic (C_2) extraction of urine showed good recovery for those analytes tested, ranging from 77% to 110% (Table IV). The retention or exclusion of the immunoreactive material from each of these SPE methods was examined using the ELISA based on monoclonal antibody AM7B2.1, which showed the strongest response to the immunoreactive material. In all cases, the urine samples collected from the exposed workers excluded the immunoreactive material from the C_2 column and retained the material on the phenyl column. The SPE eluants were analyzed by GC and GC/MS (Figure 3). Only traces of parent and some quantifiable N-dealkylated products were identified with the basic SPE scheme (Table III). No triazine peaks were seen in the phenyl SPE system using GC/NPD or GC/MS. The retention of the highly immunoreactive material from acidified urine by the phenyl column when eluted and analyzed showed a strong response in the assay selective for the *N*-acetyl-L-cysteine conjugate of atrazine, while the hydroxytriazine assay indicated less than 1 μg/mL present in both the C_2 and phenyl eluants. This not only corroborated the hypothesis that the metabolite(s) was(were) the mercapturate conjugate, but also indicated that the immunoreactive atrazine metabolite(s) was(were) acidic in nature.

Characterization of the immunoreactive material as a water-soluble material which behaved as an acid, and which very likely was nonvolatile (no acid SPE extract response on GC systems), brought us to narrow further the identity of this material by using reverse-phase ion pair HPLC. Using the approach of Seiber et al. (20), HPLC fractionation and retention time studies were conducted to characterize the metabolites of interest. In the initial fractionation studies with applicator's urine by HPLC, a small peak corresponding to the retention time of the mercapturic acid conjugate of atrazine could be seen

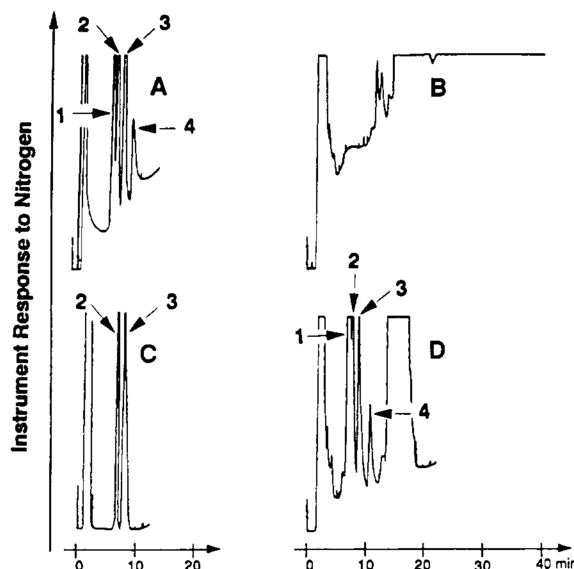


Figure 3. Representative GC chromatograms of triazine standards and extracts of human urine following the basic SPE extraction as described in the text. (A) An injection of the standards atrazine (1), deethylatrazine (2), and deisopropylatrazine (3) (5 ng of each) and the didealkylated triazine (4) (20 ng injected). This method yielded a reliable limit of detection of 10 ng/mL of urine for atrazine and the N-dealkylated metabolites except as described in (B). (B) Extract of the urine of a person not exposed to atrazine who chronically consumed over 2 L/day of a carbonated beverage. The limit of detection of compounds 1–3 by GC remained the same as for urines from other donors, but the high background required 30 min between injection. (C) Extract of urine from a sample showing 500 ng/mL of deethylatrazine and 70 ng/mL of deisopropylatrazine. (D) Extract of the urine of a person not exposed to atrazine which was spiked with 200 ng/mL standard compounds before analysis. Little interference was observed in extracts of urine samples in the 5–11.5-min region where standards appear. All positive samples were confirmed by GC/MS. Sample preparation and instrument conditions are detailed in the text.

