Development of an Enzyme-Linked Immunosorbent Assay for Atrazine Mercapturic Acid in Human Urine

Lynn L. Jaeger,[†,§] A. Daniel Jones,[§] and Bruce D. Hammock*[,†]

Departments of Entomology and Environmental Toxicology and Facility for Advanced Instrumentation, University of California, Davis, California 95616

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Improved assessments of human exposure to electrophilic chemicals require rapid and inexpensive analytical techniques that can detect specific urinary metabolites at low levels as needed for epidemiological screenings of large populations. The first aim of this study has been to apply rational hapten design strategies to develop a more sensitive and selective enzyme-linked immunosorbent assay for atrazine mercapturic acid. Polyclonal sheep antiserum was generated against an improved hapten, numerous coating antigen chemistries were evaluated, and assay conditions were optimized. An assay was developed with an IC_{50} of 0.08 ± 0.02 μg/L ($K \cong 10^{-10}$ M) for atrazine mercapturic acid. The assay exhibited greatest recognition of atrazine mercapturic acid relative to other known urinary metabolites of atrazine as well as other triazine herbicides. The assay was surprisingly selective to atrazine mercapturic acid over the structurally similar simazine mercapturic acid. Urine samples presented matrix effects due in part to the nonspecific effects of urinary salts, but 4-fold dilution of urine achieved an overall method limit of quantitation of 0.3 μg/L. Solid-phase extraction strategies were also developed in an attempt to increase the sensitivity of the overall method. However, a weak positive assay response was present in the solid-phase extracts of unspiked urines, resulting in accurate recovery of atrazine mercapturic acid at 0.1 μg/L.

Introduction

Human exposure to electrophilic xenobiotics, such as drugs, pesticides, or industrial chemicals, can be indicated by the detection of elevated levels of urinary metabolites such as mercapturic acids (*1*−*3*). These excreted *N*-acetylcysteine conjugates are formed via enzyme-catalyzed addition of the tripeptide glutathione to electrophilic chemicals, followed by metabolic hydrolysis and acetylation (*4*). Our understanding of the extent of human exposure to environmental and occupational chemicals is limited, and improved analytical methods for biomarkers of exposure (*5*), such as mercapturic acids, are needed for both toxicological and epidemiological monitoring (*6*−*9*).

Atrazine is one of the most heavily used herbicides in the world (*10*). In 1993, over 70 million pounds of atrazine were applied to field crops in the United States alone (*11*). Agricultural and manufacturing workers are exposed to atrazine during application and production. Furthermore, the widespread use of atrazine, resulting in contaminated drinking water and food products, poses risks of exposure to the general public (*10*, *12*, *13*). The mercapturic acid conjugate of atrazine (**1**, Table 1) is one of several atrazine metabolites excreted in urine (*14*−*18*), and it is a specific biomarker for atrazine exposure in humans.

Immunochemical methods of detection were originally developed for clinical applications and are still widely used for detecting hormones, markers of disease, drugs of abuse, and therapeutic drugs (*19*). One type of immunoassay, enzyme-linked immunosorbent assay (ELISA),[1] emerged during the 1980s as a sensitive and inexpensive analytical method in both clinical and environmental applications (*20*−*22*). More recently, ELISAs have been developed for detecting biomarkers of human xenobiotic exposure in blood, urine, and other biological fluids (*23*−*28*). ELISA offers the advantages of minimal sample preparation, low cost per analysis, parallel processing, simplicity, high sensitivity, and high selectivity, making it one of few analytical methods capable of meeting the needs of clinical, environmental, and epidemiological monitoring efforts.

A previously reported ELISA for atrazine mercapturic acid (AM) utilized a monoclonal antibody (*18*), developed by Karu et al. (*29*), originally designed for an atrazine ELISA (*30*). A thiopropionic acid derivative of atrazine (**2**, Table 1), coupled via the carboxylic acid to keyhole limpet hemocyanin (KLH), was used as the immunizing conjugate to generate the monoclonal antibody AM7B2. The monoclonal antibody recognized not only atrazine but also AM and was subsequently used to elucidate an immunoreactive material present in the urine of farm workers exposed to atrazine (*18*).

* Corresponding author. Tel: (530) 752-7519. Fax: (530) 752-1537. E-mail: bdhammock@ucdavis.edu.
[†] Departments of Entomology and Environmental Toxicology.
[§] Facility for Advanced Instrumentation.

[1] Abbreviations: AM, atrazine mercapturic acid; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; IC_{50}, median inhibition concentration; IC_{15}, concentration that inhibited signal by 15%; IgG, immunoglobulin class G; KLH, keyhole limpet hemocyanin; LOD, limit of detection; LOQ, limit of quantitation; MDL, method detection limit; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline plus 0.5% Tween-20; RSD, relative standard deviation; SPE, solid-phase extraction.

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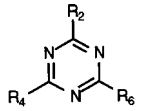
Table 1. Structure of Haptens and Hapten−Protein Conjugates



hapten	R_2	R_4	R_6	hapten−protein conjugate[a]
1	$-SCH_2CH(NHAc)COOH$	$-NHCH_2CH_3$	$-NHCH(CH_3)_2$	**1**-BSA, **1**-KLH
2	$-SCH_2CH_2COOH$	$-NHCH_2CH_3$	$-NHCH(CH_3)_2$	**2**-BSA, **2**-CONA
3	$-SCH_2CH_2COOH$	$-NHCH_2CH_3$	$-NHCH_2CH_3$	**3**-BSA, **3**-CONA
4	$-Cl$	$-NH(CH_2)_5COOH$	$-NHCH(CH_3)_2$	**4**-BSA, **4**-CONA
5	$-Cl$	$-NH(CH_2)_5COOH$	$-NHCH_2CH_3$	**5**-BSA, **5**-CONA
6	$-Cl$	$-NH(CH_2)_2COOH$	$-NHCH(CH_3)_2$	**6**-BSA
7	$-Cl$	$-NH(CH_2)_2COOH$	$-NHCH_2CH_3$	**7**-BSA
8	$-Cl$	$-NH(CH_2)_3COOH$	$-NHCH_2CH_3$	**8**-BSA

[a] Haptens **2**−**8** were synthesized by Goodrow (*34*). BSA, bovine serum albumin; KLH, keyhole limpet hemocyanin; CONA, conalbumin; Ac, acetyl.

In the present study, we describe the development of an improved ELISA for AM and the evaluation of the assay's performance. Our first objective was to rationally design, develop, and optimize a polyclonal antiserum-based ELISA for AM. We utilized an immunogen that more closely resembled AM than the immunogen for AM7B2 and hypothesized that the improved immunogen would generate a polyclonal antiserum that would recognize AM with equivalent or higher sensitivity and specificity than the monoclonal antibody. Our second objective was to determine whether components of urine interfered with accurate AM quantitation. Our third objective was to design efficient sample preparation methods to remove matrix interferences and to increase assay sensitivity.

