Development of a Class-Selective Enzyme-Linked Immunosorbent Assay for Mercapturic Acids in Human Urine

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Epidemiological and toxicological studies often require the analysis of large numbers of samples for biological markers of exposure. The goal of this work was to develop a class-selective ELISA to detect groups of structurally closely related mercapturic acids with small nonpolar *S*-substituents. An assay was developed with strong recognition for mercapturates including *S*-benzylmercapturic acid (IC_{50} = 0.018 μmol/L), *S*-*n*-hexylmercapturic acid (IC_{50} = 0.021 μmol/L), *S*-phenylmercapturic acid (IC_{50} = 0.024 μmol/L), and *S*-cyclohexylmethylmercapturic acid (IC_{50} = 0.042 μmol/L). The same assay also showed weaker recognition for *S*-(1-hydroxynaphthal-2-yl)mercapturic acid and *S*-allylmercapturic acid (IC_{50} = 1.1 and 1.7 μmol/L, respectively). Subtle modifications to the hapten linker structure of the coating antigen proved to have a strong impact on the selectivity and the specificity of the assay. A slightly modified assay showed high recognition for *S*-benzylmercapturic acid (IC_{50} = 0.018 μmol/L) and weaker recognition for seven other mercapturic acids (IC_{50} = 0.021−10 μmol/L). Strong positive assay responses were detected in 12 urine samples obtained from persons with no known occupational exposure to exogenous electrophilic xenobiotics. Solid phase extraction and cross-reactivity indicated that the presumptive immunoreactive materials were similar in size and polarity to *S*-benzylmercapturic acid. The assay was more selective to mercapturic acids than the spectrophotometric thioether assay.

Keywords: *ELISA; mercapturic acid; biological monitoring; urine*

INTRODUCTION

Improved analytical methods are required to accommodate the analysis of large numbers of samples for biological and epidemiological monitoring (Tardiff and Goldstein, 1991; Straight et al., 1995). GC, HPLC, and MS methods typically require sample extraction and derivatization prior to chromatographic separation and detection of the target analyte (Sheldon et al., 1986). Although these methods can yield quantitative and structural information, they are too time-consuming and expensive for the screening of large populations. Enzyme-linked immunosorbent assays (ELISAs), used widely since the 1970s for clinical analyses (Oellerich, 1980; Haasnoot et al., 2000) and more recently for environmental analyses (Rosner et al., 1991; de Zoysa et al., 1998), have been developed for several biomarkers of exposure found in human urine. Among them are 3-methyladenine (Prevost et al., 1990), toluene (Inagaki and Minami, 1994), atrazine, methyl parathion (Harris et al., 2000), fenitrothion, carbaryl, and naphthalene (Marco et al., 1993a; Gee et al., 1995). Samples usually require minimal preparation, such as dilution or solid phase extraction (SPE) prior to analysis. In addition, ELISAs are fast, simple, and inexpensive. Many ELISAs are quantitative assays with high selectivity for the target analyte and with low limit of detection. In some cases, ELISA can be the least expensive and most sensitive analytical method for polar compounds that are thermally labile, nonvolatile, difficult to extract, or difficult to derivatize, such as paraquat (Sherry, 1992).

Mercapturic acids (MAs; *N*-acetyl-L-cysteine *S*-conjugates) have been used as biomarkers of exposure for numerous electrophilic xenobiotics (Henderson et al., 1989; Nelson, 1992; van Welie et al., 1992; Bernauer et al., 1996; Maestri et al., 1997). MAs are metabolic end products of conjugates formed from spontaneous or enzyme-catalyzed reactions of endogenous glutathione with electrophilic xenobiotics or their metabolites (Chasseaud, 1979). Sources of electrophiles are many, including foods (Delbressine et al., 1981; Aringer and Lidums, 1988; de Rooij et al., 1996), environmental pollutants (Chasseaud, 1979), industrial chemicals (de Rooij et al., 1998), drugs, and smoke (Henderson et al., 1984). Analytical methods (GC, HPLC, GC-MS and MS) have typically been designed for individual MAs rather than for groups or classes of MAs due to different extraction or derivatization requirements (Hoffmann and Baillie, 1988; Vermeulen, 1989; Nelson, 1992; van Welie et al.,

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10.1021/jf000621b CCC: $19.00
Published on Web 11/16/2000

1992). Extraction is commonly employed to isolate and concentrate MAs with *S*-substituents of low polarity, but extraction efficiencies are often marginal for MAs with polar *S*-substituents (Giles, 1979). Thus, few methods have been developed for polar MAs.