(Figure 4). Analysis of the fractions showed over 80% of the total amount of immunoreactive material injected was recovered in the mercapturic acid fraction. The remaining fractions eluted from the HPLC were at the background level of the ELISAs. It would be misleading to imply all positive urine samples generated such a clear chromatogram. After a short period of use, human urine samples placed on the column showed no clear peaks above the broad, continuous background. In addition, analysis of HPLC fractions for immunoreactive material began to shift and spread. However, a major portion of the immunoreactive material (at least 50%) corresponded to the retention volume of atrazine mercapturate. Frequent cleaning of the column and the lines with large volumes of water and acetonitrile was necessary to recover all the immunoreactive material injected and prevent subsequent bleeding of immunoreactive material. This HPLC system deteriorated rapidly with use, indicating that it is not appropriate for routine analysis. Combined SPE, HPLC, and immunoassay yielded a limit of detection of approximately 20 ng/mL for atrazine and its mercapturic acid conjugate.

No evidence for the existence of hydroxyatrazine was seen in the expected HPLC fraction using the anti-hydroxy-*s*-triazine ELISA described above with a detection limit of 1 μg/mL. Subsequent ELISA analysis of the HPLC fractions with this assay required dilution in excess of 50-fold in order to negate the matrix effects, due primarily to the concentration of the urine constituents by the HPLC

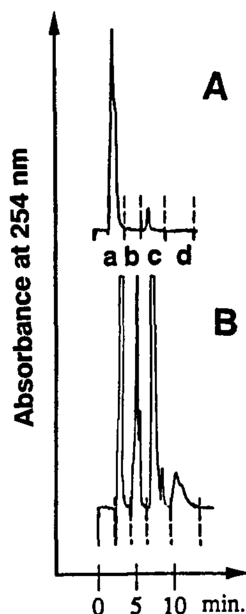


Figure 4. Representative HPLC chromatograms of human urine following solid-phase extraction. (A) A urine sample (1.2 mL of urine equivalents) from an exposed worker containing 500 ng/mL atrazine mercapturate and 70 ng/mL combined mono- and didealkylated products (by GC). Immunoassay of fractions a–d demonstrated immunoreactivity only in fraction c corresponding to the atrazine mercapturate. In this case 80% of the immunoreactivity injected on the column was detected in peak c, and peak c accounted for 87% of the recovered immunoreactivity from the column. (B) Elution profile of triazine standards. Fraction a contains all of the mono- and didealkylated products (compounds **2–4**), fraction b contains atrazine (compound **1**), fraction c contains the mercapturate, and fraction d contains hydroxyatrazine. The tracing corresponds to approximately 2 μg of each compound except for 0.2 μg of hydroxyatrazine. No clear UV-absorbing peaks or immunoreactive material was detected in urine from unexposed individuals.

system. FAB analysis of all fractions yielded no clear identification of any triazine compound in any fraction, again due to the concentration of the urine matrix components.

The coelution of the majority of the immunoreactive material with a standard of the mercapturic acid conjugate of atrazine provided strong support for our initial hypothesis, but we wanted to obtain structural identity. A series of derivatizing agents (diazomethane, pentafluoropropanol, and trimethylsilylating reagents) was used in an attempt to make the mercapturic acid derivative volatile and amenable to GC and GC/MS analysis. No structurally identifying peaks were obtained, despite derivatizing microgram quantities of the mercapturic acid atrazine standard, decreasing the column length (5 m), increasing the flow rate (from 3–10 to 80–100 mL/min), and reducing the inlet and column temperature to below 150 °C. The lack of a definitive melting point (see Materials and Methods section) indicated thermal instability of the molecule. Soft ionization (FAB-MS) of the derivatized and underivatized mercapturic acid standard provided the characteristic spectra, demonstrating that the derivatization was successful. However, when spiked or applicator urine samples were placed through SPE and/or HPLC fractionation, with subsequent derivatization and C_{18} SPE cleanup, no clear FAB spectra emerged due to the coextracted components of the matrix. It was clear to us at this point that extensive sample cleanup would be necessary to obtain clear spectra of the immunoreactive material in urine.