Experimental Procedures

Chemicals. Proteins, adjuvants, immunochemicals, Tween-20, and *N*-acetylcysteine were purchased from Sigma Chemical Co. (St. Louis, MO); keyhole limpet hemocyanin (KLH) was from Calbiochem-Novabiochem Corp. (La Jolla, CA); and other buffers, solvents, and reagents were from Fisher Scientific Co. (Pittsburgh, PA), Pierce Chemical Co. (Rockford, IL), or Aldrich Chemical Co. (Milwaukee, WI). Technical grade atrazine was provided by Shell Agricultural Chemical Co. (Modesto, CA). Analytical triazine standards and ^{14}C-labeled AM (25 μCi/mg) were provided by Ciba-Geigy Corp. (Greensboro, NC). Bradford protein assay dye was purchased from BioRad Laboratories (Richmond, CA).

Analytical Equipment. ELISA absorbances were measured with a Spectra Max 250 96-well plate reader (Molecular Devices, Sunnyvale, CA). UV spectra and end points were measured on a UV-2101PC scanning spectrophotometer (Shimadzu Scientific Instruments Inc., Columbia, MD). Two brands of 96-well microtiter plates were used: (1) Maxisorb Immuno Plates (Nunc, Roskilde, Denmark, part no. 442404) were used for ELISAs, and (2) Microtiter Plates (Dynatech Laboratories, Inc., Chantilly, VA, part no. 001-012-9205) were used for performing serial dilutions and mixing. Kieselgel 60 F_{254} silica gel (0.2 mm) plastic sheets were used for TLC (EM Separations Technology, Gibbstown, NJ).

Atrazine Mercapturic Acid. Atrazine mercapturic acid [*N*-acetyl-*S*-{4-(ethylamino)-6-[(1-methylethyl)amino]-1,3,5-triazin-2-yl}-L-cysteine] was synthesized according to a method described previously (*18*). Melting point determinations, TLC R_f values, and NMR spectra for our AM were similar to those previously reported. Additional structural support was obtained using mass spectrometry. A high-resolution fast atom bombardment ionization mass spectrum (ZAB-HS-2F mass spectrometer, VG Analytical, Wythenshawe, U.K.) showed a base peak of m/z = 343.1580 (calcd m/z 343.1552 for $[M + H]^+ = C_{13}H_{22}N_6O_3SH$). An electrospray mass spectrum (VG Quattro-BQ mass spectrometer, VG Biotech, Altrincham, U.K.) generated under the following conditions: positive mode, 50% aqueous methanol + 1% formic acid, 20-μL direct loop injection, 10 μL/min, source temperature = 65 °C, showed a strong peak at m/z = 343, corresponding to $[M + H]^+$, and a base peak at m/z = 214, corresponding to a thioether bond-cleaved fragment $[M - C_5H_6NO_3]^+$. Purity was assessed using capillary electrophoresis. A 2 mg/mL solution of AM was analyzed by a model 270A capillary electrophoresis instrument (Applied Biosystems, Foster City, CA) under the following conditions: 100 mM borate buffer (pH 9.3), 10 mM β-cyclodextrin, total column length (L_t) = 72 cm, length to the detector (L_d) = 50 cm, voltage = 20 kV, spectrophotometric detection (λ) = 214 nm. A peak eluting at 14.5 min represented 97% of the total integrated area of the electropherogram.

Atrazine Mercapturate Methyl Ester. The methyl ester of AM was prepared as previously described (*31*). Atrazine mercapturic acid (105 mg) was dissolved in 1 of mL methanol. (Trimethylsilyl)diazomethane (800 μL of 2.0 M solution in hexanes) was added and the solution remained at room temperature for 40 min. The reaction product was dried with nitrogen and flash-chromatographed with ethyl acetate elution. The final product showed a single UV dense spot (R_f = 0.87 with 1:1 ethanol−ethyl acetate). By electrospray mass spectrometry, the product showed a strong peak at m/z = 357, corresponding to $[M + H]^+$, and a base peak at m/z = 214, corresponding to the thioether bond-cleaved fragment.

Atrazine Mercapturic Acid−Protein Conjugates. The immunogen and one coating antigen were prepared using *N*-hydroxysulfosuccinimide (*32*, *33*). Briefly, 40 mg of AM (120 μmol), 35 mg of *N*-hydroxysulfosuccinimide (160 μmol), and 27 of mg 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (140 μmol) were added to 8 mL of dry dimethylformamide and stirred overnight at room temperature. Forty milligrams of bovine serum albumin (BSA) or KLH was dissolved in 0.13 M $NaHCO_3$ (30 mL) and stirred in a 4 °C water bath. Aliquots (2 mL) of the active ester solution were added dropwise to the two protein solutions. The reaction mixture was stirred at 4 °C for 30 min and then at room temperature for 16 h. Conjugates were exhaustively dialyzed against 1× PBS (8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.5) and stored at −80 °C. Protein concentrations (measured by Bradford protein assay) of the immunogen (**1**-KLH, Table 1) and the homologous coating antigen (**1**-BSA, Table 1) were 1.3 and 1.6 mg/mL, respectively.

Hapten Density Determination. Hapten and protein concentrations of conjugates **1**-BSA and **1**-KLH were individually determined by UV spectroscopy methods (*34*−*36*). Absorbances at two wavelengths (A_{245} and A_{275}) were measured for **1**-KLH, **1**-BSA, and standard solutions of AM, KLH, and BSA. The hapten densities were calculated using Beer's law, the

measured absorbances, and the known concentrations of AM and protein.

A second method was used to calculate the hapten density for comparison. First, the hapten concentration of the conjugates was estimated by ELISA (see Coating Antigen ELISA below) using **7**-BSA (Table 1) as the coating antigen. Serially diluted solutions of AM methyl ester were used to generate a standard curve, and **1**-KLH and **1**-BSA were analyzed as samples. The methyl ester was used as the standard instead of the acid to better mimic the epitope of the hapten bound to protein. Second, protein concentrations were determined by Bradford assay. Third, using the hapten concentration measured by ELISA and the protein concentration measured by Bradford assay, the hapten densities were calculated.

Anti-Atrazine Mercapturic Acid Antiserum. One Hampshire Cross ewe (100 kg) was immunized every 30 days with **1**-KLH to generate an anti-AM polyclonal antiserum. Approximately 2 mg of **1**-KLH, diluted to 2 mL with 1× PBS, pH 7.5, was emulsified with 2 mL of Freund's complete adjuvant. The sheep was immunized intramuscularly in two locations on the hind leg. Freund's incomplete adjuvant was used in all subsequent immunizations. Test bleeds (30 mL) were collected from the jugular vein 7−10 days after the first through fourth immunizations, and 200-mL volumes were collected 7−10 days after the fifth through seventh immunizations.

All blood samples were collected into evacuated test tubes and coagulated overnight at 4 °C. The blood clots were removed from the antisera with cotton-tipped swabs, and the residual red blood cells were separated with centrifugation. Antiserum was preserved with sodium azide (0.02% final concentration) and stored at −20 °C.