A spectrophotometric thioether assay (STA) has been used to estimate total MA concentration in human urine (Henderson et al., 1984; van Welie et al., 1991). The STA can aid in early exposure monitoring to indicate increased urinary excretion of total thiol-forming compounds from persons occupationally exposed to exogenous electrophilic chemicals. However, because the assay responds to urinary disulfides, thioesters, and other non-MA thioethers, the STA lacks the desired specificity for MAs. Additionally, the STA is limited because extraction and conversion to thiols can be inefficient and variable for some MAs (Henderson et al., 1984). Rapid methods that are more selective than the STA are needed for monitoring total MAs or classes of MAs in large numbers of urine samples.

Most ELISAs are designed to be selective for the analysis of a single compound (compound selective). This is accomplished by coupling a mimic of the target analyte (hapten) to an immunizing protein with the unique functional groups of the target analyte distal to the spacer arm (Goodrow et al., 1995). Compound-selective ELISAs have been developed in this manner to detect a variety of compounds in urine such as drugs and drug metabolites (Aoki et al., 1996), hormones and tumor markers (Gosling and Basso, 1994), domoic acid (Newsome et al., 1991), hippuric acid (Inagaki and Minami, 1994), and biomarkers of exposure (Gomes and Santella, 1990; Gee et al., 1995; Harris et al., 1995). In addition, ELISAs have been developed for some MAs, such as the MA of alachlor (Feng et al., 1994), the MA of 1,2-naphthalene oxide (Marco et al., 1993b,c), and the MA of atrazine (Lucas et al., 1993).

In the present study, we describe the design, development, and evaluation of a class-selective ELISA for the detection of structurally similar aliphatic and aromatic MAs. The first objective was to generate antisera that would recognize the common functional groups of MAs [R−SCH_2CH(NHAcetyl)COOH]. For this purpose, a series of immunogens was synthesized. *N*-Acetyl-L-cysteine was coupled to carrier proteins via structurally diverse linker arms. The second objective was to develop and optimize an ELISA and to probe the selectivity of the assay for MAs. The third objective was to compare the ELISA response to the STA response on a set of human urine samples from persons with no documented occupational exposure to electrophilic xenobiotics.