Purification and Identification of Urinary Metabolites. Analytes closely related structurally to the immunizing agents (shown in Figure 1 and Table I) are expected to be well recognized by the antibodies generated using this immunogen. The monoclonal antibody (AM7B2.1) coated on a microtiter plate was found to be very efficient in trapping authentic atrazine mercapturate conjugate standard from human urine as well as immunoreactive material from the urine of exposed workers. No solvent (acetonitrile and methanol up to 100%; more hydrophobic solvents were not used as the ELISA plates are made of soft plastic), salt concentration (up to 2 M PBSTA), water pH (0–12), or heating of the plate (to 50 °C) could elute the (alkylthio)-*s*-triazine from the antibody. Reasoning that this antibody was binding the analyte strong enough to resist these elution attempts, 10 anti-triazine polyclonal antibodies were also tested in the same fashion for affinity extraction of the presumed mercapturate conjugate. Efficient trapping and subsequent extraction of the mercapturic acid conjugate of atrazine were successful when an antibody raised against a 2-hydroxy-4-(ethylamino)-*s*-triazine structure (2266) was employed (Figure 1). After the initial extraction of the urine sample by the antibodies coated in an ELISA plate occurred, the diluted urine and subsequent washes were combined, analyzed by ELISA using antibody AM7B2.1, and termed unbound material. Following acid extraction all solutions were again analyzed by ELISA to determine the amount of affinity-purified mercapturate. The efficiency of the C_2 and C_{18} SPE workup procedures was monitored by ELISA. The solutions excluded by the C_2 and C_{18} SPE procedures were found to contain little if any immunoreactive material (<20 ng/mL). Analysis of the material washed from the antibody-coated plate just prior to acid extraction, along with the material after extracting the plate and phenyl SPE extraction, indicated the trapping efficiency of the wells (coated with goat anti-rabbit, 2266, as described in the Materials and Methods section) to be 0.0108 ng/well or 0.108 ng/mL. In scaling up to 15-mL polystyrene test tubes, which were coated in an identical fashion to the microtiter well used to extract urine, the volume extracted increased proportionally. Use of higher concentrations of urine (>3%) or increasing the amount of antibody 2266 coated in the well or test tube either decreased or did not change the extraction capacity of the system. Although the maximum loading of the coated test tube was the same as the wells (0.108 ng/mL), the total amount of urine, and subsequently analyte, which could be extracted by this affinity procedure was dramatically increased.

Following the addition of acidified water (pH 2.8) to the extraction wells, the solution was then removed and placed directly through a phenyl SPE column as previously described. The phenyl extraction and subsequent pentafluoropropyl esterification of the eluant followed by C_{18} SPE cleanup were necessary to obtain FAB-MS spectra free from interfering substances. The ELISA selective for the mercapturic acid conjugate of atrazine was used at each eluting and derivatizing step to track the location of the immunoreactive material. Confirmation of the pentafluoropropyl ester derivative of atrazine mercapturic acid was performed using CAD/MIKE. Daughter ion spectra were obtained by manually adjusting the magnetic sector of the mass spectrometer to the mass of the parent ion of interest (m/z 475, $[M + H]^+$ of the atrazine

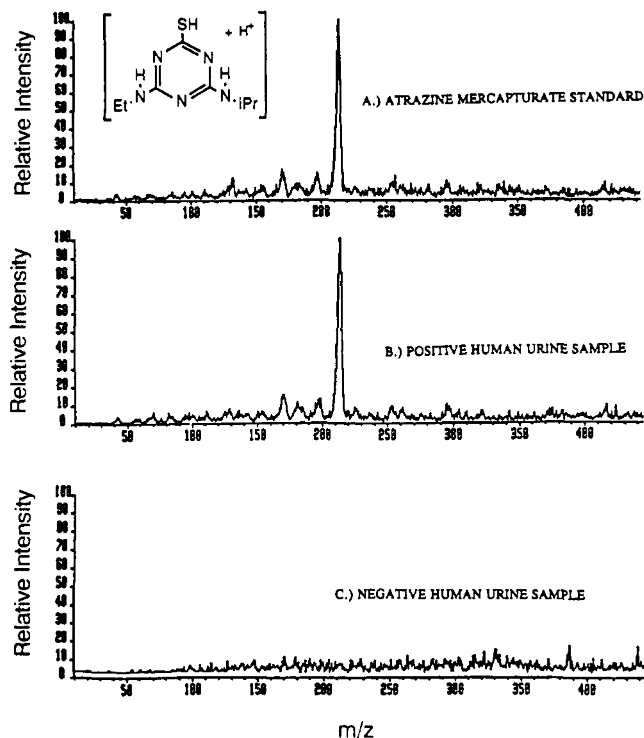


Figure 5. FAB-MIKE spectra of (A) a negative human urine extract, (B) atrazine mercapturate standard in control urine, and (C) a positive human urine sample extract. Raw urine samples were diluted to 3% in PBSTA and extracted with a triazine-selective antibody as described in the text.

mercapturate pentafluoropropyl ester) and scanning the electric sector at 20 s/scan. Full FAB scans provided no further structural details.