The change in antibody titer after each test bleed was monitored by conducting two-dimensional titration ELISA experiments (*37*) using coating antigen **1**-BSA (heterologous in protein and homologous in hapten and spacer arm). After four immunizations, the titer for the anti-AM antiserum reached a maximum. Because a large volume of antiserum was desired, the sheep was immunized three additional times. Antiserum from the seventh (final) bleed was used in subsequent ELISA experiments. The sheep produced over 600 mL of anti-AM antiserum over 7 months.

ELISA Buffers and Substrate Solution. Water from a Sybron/Barnstead Nanopure II water system (Newton, MA) was used to prepare all buffers. Coating buffer comprised 0.05 M carbonate−bicarbonate (pH 9.6). Assay buffer comprised 1× PBS + 0.05% Tween-20 (pH 7.5) (PBST). Wash buffer comprised 0.5× PBS (pH 7.5) + 0.05% Tween-20. Tetramethylbenzidine substrate solution comprised 0.05 M citrate−acetate (pH 5.5) (25 mL), 1% H_2O_2 (100 μL), and tetramethylbenzidine (0.6% in dimethyl sulfoxide) (400 μL).

Coating Antigen ELISA. The ELISA format was based on methods described by Voller (*38*). Plates were coated with coating antigen (100 μL/well), diluted in coating buffer. After incubating overnight at 4 °C, the coated plate was washed five times with wash buffer. Calibration curve solutions and samples were diluted with assay buffer in a separate, uncoated Dynatech plate. An equal volume of anti-AM antiserum, diluted in assay buffer, was added to the mixing plate. Solutions were mixed thoroughly, and aliquots (50 μL/well) were transferred (in triplicate or quadruplicate) from the mixing plate to the coated plate. The plate was incubated for 1 h and was washed. Donkey anti-sheep immunoglobulin G−horseradish peroxidase (IgG-HRP) (50 μL/well) was added. The plate was incubated at room temperature for 1 h and was washed. Tetramethylbenzidine substrate solution (100 μL/well) was added. The substrate reaction was stopped after 30 min with 4 N H_2SO_4 (50 μL/well), and absorbances were measured at 450 and 650 nm. SoftMax Pro software (Molecular Devices) utilized a four-parameter algorithm (*39*) for curve fitting and interpolation.

Antiserum and Coating Antigen Screening. One homologous hapten−protein conjugate (**1**-BSA, Table 1) and 11 heterologous hapten−protein conjugates (Table 1) were screened as potential coating antigens by two-dimensional titration, noncompetitive ELISA (*37*). The heterologous conjugates were previously synthesized in our laboratory for the development of ELISAs for atrazine and other *s*-triazine herbicides (*34*). Next, the 12 conjugates were screened by competitive coating antigen ELISA using AM as the inhibitor. Finally, the selectivity of three of the conjugates—**3**-BSA, **5**-BSA, and **7**-BSA—was screened by comparing the cross-reactivity pattern of potential triazine inhibitors: three triazine herbicides (atrazine, simazine, and ametryn) and three triazine metabolites (hydroxyatrazine, didealkylated atrazine, and simazine mercapturic acid).

Cross-Reactivity. The selectivity of one of the conjugates, **7**-BSA, was conducted by analyzing standard solutions of AM, triazine herbicides, atrazine and simazine metabolites, and other structurally related compounds. Cross-reactivity values were calculated from AM and analyte IC_{50} values from the same plate: % cross-reactivity = [(IC_{50} of AM)/(IC_{50} of triazine inhibitor)] × 100.

Optimization. Reagents were evaluated at different concentrations to achieve the most sensitive assay: coating antigen **7**-BSA (1:200000, 1:600000, and 1:1000000), anti-AM antiserum (1:20000, 1:40000, 1:60000, and 1:80000), and donkey anti-sheep IgG-HRP (1:2500, 1:5000, and 1:15000). Buffer composition was not optimized.

Matrix Effects. (a) pH. The effect of pH on the quantitation of AM was evaluated by analyzing standard solutions of AM prepared in buffer at pH 4, 6, and 8. McIlvaine buffers were used at constant ionic strength, 0.1 M (sodium phosphate/citric acid/potassium chloride) (*40*).

(b) Ionic Strength. The effect of ionic strength on the quantitation of AM was evaluated by analyzing standard solutions of AM prepared in 1× PBS + 0.05% Tween-20 (approximately 300 mOsm/L), 3× PBS + 0.05% Tween-20 (approximately 900 mOsm/L), 5× PBS + 0.05% Tween-20 (approximately 1500 mOsm/L), and 8× PBS + 0.05% Tween-20 (approximately 2400 mOsm/L), all at pH 7.5.

(c) Urine. The effect of urine on the quantitation of AM was evaluated by analyzing standard solutions of AM prepared in one urine sample (either undiluted or diluted 1:2, 1:4, or 1:10 with PBST). In another experiment, urines with low (250 mOsm/L, urine 1), medium (570 mOsm/L, urine 2), and high (970 mOsm/L, urine 3) osmolarity were spiked with AM to evaluate the effect of osmolarity (ionic strength) on AM quantitation. The three urines were selected from a group of 17 urine samples obtained from individuals with no documented exposure to triazines. The osmolarity of these 17 samples, measured with an Osmette A automatic osmometer (Precision Systems Inc., Natick, MA), was evenly distributed between 200 and 1000 mOsm/L. Each urine was spiked in triplicate with either 0.05, 0.08, 0.1, 1, or 10 μg/L AM and analyzed by the ELISA. Unspiked urines were also analyzed. Samples were analyzed either undiluted or diluted (1:2, 1:10, or 1:50) depending on the spiked concentration.

Liquid Scintillation Counting. Radiolabeled AM was used during solid-phase extraction (SPE) method development. The activity of spiked urine and eluates was determined with a 1409 liquid scintillation counter (Wallac, Gaithersburg, MD). Urine, spiked with ^{14}C-labeled AM to 635 dpm (12 μg/L), was used to determine conditions for AM retention, washing, and elution. Urine and SPE eluates (0.5−1 mL) were mixed with Scintiverse BD cocktail (9 mL) (Fisher) prior to counting. Because solution composition of the urine and SPE eluates varied, quench correction was performed with an europium external standard and Wallac's digital overlay technique (DOT-DPM) method. Satisfactory quench correction was indicated by a sample quality monitor value greater than 95.

Solid-Phase Extraction. An SPE method was developed using C18 columns (10 cm^3/500 mg; part no. 1211-3027, Varian Sample Preparation Products, Harbor City, CA) and a 10-position VacElut manifold equipped with stopcocks (Varian). The method was based on previously reported methods for isolating small acidic molecules from human urine using C18

SPE (*41*−*43*). Columns were conditioned with 2-mL volumes of ethyl acetate, methanol, deionized water, and 0.1 M phosphate buffer (pH 2.2). Urine (10 or 20 mL) was mixed with an equal volume of 0.1 M phosphate buffer (pH 2.2) to normalize ionic strength and adjust pH. Buffered urines were loaded on the column and eluted with 2−4 mL/min flows. Columns were washed with 0.1 M phosphate buffer (pH 2.2) containing either 25%, 30%, 40%, or 50% methanol. Columns were dried with high vacuum flow for 15 min and then eluted with 100% methanol (2 or 3 mL). Loading eluates, wash eluates, and final eluates were collected and analyzed by liquid scintillation counting to monitor AM retention and elution behavior.