MATERIALS AND METHODS

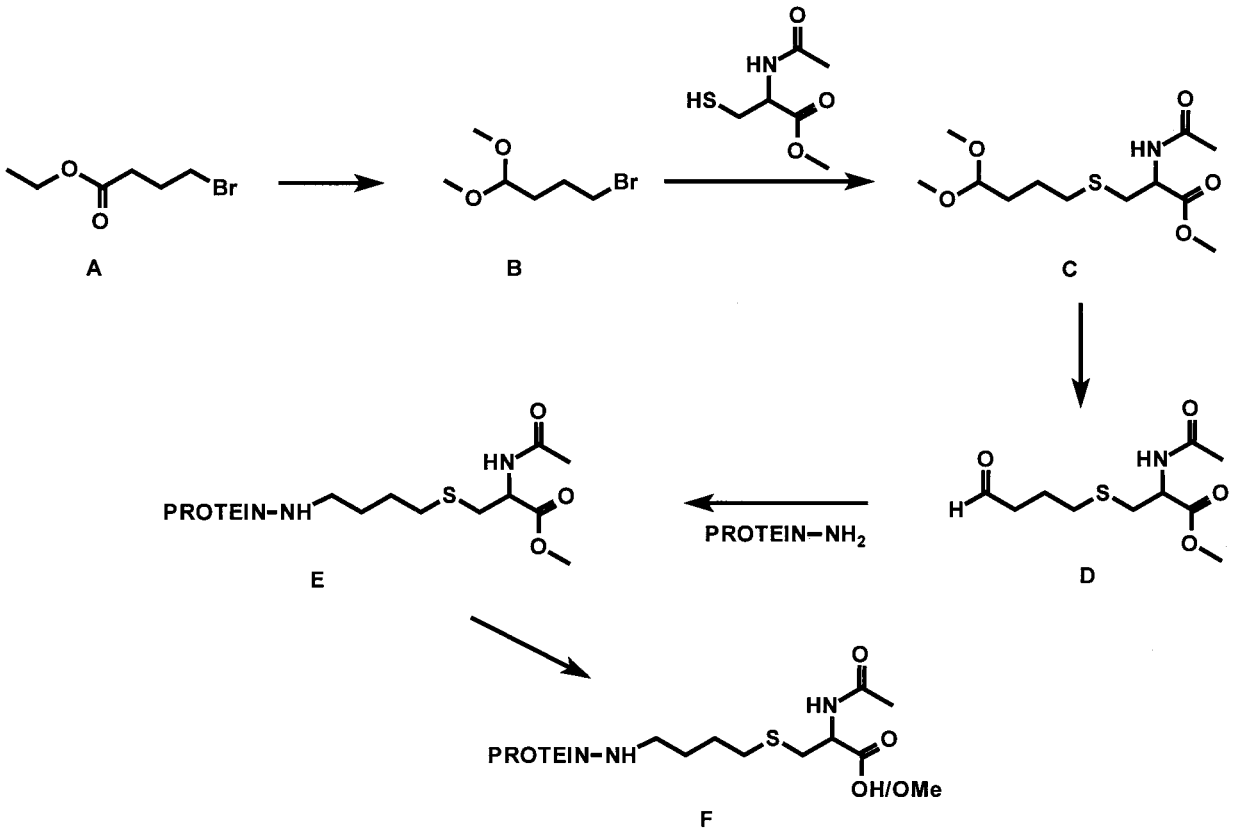
Chemicals. Bovine serum albumin (BSA), conalbumin (CONA), adjuvants, Tween 20, 3,3′,5,5′-tetramethylbenzidine (TMB), goat anti-rabbit immunoglobulin G−horseradish peroxidase (IgG-HRP), and myoglobin (from horse heart) were purchased from Sigma Chemical Co. (St. Louis, MO). Keyhole limpet hemocyanin (KLH) was from Calbiochem-Novabiochem Corp. (La Jolla, CA). Ethyl 4-bromobutyrate, DIBAL-H, and sodium cyanoborohydride ($NaCNBH_3$; caution: highly toxic!) were purchased from Aldrich Chemical Co. (Milwaukee, WI). *N*-Acetyl-L-cysteine methyl ester was ordered from Fluka Chemie AG (Buchs, Switzerland). *S*-2,4-Dinitrophenylmercapturic acid was a gift from Dr. David Grant (Department of Pharmacology and Toxicology, University of Arkansas, Little Rock, AR) and was one dense spot on TLC detected by UV absorption, R_f 0.68 (system 2). *S*-(1-Hydroxynaphthal-2-yl)-mercapturic acid was a gift from the laboratory of Dr. Alan Buckpitt (Department of Molecular Biosciences, University of California, Davis, CA). 2-*N*-Acetylaminophenylmercapturic acid was a gift from Dr. Pilar Marco (Department of Biological Organic Chemistry, CID-CSIC, Barcelona, Spain). 4-Hydroxynonenal-1-ylmercapturic acid was a gift from Dr. Bernd Bruenner (Facility for Advanced Instrumentation, University of California, Davis, CA). *S*-Atrazinylmercapturic acid was synthesized according to methods described in Lucas et al. (1993). All other chemicals were of reagent grade. Kieselgel 60 F_{254} silica gel (0.2 mm) plastic sheets were used for TLC (EM Separations Technology, Gibbstown, NJ). Solvent systems were ethanol/ethyl acetate 1:1 + 1% acetic acid (system 1) and acetonitrile/water/acetic acid 44:5:1 (system 2). Spots were visualized by UV light, in iodine vapor or 1% $KMnO_4$ (in water). Preparative column chromatography was performed on silica gel (o.d. = 40 μm, J. T. Baker, Philipsburg, NJ). Bradford protein assay dye reagent was from Bio-Rad Laboratories (Richmond, CA.).