The CAD/MIKE daughter ion spectra of the pentafluoropropyl ester of an authentic standard of atrazine mercapturic acid and a field urine sample are shown in Figure 5. The fragmentation of $[M + H]^+$ is simple, with a fragment at m/z 214 (arising from cleavage of the sulfur-aliphatic carbon bond) accounting for most of the fragment ion intensity. Similar fragmentation has been observed for other thioether conjugates (*21, 22*).

These cumulative data strongly supported the atrazine mercapturate as accounting for the majority of the immunoreactive material initially seen in our ELISA. FAB scans for other conjugates (GSH, cysteine, mercaptopyruvic acid, mercaptoacetic acid, and mercaptolactic acid) existing as either parent or as any one of the three N-dealkylated forms indicated only the mercapturic acid conjugate of atrazine to be present. The nature of the extraction scheme does not rule out that other such alkylthio metabolites are not created, but that their presence in urine for a limited number of human samples has not been confirmed. Subsequent analysis of the urine of over 40 other triazine-exposed individuals has yielded confirmation of the mercapturic acid conjugate as a primary urinary metabolite in addition to a mercapturic acid conjugate of an N-dealkylated *s*-triazine.

In addition to identification of the immunoreactive material in human urine after exposure to atrazine, the possibility of conjugates other than alkylthio was addressed. Since no 2-hydroxy-*s*-triazine products were observed using the HPLC, or an ELISA selective for 2-hydroxy-*s*-triazines (the polyclonal ELISA detailed previously), or in any FAB scan of sample extracts, it is unlikely that 2-hydroxy-*s*-atriazine is an important metabolite in human urine. However, the possibility of conjugates (glucuronide or sulfate) formed after hydroxylation still needed to be examined.

The ferric chloride test for phenolic compounds (*23*) was performed to test whether of the hydroxyl group of a hydroxyatrazine standard was ionizable. A control compound, 2-methoxyphenol, was run simultaneously with the hydroxy-*s*-triazine standard, as well as a blank water sample. The hydroxyatrazine failed to produce a positive response, indicating the hydroxy group does not exhibit phenolic characteristics in a neutral (pH 7) solution. As the glucuronide and sulfate transferase enzymes usually require an ionizable hydrogen to transform compounds (*24*), these particular conjugates are not likely to be produced.

Human Sample Results. Samples from field workers who had applied atrazine were analyzed. Partitioning their urine with chloroform showed essentially all the immunoreactive material to be contained in the aqueous phase. In all strongly positive samples analyzed (50 μg/L or greater), deethylatrazine could also be found. Smaller amounts of deisopropylatrazine were also seen in these samples by GC. No didealkylated atrazine was detected. Because this metabolite is reported to degrade when stored for long periods of time or if subjected to freezing and thawing (*11*), we did not expect to find didealkylated atrazine. The instability of this metabolite does not make it an ideal candidate for use as a biomarker of exposure.

During the time period of this study, the applicator had total atrazine exposures ranging from 1 mg to 21 mg per application day (Table V). Inhalation exposures contributed only 0.075% or less to the overall exposure. This level of contribution of inhalation exposure to total exposure is consistent with many previous studies (*25*). We have no reliable estimate of how exposure relates the external exposure of this compound to an internal dose.

Atrazine residues were not detected in any of the field urine samples before or during this study (minimum detectable level = 0.02 μg/L). No detectable atrazine metabolites were found in the preexposure urine sample or numerous control urine samples. Following exposure, most samples had measurable levels of metabolites, with the atrazine mercapturate being the most prevalent in frequency and amount. Hence, only atrazine mercapturate was used to estimate atrazine exposure, with all values being converted to atrazine equivalents by using a molar

Table V. Dermal and Inhalation Exposure and Monitoring Conditions of a Single Mixer/Loader/Applicator Applying Atrazine for Preemergent Control of Weeds in Sudan Grass Using an Enclosed Cab Spray Vehicle[a]

date	a.i. applied (kg)	area treated (ha)	exposure time (h)	upper body (μg)	hands (μg)	inhalation exposure (μg)	total exposure (μg)
05/16/90	54	24	1.8	278	780	8	1066
05/22/90	44	17	2.2	2670	11600	5	14275
05/24/90	108	41	8.8	9320	12300	35	21655
05/25/90	40	12	1.8	222	2152	7	2381

[a] Estimated total exposure is the sum of upper body, hand, and inhalation exposures as measured by T-shirt dosimetry, hand dosimetry, and personal breathing zone air monitoring.