A phenyl SPE column (10 cm^3/500 mg; Varian part no. 1211-3031) was evaluated in a similar manner. In addition, a mixed-mode SPE column (Certify II, 10 cm^3/200 mg; Varian part no. 1211-3051) was evaluated with the following differences: (a) 0.1 M phosphate buffer (pH 7) was used to dilute urine and to condition and wash the columns, and (b) methanol + 2% acetic acid was used for final elution. This method was based on previously reported SPE methods for isolating small acidic molecules from human urine using mixed-mode solid phases (*41*, *44*).

Solvent Effects. The effect of organic solvents on the quantitation of AM was determined by analyzing standard solutions of AM prepared in buffer containing 0%, 5%, 10%, or 25% methanol or acetonitrile (two water-miscible, volatile solvents likely to be used for SPE final elution).

Sample Preparation. Twelve urine samples were collected from individuals with no documented exposure to triazines and were used to evaluate and validate two sample preparation methods.

(a) Dilution. The 12 unspiked urines and 1 blank buffer were diluted 4-fold and analyzed by the ELISA to determine whether this dilution was sufficient to reduce the signal from the sample below the assay limit of detection (reduce matrix effects). In addition, urine samples and one PBST control buffer were spiked in duplicate with either 0.12 or 0.2 μg/L AM, diluted 4-fold with PBST, and analyzed by the ELISA.

(b) Solid-Phase Extraction. The 12 unspiked urines and 1 blank buffer were extracted by C18 SPE, evaporated to dryness with a model 10.22 concentrator evaporator (Jouan, Winchester, VA), and redissolved in 0.5 mL of PBST. Extracts were diluted (1:3, 1:9, 1:27, and 1:81) and analyzed by the ELISA. In another experiment, four urines were spiked with 0.1 μg/L AM and one PBST control was spiked in duplicate with 0.01 and 0.1 μg/L AM. All samples were extracted by the C18 SPE method, evaporated to dryness, and redissolved in 1 mL of PBST. The pH of the dissolved extract was adjusted to 7.5, and the extracts were analyzed by ELISA undiluted and diluted (1:3 and 1:9).

Results

Hapten Density. Hapten density was determined to confirm the synthesis of the immunogen. Hapten density is a ratio of the hapten concentration and the protein concentration. Because KLH can be poorly soluble, the densities of both **1**-KLH and **1**-BSA were determined. Methods to determine these individual concentrations for chemically modified proteins are limited (*36*). For example, UV absorbance of proteins can change under conjugation reaction conditions, with or without hapten modification. Also, UV absorbance of haptens can change when coupled to proteins. Therefore, two different hapten density determination methods were compared. Protein concentrations determined by protein assay and UV spectroscopy were similar for both conjugates. Protein concentrations for **1**-KLH were 2.2×10^{-4} nmol/L (by protein assay) and 1.2×10^{-4} nmol/L (by UV) and for **1**-BSA were 0.025 nmol/L (by protein assay) and 0.026 nmol/L (by UV). Unlike protein concentrations, hapten concentrations determined by ELISA and UV spectroscopy were different for both conjugates. Hapten concentrations for **1**-KLH were 2.9×10^{-5} nmol/L (by ELISA) and 6.3×10^{-4} nmol/L (by UV) and for **1**-BSA were 0.01 nmol/L (by ELISA) and 0.03 nmol/L (by UV). ELISA may have underestimated hapten concentration if the anti-AM antibody were sterically prevented from binding to some of the AM haptens bound to the proteins or if the methyl ester of AM were not an appropriate standard. Enzymatic digestion of the immunogen prior to hapten detection may have produced a more accurate hapten concentration value. Conversely, UV spectroscopy may have overestimated hapten concentration because UV absorbance cannot distinguish between covalent and noncovalent adducts. The hapten density for **1**-KLH was estimated to be 5.3 haptens/protein molecule (by the UV method) and 0.13 hapten/protein molecule (by the ELISA + protein assay method), and the hapten density for **1**-BSA was estimated to be 1.1 hapten/protein molecule (by the UV method) and 0.4 hapten/protein molecule (by the ELISA method + protein assay). Both techniques indicated that the hapten densities were lower than optimal for strong immune response (*36*).

Table 2. Competitive ELISA Results for One Homologous and Three Heterologous Coating Antigens

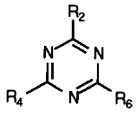
coating antigen[a]	dilution: coating antigen	dilution: antiserum	curve parameters[d] (μg/L): *A*	*B*	*C*	*D*	R^2
1-BSA[b]	1/20000	1/80000	1.5	0.20	800	0.05	0.97
3-BSA[c]	1/1000000	1/30000	0.44	0.92	0.2	0.05	1.0
5-BSA[c]	1/300000	1/10000	0.45	1.4	0.3	0.06	1.0
7-BSA[c]	1/1000000	1/30000	0.48	1.1	0.12	0.05	1.0

[a] See Table 1 for structures. [b] Homologous (hapten same as immunizing hapten). [c] Heterologous (hapten different than immunizing hapten). [d] *A*, maximum absorbance; *B*, slope; *C*, IC_{50}; *D*, minimum absorbance.

Coating Antigen Screening. The anti-AM antiserum recognized all of the coating antigens in the noncompetitive ELISA (data not shown), and AM competed with all of the coating antigens in the competitive ELISA (Table 2, selected data). The IC_{50} values for the heterologous competitive assays were all below 0.3 μg/L, whereas the IC_{50} value for the homologous assay was 800 μg/L. In addition, the curve slopes for the heterologous assays were all near 1, whereas the curve slope for the homologous assay was only 0.2.