Table VI. Biological Monitoring of a Single Mixer/Loader/Applicator within a 10-Day Work Period[a]

date	void times (h)	urine volumes (mL)	atrazine mercapturate (ng/mL)	atrazine equiv (μg)
05/15/90	24	600	ND	ND
05/16/90	24	138	32	2.8
05/22/90	24	1562	276	271
05/23/90	24	825	49	26
05/24/90	24	1895	127	16
05/25/90	6	885	0.5	0.3

[a] Days when more than one sample were collected show the sum of all samples collected. ND indicates not detected, or below 0.1 ng/mL. Exposure conditions are listed in the text.

ratio of 0.628. Atrazine mercapturate levels ranged from trace levels to 276 ng/mL (Table VI).

For this pilot study, simultaneous urine collection and passive exposure monitoring occurred four times. Measurable atrazine equivalents were detected in the worker's urine on each application day as well as 1 day after the second application (Table VI). This indicates that small amounts of atrazine metabolites continue to be excreted beyond 24 h. The geometric mean of daily urinary atrazine equivalents was 8.1 μg (SE = 286%), and the geometric mean of total external exposure was 5.3 mg (SE = 205%). At this point external exposure in humans cannot be correlated with internal dose. These results suggest mercapturate conjugates, at least in the case of atrazine, are a potentially effective indicator of atrazine exposure.

Conclusion

We found that a prevalent urinary product of atrazine metabolism in humans is the mercapturic acid conjugate. Atrazine and its N-dealkylated metabolites are relatively minor urinary metabolites, and no hydroxylated or other conjugages were found. We are currently developing immunoassays for these metabolites and/or degradation products of atrazine, but on the basis of limited human studies, they do not appear to be as useful in indicating human exposure as the atrazine mercapturate. These other metabolites may prove useful for the monitoring of human exposure to other triazine herbicides or monitoring exposure in other species, or in environmental samples.

The strong ELISA recognition coupled with the well-characterized across reactivity of the antibody indicated an (alkylthio)-*s*-triazine as a major metabolite of atrazine. Chloroform partitioning experiments demonstrated the majority of the immunoreactive material to be hydrophilic. Analysis for the parent and N-dealkylated compounds using C_2 SPE extraction generated values which comprised only approximately 10% of the total immunoreactive response. Phenyl SPE and chloroform partitioning experiments indicated this immunoreactive material to be hydrophilic and acidic. Ion pairing reverse-phase HPLC showed the majority of the immunoreactive material eluted with the same retention time as a standard of the mercapturic acid conjugate of atrazine.

Our data indicate that at least 80% of the immunoreactive material in the urine is the mercapturate and the parent compound accounts for less than 1% of the immunoreactive material detected. However, the presence of parent compound can be exclude by simple partitioning with an organic solvent or by SPE.

An antibody developed for 2-hydroxy-*s*-triazines, which weakly recognized the 2-chloro- and 2-(alkylthio)triazines, was used to extract the immunoreactive material from urine. Subsequent phenyl solid-phase extraction, derivatization with pentafluoropropanol, and FAB MS/MS analysis identified the mercapturate conjugate of atrazine as the highly immunoreactive material in the urine samples.

The use of ELISA plates not only to analyze but also to extract samples provided valuable tools in obtaining structural confirmation of the metabolites of atrazine. The presence of urinary hippuric acid, as well as contaminants intrinsic to SPE and HPLC schemes (e.g., detergents, plasticizers, etc.) failed to provide a clean enough extract for direct-probe MS analysis. The thermal instability of the 2-thio-*s*-triazines also eliminated the possibility and explained the failure of chemical derivatization followed by GC or GC/MS analysis. The affinity extraction using "low-affinity" antibodies provided a convenient means to isolate and definitely characterize the compounds of interest. This affinity extraction step coupled with subsequent analysis using the atrazine mercapturate-selective antibody led to detection limits of 0.25 ng/mL, indicating that the atrazine mercapturate can be followed for multiple half-lives following occupational exposure.