Cross-Reactivity. Cross-reactivity experiments were conducted to determine the selectivity of the assay for AM compared to compounds with similar structure. Three of the coating antigens—**3**-BSA, **5**-BSA, and **7**-BSA—were screened in a small-scale cross-reactivity experiment (with atrazine, simazine, ametryn, hydroxyatrazine, didealkylated atrazine, and simazine mercapturic acid as inhibitors) to determine whether one of the coating antigens was more selective for AM than the other two. However, results from the small-scale cross-reactivity experiments revealed only minor differences in selectivity for AM compared to the inhibitors (data not shown). The expanded cross-reactivity experiments (with the **7**-BSA ELISA) showed that the assay was more selective to AM than to any other compound tested (Table 3). Analytes inhibited the assay strongly only if they comprised certain functional groups at all three positions: a thioether group or a chlorine atom at position R_2, an aminoisopropyl group at position R_4, and a

Table 3. Percent Cross-Reactivity of Inhibitors



analyte	R_2	R_4	R_6	% cross-reactivity[a] (*n*)
		Atrazine and Atrazine Metabolites		
AM (hapten **1**)	−NAcCys	$-NHCH_2CH_3$	$-NHCH(CH_3)_2$	100 (5)
atrazine	−Cl	$-NHCH_2CH_3$	$-NHCH(CH_3)_2$	35 ± 2 (4)[b]
desethyl AM	−NAcCys	$-NH_2$	$-NHCH(CH_3)_2$	1.1 ± 0.4 (2)
hydroxyatrazine	−OH	$-NHCH_2CH_3$	$-NHCH(CH_3)_2$	0.77 ± 0.4 (3)
desethylatrazine	−Cl	$-NH_2$	$-NHCH(CH_3)_2$	0.38 ± 0.1 (2)
desisopropyl AM	−NAcCys	$-NHCH_2CH_3$	$-NH_2$	0.030 ± 0.02 (2)
desisopropylatrazine	−Cl	$-NHCH_2CH_3$	$-NH_2$	0.023 ± 0.01 (2)
didealkylated AM	−NAcCys	$-NH_2$	$-NH_2$	<0.01 (1)
didealkylatedatrazine	−Cl	$-NH_2$	$-NH_2$	<0.01 (2)
		Simazine and Simazine Metabolites		
simazine mercapturate	−NAcCys	$-NHCH_2CH_3$	$-NHCH_2CH_3$	8.5 ± 2 (4)
simazine	−Cl	$-NHCH_2CH_3$	$-NHCH_2CH_3$	0.69 ± 0.2 (3)
		Other Triazines		
ametryn	$-SCH_3$	$-NHCH_2CH_3$	$-NHCH(CH_3)_2$	31 ± 5 (4)
prometryn	$-SCH_3$	$-NHCH(CH_3)_2$	$-NHCH(CH_3)_2$	21 ± 7 (2)
simetryn	$-SCH_3$	$-NHCH_2CH_3$	$-NHCH_2CH_3$	0.93 ± 0.06 (2)
terbutryn	$-SCH_3$	$-NHC(CH_3)_3$	$-NHCH_2CH_3$	0.18 ± 0.02 (2)
atraton	$-OCH_3$	$-NHCH_2CH_3$	$-NHCH(CH_3)_2$	11 ± 4 (2)
prometon	$-OCH_3$	$-NHCH(CH_3)_2$	$-NHCH(CH_3)_2$	10 ± 2 (2)
propazine	−Cl	$-NHCH(CH_3)_2$	$-NHCH(CH_3)_2$	6.9 ± 3 (4)
		Haptens		
hapten **2**	$-S(CH_2)_2COOH$	$-NHCH_2CH_3$	$-NHCH(CH_3)_2$	38 ± 20 (3)
hapten **3**	$-S(CH_2)_2COOH$	$-NHCH_2CH_3$	$-NHCH_2CH_3$	1.7 ± 0.7 (4)
hapten **6**	−Cl	$-NHCH(CH_3)_2$	$-NH(CH_2)_2COOH$	1.2 ± 0.5 (3)
hapten **7** (coating Ag)	−Cl	$-NHCH_2CH_3$	$-NH(CH_2)_2COOH$	0.024 ± 0.02 (2)
		Miscellaneous		
AM methyl ester	−NAcCysME	$-NHCH_2CH_3$	$-NHCH(CH_3)_2$	20 (1)
N-acetylcysteine				<0.01 (2)
S-benzyl mercapturate				<0.01 (1)

[a] Cross-reactivity was calculated as (IC_{50} of AM on the same plate as inhibitor/IC_{50} of inhibitor) × 100. [b] IC_{50} for atrazine was 0.22 μg/L. *n*, number of times cross-reactivity experiment was performed; NAcCys, $-SCH_2CH(NHAc)COOH$; NAcCysME, $-SCH_2CH(NHAc)COOCH_3$.

secondary amine at position R_6. Compounds that lacked just one of these groups cross-reacted by ≤11%. The one exception to this trend was hapten **6** (1.2%), comprising Cl at R_2, an aminoisopropyl at R_4, and an aminoalkyl acid at R_6. The coating antigen hapten (hapten **7**) only showed 0.024% cross-reactivity compared to the hapten used for immunization (hapten **1**). Simazine mercapturic acid, differing from AM by only one methyl group, only cross-reacted by 9%. Dealkylated metabolites of atrazine did not cross-react significantly. *N*-Acetylcysteine and *S*-benzylmercapturic acid did not cross-react below 800 μg/L.

Optimization. Reagent concentrations were optimized to yield the lowest IC_{50} value but to maintain a maximum absorbance at least 0.5 absorbance unit greater than the background. A high concentration of donkey anti-sheep IgG-HRP (1:2500) and long incubation times (1 h or 30 min) were used in order to conserve both the coating antigen (**7**-BSA) and the antiserum (during competitive incubation) used at 1:600000 and 1:80000, respectively.

Sensitivity, Precision, and Limit of Detection. The assay reproducibly achieved low IC_{50} values. The average IC_{50} of 68 standard curves generated on 13 different days was 0.08 ± 0.02 μg/L. Precision was improved (SD ± 0.01 μg/L) for curves generated on the same day due to fewer variations in reagent concentrations, incubation times, and pipetting techniques, variables inherent to all ELISAs. A typical standard curve, obtained from triplicate analyses at each concentration, had plateaus at both maximum and minimum absorbance to ensure good curve fits. Additionally, curves had maximum absorbances near 0.7 (A_{450} minus A_{650}), slopes near 1, and minimum absorbances near 0.1 (A_{450} minus A_{650}). Blank absorbances were not subtracted from standard or sample absorbances. The limit of detection (LOD) was estimated statistically as the concentration that corresponded to the maximum absorbance minus 3 times the standard deviation of the zero dose ($n = 3$) (*45*, *46*). The mean LOD for 20 curves was 0.019 ± 0.011 μg/L. The LOD was also estimated empirically as the IC_{15} (*46*). For the same 20 curves, the mean IC_{15} value was 0.02 ± 0.005 μg/L.

Matrix Effects. Because the assay was intended for the analysis of urine samples, it was necessary to determine whether pH or ionic strength of urine samples required adjustment prior to ELISA detection. One delimitation for this assay was that the standard curve solutions were prepared in 1× PBS + 0.05% Tween (the assay buffer). Sample pH did not significantly affect AM quantitation (Figure 1A). However, the AM response at all concentrations below 1 μg/L was progressively suppressed with increasing ionic strength of the assay buffer (Figure 1B).

Additional experiments were conducted to evaluate the effect of urine on the assay. Dilution (1:2) prior to ELISA

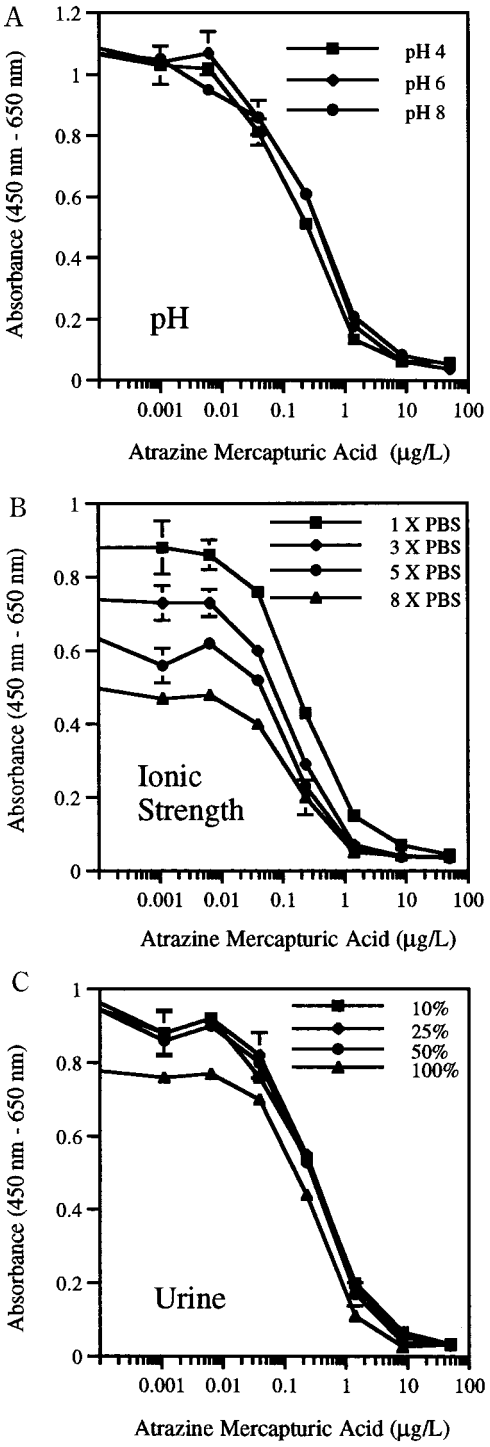


Figure 1. Atrazine mercapturic acid competition curves in buffers of varying (A) pH, (B) ionic strength, and (C) % urine. All concentrations were analyzed in quadruplicate on 1 day. Reagent concentrations: coating antigen (**7**-BSA) (1/200000); anti-AM antiserum (1/12000); donkey anti-sheep IgG-HRP (1/10000).

was sufficient to reduce the effect of matrix interferences from the one urine analyzed (Figure 1C). In another experiment, urines with high osmolarity (>570 mOsm/L) were found to inhibit the assay at low AM concentrations (Table 4). Urinary salts had little effect on accurate quantitation for urines spiked with AM ≥ 1 µg/L. Recoveries were 110 ± 3% (relative standard deviation, RSD, 3%), 110 ± 1% (RSD 1%), and 95 ± 6% (RSD 6%) for urines 1−3, respectively. However, urinary salts interfered significantly with AM quantitation for unspiked urines and urines spiked with AM < 1 µg/L. This established an approximate limit of quantitation (LOQ) of 1 µg/L, in accordance with a recommended LOQ determination guideline (recovery between 70% and 120% with RSD ≤ 20%) (*47*).

Solid-Phase Extraction Method Development. Because salts in urine interfered with the ELISA and because urine osmolarity varies so greatly, SPE was evaluated as a desalting and concentrating method. Atrazine mercapturic acid ([^{14}C]AM) was not detected in the C18, phenyl, or Certify II loading eluants, indicating that the loading conditions used were suitable for AM retention. Atrazine mercapturic acid eluted completely in 1−2 mL of methanol (C18 and phenyl) or acidic methanol (Certify II), indicating that final elution conditions were also appropriate. For C18 and phenyl columns, AM was retained with wash buffer containing 25% methanol (Table 5, C18 only) but eluted with higher concentrations of methanol. However, with the Certify II columns, AM eluted with all methanolic wash buffers (data not shown). Therefore, the SPE method selected for subsequent urinary analysis utilized C18 solid-phase, with 20-mL urine sample size (diluted with equal volume of buffer prior to loading), 20% methanolic buffer (10 mL) for washing, and 100% methanol (2 mL) for final elution. The phenyl and Certify II columns were not evaluated further.

Solvent Effects. The ELISA was tolerant to higher concentrations of methanol (to a least 25%) (Figure 2A) than to acetonitrile (to approximately 10%) (Figure 2B). Therefore, methanol or acetonitrile extracts can be diluted and analyzed directly by this ELISA, as long as the solvent concentration in the diluted extract is equivalent to the solvent concentration in the standard.

Sample Preparation. (a) Dilution. Fourfold dilution was sufficient to reduce the signal below the LOD (0.08 or 0.12 µg/L) for the buffer and for 11 of the 12 unspiked urine samples. (One unspiked urine sample, diluted 4-fold, gave a weak positive assay response of 0.16 µg/L AM equivalents.) Recovery of AM from buffer spiked with 0.12 and 0.2 µg/L was 100% for both, indicating low systematic error. Recovery of AM from the 12 urines spiked at 0.12 and 0.2 µg/L was 0.17 ± 0.04 µg/L (139 ± 31%, RSD 22%) and 0.22 ± 0.05 µg/L (109 ± 25%, RSD 23%), respectively. This established an approximate LOQ of 0.2 µg/L. Limit of quantitation was also determined as 3 times the method detection limit (MDL). The MDL values were calculated according to Environmental Protection Agency guidelines (*48*), using the SD of duplicates of 12 urine samples rather than the SD of 7 replicates of 1 urine sample. The MDL values for the 0.12 and 0.2 µg/L spiked samples were 0.092 and 0.12 µg/L, respectively. Thus, the statistically derived LOQ was 0.3 µg/L.

(b) Solid-Phase Extraction. The SPE eluates of all 12 unspiked urine samples gave a weak positive assay response (0.04 ± 0.02 µg/L AM equivalents). In subsequent experiments, using slightly different SPE conditions, a weak positive assay response was observed in the SPE eluate of an unspiked buffer (0.004 µg/L AM equivalents) and was still present in an unspiked urine sample (0.014 µg/L AM equivalents). Recovery of AM from buffer spiked with 0.01 and 0.1 µg/L was 0.013 ± 0.001 µg/L (125 ± 5%) and 0.093 ± 0.01 µg/L (93 ± 8%), respectively, indicating lower systematic error at the higher concentration. On the basis of these results, the

Table 4. Effect of Osmolarity on Atrazine Mercapturic Acid Recovery

spike concn (μg/L)	dilution factor	atrazine mercapturate equivalents (μg/L)[a] (RSD) urine 1, 250 mOsm/L (n = 3)	urine 2, 570 mOsm/L (n = 3)	urine 3, 970 mOsm/L (n = 3)
0	undiluted	<0.03	0.048 ± 0.003	0.11 ± 0.008
0.05	undiluted	0.036 ± 0.002	0.086 ± 0.005	0.16 ± 0.006
0.08	undiluted	0.055 ± 0.0005	0.11 ± 0.003	0.18 ± 0.005
0.1	undiluted	0.069 ± 0.002	0.12 ± 0.004	0.20 ± 0.009
1	1:2	1.1 ± 0.03 (3%)	1.1 ± 0.01 (1%)	0.95 ± 0.06 (6%)
10	1:50	12 ± 0.4 (3%)	12 ± 0.4 (3%)	11 ± 0.2 (1%)

[a] All replicates were analyzed in quadruplicate. RSD, relative standard deviation.