Comparison of cumulative atrazine exposure to the total amount of atrazine equivalents excreted (as the mercapturate conjugate) tentatively demonstrates the value of using this metabolite as a biomarker of internal exposure to this herbicide. Further field studies investigating the use of this metabolite as a urinary marker of exposure are currently being perused.

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References

(1) Eisler, R. (1989) *Atrazine Hazards to Fish, Wildlife, and Invertebrates: A Synoptic Review*, Fish and Wildlife Service, U.S. Dept. of the Interior, Contaminant Hazard Review Report No. 18.

(2) Parsons, D. W., and Witt, J. M. (1988) *Pesticides in Groundwater in the United States of America*, Oregon Pesticide Impact Assessment Program, Oregon State University Extension Service.

(3) Belluck, D. A., Benjamin, S. L., and Dawson, T. (1991) Groundwater contamination by Atrazine and Its Metabolites. In *Pesticide Transformation Products, Fate and Significance in the Environment* (Somasundaram, L., and Coats, J. R., Eds.) ACS Symposium Series 459, pp 254–273, American Chemical Society, Washington, DC.

(4) Esser, H. O., Dupuis, G., Ebert, E., Marco, G. J., and Vogel, C. (1976) S-Triazines. In *Herbicides: Chemistry, Degradation and Mode of Action* (Kearney, P., and Kaufman, D., Eds.) pp 129–208, Marcel Dekker, New York.

(5) Reed, J. P., Hall, F. R., and Krueger, H. R. (1990) Measurement of ATV Applicator Exposure to Atrazine Using an ELISA Method. *Bull. Environ. Contam. Toxicol.* **44**, 8–12.