Table 5. Solid-Phase Extraction Method Development

methanol (%)[a]	percent atrazine mercapturic acid recovered (RSD) wash eluate fraction 1 (1.2 mL)	wash eluate fraction 2 (2 mL)	analyte eluate[b] 1 mL
50	26	72	ND
40	84	16	ND
30	ND	16	82
25	ND	ND	94

[a] In 0.1 M phosphate buffer, pH 2.2. [b] Analytes were eluted with 100% methanol. ND, none detected.

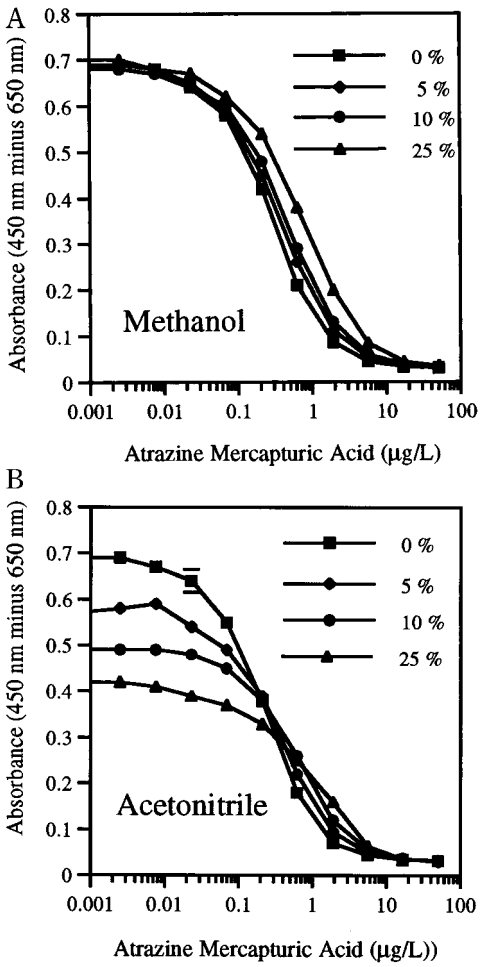


Figure 2. Atrazine mercapturic acid competition curves in PBST with increasing concentrations of (A) methanol and (B) acetonitrile.

feasibility of SPE for concentrating AM from urine was evaluated by spiking four urines at 0.1 μg/L AM, a concentration below the dilution + ELISA method LOQ (0.3 μg/L) but greater than the concentration of the weak positive assay response previously observed in urine (0.04 ± 0.02 μg/L AM equivalents). Recovery of AM from four urines spiked at 0.1 μg/L was 0.083 ± 0.008 (83 ± 8%, RSD 9%). These results established an empirically derived LOQ of 0.1 μg/L (between 75% and 120% recovery with RSD ≤ 20%) that was equivalent to a statistically derived LOQ (3 times the MDL = 0.032).

Discussion

Our first objective was to design, develop, and optimize an improved ELISA for AM. The availability of numerous hapten−protein conjugates in the triazine series (Table 1) facilitated the selection of a coating antigen yielding a highly sensitive and selective assay. The coating antigen was selected based on the results of several screening experiments. Results from the competitive ELISA experiments (Table 2) showed that the heterologous conjugates produced assays with lower IC_{50} values and steeper slopes than the homologous conjugate (**1**-BSA); therefore, the homologous conjugate was not evaluated further. These results were consistent with previous reports wherein small-molecule ELISAs that utilize coating antigens with a hapten slightly different than the immunizing hapten (heterologous formats) produce assays with lower LOD and steeper calibration curve slopes than ELISAs that utilize homologous formats (*34*, *49*). Because all of the heterologous conjugates (Table 1) were recognized by the anti-AM antiserum in the competitive ELISA, all were candidates for further examination. However, only three conjugates—**3**-BSA, **5**-BSA, and **7**-BSA—were selected for further screening. These conjugates were chosen because their competition curves met the following criteria: (1) an IC_{50} value less than 0.4 μg/L, (2) a slope greater than 0.7, (3) a maximum absorbance greater than 0.5, and (4) a minimum absorbance less than 0.1. In addition, the three conjugates were selected because they comprised three different haptens. We surmised that one of the conjugates would be more selective for AM than the other two.

However, results from the small-scale cross-reactivity experiment revealed no significant differences among the cross-reactivity patterns for the three conjugates (data not shown). This indicated that the binding characteristics of the antiserum influenced the assay selectivity more than the structure of the haptens on the conjugates. Because screening by cross-reactivity did not reveal an assay with superior selectivity, **7**-BSA was chosen to be the coating antigen for the ELISA because it produced a curve with the lowest IC_{50} value.

Results from the cross-reactivity experiments (Table 3) and results from previous reports on atrazine metabolism (*14*−*17*, *30*), suggested that a positive ELISA response from the urine of atrazine-exposed people would

primarily be due to AM, with little contribution from unmetabolized atrazine [present at concentrations 500−1000 times lower than the AM concentration (*18*)] and no contribution from hydroxyatrazine or the dealkylated metabolites. The cross-reactivity of simazine mercapturic acid (9%) and simazine (0.69%), differing from AM and atrazine by only one methyl group, also indicated the high degree of assay selectivity. These data supported the trend that analyte selectivity in triazine ELISAs is strongly influenced by the structure of the R_4 and R_6 alkyl groups (*50*, *51*).

In general, ELISAs with high selectivity are more quantitative (for the analyte used as the standard) than ELISAs with broad selectivity. More quantitative assays are useful in biological monitoring because they can more accurately measure the concentration of a specific metabolite and thereby allow better estimates of exposed doses (assuming the metabolism of the pollutant has been elucidated in humans). Less quantitative assays can also be useful in biological monitoring to screen for evidence of exposure by detecting multiple metabolites. In addition, ELISAs that are highly selective for a specific metabolite can be used for metabolism studies. For example, this AM-selective ELISA may be a potential tool for GSH atrazine metabolism research. Clearer assessment of xenobiotic metabolism and exposure is possible when both quantitative and qualitative analytical methods are employed.

The cross-reactivity of *N*-acetylcysteine and *S*-benzylmercapturic acid indicated that other miscellaneous N-acetylated compounds or mercapturic acids, if present at or below approximately 1 mg/L, would not interfere significantly. In addition, the cross-reactivity of several parent triazine herbicides indicated that the ELISA could be reconfigured for environmental monitoring of atrazine (IC_{50} = 0.22 μg/L, calcd), ametryn (IC_{50} = 0.25 μg/L, calcd), or prometryn (IC_{50} = 0.4 μg/L, calcd).

The sensitivity and selectivity of the present ELISA and the Lucas et al. ELISA were compared. Only a qualitative comparison could be drawn between the two ELISAs because they used different reagents and formats, and they also had different objectives. The present ELISA and Lucas' ELISA had IC_{50} values for AM of 0.08 and 0.4 μg/L, respectively. This suggested that the new ELISA was more sensitive than Lucas' assay. However, Lucas' assay may not have been as rigorously optimized as the present assay because the main objective was to identify an immunoreactive material in urine, rather than to optimize and validate an ELISA for analysis of large numbers of urine samples for AM. Both assays recognized AM with highest selectivity among the other compounds tested. The two ELISAs differed slightly in their cross-reactivities for parent triazines but were similar for the atrazine metabolites. These results demonstrated that sometimes polyclonal antiserum-based ELISAs can have comparable selectivity and superior limit of detection to monoclonal antibody-based ELISAs. Both monoclonal and polyclonal assays have advantages. Monoclonal antibody-based ELISAs are conducive to molecular cloning efforts (*52*, *53*), whereas polyclonal antiserum-based ELISAs are faster and more economical to produce. Overall, we concluded that we had developed a new ELISA for AM, more sensitive but equally selective for atrazine metabolites than the ELISA developed previously.

Our second objective was to evaluate urinary matrix effects on the ELISA. Although urine is aqueous and thus miscible with the assay buffer, it is a complex matrix containing high concentrations of salts and various organic compounds (*54*). Results from the effect of pH experiment (Figure 1A) indicated that slight differences in sample or buffer pH would not affect accurate AM quantitation. However, results from the effect of ionic strength experiment (Figure 1B) suggested that high concentrations of urinary salts would interfere with accurate AM quantitation. These results were consistent with previous reports regarding disruption of antibody−antigen interactions (*55*). Results from the effect of urine experiment (Figure 1C) indicated that endogenous urinary compounds did not significantly interfere and that a 2-fold dilution was sufficient to minimize matrix effects (presumably due to salts) for that one urine sample. Results from the effect of urinary osmolarity experiment (Table 4) indicated an approximate LOQ of 1 μg/L (dilution 1:2 + ELISA method) for all urine samples. In addition, these results suggested that the assay was not significantly inhibited by endogenous urinary compounds, presumably because the hapten did not resemble endogenous materials. In contrast, other ELISAs for urinary biomarkers of exposure reported that urine required 50−100-fold dilution to reduce the concentration of unidentified urinary interferences (*56*, *57*). Collectively, these results indicated that variable ionic strength was the primary urinary matrix problem and that a desalting method (dilution or other) was required.

Our third objective was to devise sample preparation methods to normalize the ionic strength of urine samples and to reduce the overall method LOQ. Dilution was one option for sample preparation. Many clinical ELISAs require dilution of urine samples prior to analysis because the target analyte concentration is greater than the dynamic range of the assay (*25*, *33*, *58*−*62*). In these assays, urine is present at or below 2% during competitive incubation; thus urinary matrix interferences, such as salts, are diluted out. However, because we were targeting a biomarker of exposure known to be present at trace concentrations (*18*), it was important for us to determine the smallest dilution factor required to minimize interference from salts but also to maintain a low LOQ. Results from the spike and recovery experiments (for the 1:4 dilution + ELISA method) indicated that AM could be detected in most urine samples at concentrations $\geq$ 0.3 μg/L. At this dilution factor, urine comprised 12.5% of the assay buffer solution during competitive incubation, demonstrating a high degree of tolerance for the complex matrix. Other ELISAs for trace levels of urinary biomarkers of exposure will also require an approximate 4-fold dilution to reduce salt concentrations. Therefore, we concluded that 4-fold dilution was a simple and fast method for normalizing the ionic strength of urine samples and thus would be an appropriate sample preparation method for measuring AM at concentrations $\geq$ 0.3 μg/L.

Another option for sample preparation was SPE, used widely in both environmental and clinical applications for water and biological fluids (*63*). Solid phase extraction was evaluated for its ability to normalize ionic strength and to concentrate AM from urine. The weak positive assay response observed in the unspiked urine SPE extracts was unexpected based on the assay's high degree of selectivity (Table 3) and because the urines

were from individuals with no known exposure to triazines. The responses in each urine sample were equivalent at multiple dilutions, suggesting the presence of compounds specifically recognized by the antiserum and not compounds that disrupted antiserum binding. Results from the analysis of spiked and unspiked buffer samples indicated that a small portion of the weak positive assay response in the unspiked urine samples was due to materials from the SPE process. However, results from the analysis of unspiked urine samples indicated that another portion of the weak positive assay response was due to the presence of specific compounds in urine with similar chemical or physical properties as AM. Spike recovery results for the four urine samples (between 70% and 90%) suggested that AM could be detected in SPE-extracted urines at or above approximately 0.1 μg/L with little to no contribution from compounds that caused the weak positive assay response. However, this overall method LOQ was not significantly lower than the dilution + ELISA method LOQ. The data supported two conclusions: (1) the weak positive assay response was due to trace levels of AM or triazine metabolites, and (2) the weak positive assay response was due to interfering materials that coeluted with AM.

We concluded that both of the sample preparation methods had advantages and disadvantages. Dilution was easier, faster, and yielded an LOQ (0.3 μg/L) below previously reported concentrations of AM in human urine (*18*). Thus, dilution would be more amenable than this SPE method for the rapid analysis of large numbers of urine samples, assuming AM concentrations greater than 0.3 μg/L. Other solid phases or SPE conditions may aid in elucidating the compounds that caused the weak positive assay response. However, SPE is more time-consuming and expensive and would only be worthwhile if a lower LOQ could be achieved.

The level of sensitivity achieved with this ELISA was far superior to the reported detection limits for other chromatographic and mass spectrometric methods for mercapturic acids in urine (*2*, *3*). In addition sample preparation was easier and faster than most other reported methods for biological monitoring (*64*). No other methods for AM with a similar LOD were reported.

We have shown that a highly selective polyclonal antibody-based ELISA was developed for AM, indicating that development of ELISAs for other mercapturic acids is feasible. Because some endogenous mercapturic acids or other materials may interfere with mecapturic acid-selective ELISAs, immunizing haptens should resemble the structure of the mercapturic acid as closely as possible. Dilution was an effective method to normalize salt concentrations among urine samples; however, dilution limited the overall method LOQ to 0.3 μg/L. The SPE method used was not effective for selectively extracting and concentrating AM from urine. The presence of a weak positive assay response, from either AM, other triazine metabolites, or nontriazine interferences, resulted in accurate spike recovery of AM at 0.1 μg/L. Provided that matrix effects and sample preparation methods are evaluated thoroughly, dilution with ELISA detection is an excellent method to accurately and economically quantitate trace levels of atrazine mercapturic acid in large numbers of human urine samples.

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