(6) Catenacci, G., Maroni, M., Cottica, D., and Pozzoli, L. (1990) Assessment of Human Exposure to Atrazine Through Determination of Free Atrazine in Urine. *Bull. Environ. Contam. Toxicol.* **44**, 1–7.
(7) Ikonen, R., Kangas, J., and Savolainen, H. (1988) Urinary Atrazine Metabolites as Indicators for Rat and Human Exposure. *Toxicol. Lett.* **44**, 109–112.
(8) Adams, N. H., Levi, P. E., and Hodgson, E. (1990) In Vitro Studies of the Metabolism of Atrazine, Simazine and Terbutryn in Several Vertebrate Species. *J. Agric. Food Chem.* **6**, 1411–1417.
(9) Gojmerac, T., and Kiniewald, J. (1989) Atrazine Metabolism in Rats—A Model for Mammalian Metabolism. *Bull. Environ. Contam. Toxicol.* **43**, 199–206.
(10) Timchalk, C., Dryzga, M. D., Langvardt, P. W., Kastl, P. E., and Osborn, D. W. (1990) Determination of the Effects of Tridiphane on the Pharmacokinetics of [^{14}C] Atrazine Following Oral Administration to Male Fischer 344 Rats. *Toxicology* **61**, 27–40.
(11) Bradway, D. E., and Moseman, R. F. (1982) Determination of Urinary Residues of the N-Dealkylated Metabolites of Triazine Herbicides. *J. Agric. Food Chem.* **30**, 244–247.
(12) Erickson, M. D., Frank, C. W., and Morgan, D. P. (1979) Determination of *s*-Triazine Residues in Urine: Studies of Excretion and Metabolism in Swine as a Model for Human Metabolism. *J. Agric. Food Chem.* **27**, 743–745.
(13) Khan, S. U., Foster, T. S., and Akhtar, M. H. (1979) In Vitro Metabolism of a Mixture of Atrazine and Simazine by the Soluble Fraction (105000g) From Goose, Pig, and Sheep Liver-Homogenates. *Pestic. Sci.* **10**, 460–466.
(14) Goodrow, M. H., Harrison, R. O., and Hammock, B. D. (1990) Hapten Synthesis, Antibody Development, and Competitive Inhibition Enzyme Immunoassay for *s*-Triazine Herbicides. *J. Agric. Food Chem.* **38**, 990–996.
(15) Rodbard, D. (1981) Mathematics and Statistics of Ligand Assay. In *Ligand Assay* (Langan, J., and Clapp, J. J., Eds.) pp 45–99, Masson, New York.
(16) Karu, A. E., Harrison, R. O., Schmidt, D. J., Clarkson, C. E., Grassman, J., Goodrow, M. H., Lucas, A. D., Hammock, B. D., White, R. J., and Van Emon, J. M. (1990) Monoclonal Immunoassay of Triazine Herbicides: Development and Implementation. In *Immunoassays for Monitoring Human Exposure to Toxic Chemicals* (Vanderlaan, M., Stanker, L. H., Watkins, B. E., and Roberts, D. W., Eds.) ACS Symposium Series 451, pp 59–77, American Chemical Society, Washington, DC.
(17) Lucas, A. D., Schneider, P., Harrison, R. O., Biggar, J. W., Rolston, D. E., Seiber, J. N., and Hammock, B. D. (1991) Determination of Atrazine and Simazine in Water and Soil Using Polyclonal and Monoclonal Antibodies in Enzyme Linked Immunosorbent Assays. *Food Agric. Immunol.* **3**, 155–167.
(18) Schneider, P., and Hammock, B. D. (1992) Influence of the ELISA Format and the Hapten–Enzyme Conjugate on the Sensitivity of an Immunoassay for *s*-Triazine Herbicides Using Monoclonal Antibodies. *J. Agric. Food Chem.* **40**, 525–530.
(19) Harrison, R. O., Goodrow, M. H., and Hammock, B. D. (1991) Competitive Inhibition ELISA for the *s*-Triazine Herbicides: Assay Optimization and Antibody Characterization. *J. Agric. Food Chem.* **39**, 122–128.
(20) Seiber, J. N., Glotfelty, D. E., Lucas, A. D., McChesney, M. M., Sagebiel, J. C., and Wehner, T. A. (1990) A Multiresidue Method by High Performance Liquid Chromatography-Based Fractionation and Gas Chromatography Determination of Trace Levels of Pesticides in Air and Water. *Arch. Envrion Contam. Toxicol.* **19**, 583–592.
(21) Deterding, L. J., Srinivas, P., Mahmood, N. A., Burka, L. T., and Tomer, K. B. (1989) Fast Atom Bombardment and Tandem Mass Spectrometry for Structural Determination of Cysteine, N-Acetylcysteine, and Glutathione Adducts of Xenobiotics. *Anal. Biochem.* **183**, 94–107.
(22) Jones, A. D., Winters, C. K., Segall, H. J., and Buonarati, M. H. Analysis of Mercapturic Acid Conjugates using Negative Ionization and Tandem Mass Spectrometry. *Biol. Mass Spectrom.* (in press).
(23) Shriner, R. L., Fuson, R. C., Curtin, D. Y., and Morrull, T. C. (1980) The Detection and Confirmation of Functional Groups. In *Systematic Identification of Organic Compounds*, 6th ed., p 348, John Wiley & Sons, New York.
(24) deBethizy, J. D., and Hays, J. R. (1989) Metabolism: A Determinant of Toxicity. In *Principles and Methods of Toxicology* (Hays, A. W., Ed.) 2nd ed., pp 45–48, Raven Press Ltd., New York.
(25) Wolf, H. (1976) Field Exposure to Airborne Pesticides. In *Air Pollution from Pesticides in Agricultural Processes* (Lee, R. E., Jr., Ed.) pp 137–158, CRC Press, Cleveland, OH.

Registry Numbers Supplied by Author. Atrazine, 1912-24-9; hydroxyatrazine, 2163-68-0; simazine, 122-34-9; hydroxysimazine, 2599-11-3; ametryne 834-12-8; simetryn, 1014-70-6; propazine, 139-40-2; prometryn, 7287-19-6; prometon, 1610-18-0; terbutryne, 886-50-0; cyanazine, 21725-46-2; cyanazine acid, 36576-43-9; deethylatrazine, 6190-65-4; deisopropylatrazine, 4147-58-4; didealkylatedatrazine, 3397-62-4; melamine, 108-78-1; **2a**, 68228-19-3, **2b**, 82784-46-1; **2c**, 125454-26-4; **2d**, 125454-27-5; **2e**, 125454-28-6; **4a**, 125454-30-0.