Equipment. Electrospray ionization (ESI) mass spectra (negative ion mode, 50% aqueous methanol, 20 μL direct loop injection, 10 μL/min flow) were obtained with a VG Quattro-BQ mass spectrometer (VG Biotech, Altrincham, U.K.) and with a Qstar (PE Biosystems, Foster City, CA) hybrid (quadrupole time-of-flight, Q-TOF) mass spectrometer, using nanospray interface, 500 nL/min flow rate, in positive mode. ^{1}H/^{13}C NMR spectra were recorded on a General Electric QE-300 spectrometer (Bruker, Billerica, MA) at 300.1 and 75.5 MHz, respectively. The solvent was $CDCl_3$ if not stated otherwise. Chemical shifts are referenced to the $CDCl_3$ signal at 7.26 ppm (^{1}H) and 77.0 ppm (^{13}C), respectively. ELISA absorbances were measured with a Spectra Max 250 96-well plate reader with Softmax Pro software (Molecular Devices, Sunnyvale, CA). Water from a Sybron/Barnstead Nanopure II water system (Newton, MA) was used to prepare all buffers. Maxisorp Immuno plates (Nunc, Roskilde, Denmark, no. 442404) were used for ELISA, and microtiter plates (Dynatech Laboratories Inc., Chantilly, VA, no. 001-012-9205) were used for reagent mixing prior to ELISA. For SPE, 500 mg C18 cartridges (large reservoir capacity, part 1211-3207) from Varian (Harbor City, CA) were used. Eluate evaporation was performed in a model 10.22 concentrator evaporator (Jouan, Winchester, VA). Dialysis was conducted with 12000−14000 MW cutoff Spectra/Por membrane tubing (Spectrum Medical Industries Inc., Laguna Hills, CA).

Synthesis of Haptens. Methyl 2-(acetylamino)-3-(4-oxobutylthio)propanoate **D** was synthesized according to Scheme 1. 4-Bromo-1,1-dimethoxybutane **B** was prepared according to a procedure of Ihara et al. (1989) starting with ethyl 4-bromobutyrate **A**. Crude **B** was distilled under reduced pressure (main fraction at 40−42 °C, 0.6 Torr) to give 6.08 g (30.9 mmol, 64%) of pure **B** as a colorless liquid: ^{1}H NMR δ 4.29 (t, J = 6.0 Hz, 1H), 3.37 (t, J = 6.5 Hz, 2H), 3.25 (s, 6H), 1.87−1.78 (m, 2H), 1.71−1.62 (m, 2H); ^{13}C NMR δ 103.9, 53.0 (2×), 33.7, 31.2, 28.0.

To a stirred solution of 300 mg (1.69 mmol) of *N*-acetyl-L-cysteine methyl ester in 15 mL of methanol was added dropwise at 0 °C 1.70 mL (1 equiv) of 1 M NaOMe. The resulting solution was stirred for 20 min and then allowed to warm to ambient temperature (AT). A solution of 334 mg (1.69 mmol) of 4-bromo-1,1-dimethoxybutane **B** in 5 mL of MeOH was added drop by drop under continuous stirring. After 30 min at AT, the resulting mixture was refluxed under N_2 for 5 h. The reaction mixture was cooled to AT and the methanol evaporated. The remaining residue was partitioned between ethyl acetate and H_2O. After extraction, the organic layer was washed with brine and dried over Na_2SO_4. The crude product was subsequently chromatographed on silica gel (ethyl acetate/hexane, 5:1, TLC R_f 0.35). The purified methyl 2-(acetylamino)-3-(4,4-dimethoxybutylthio)propanoate **C** was isolated as a colorless oil (0.263 g, 896 mmol, 53%): ^{1}H NMR δ 6.39 (d br, J = 7.2 Hz, NH), 4.77 (ddd, J = 7.2, 5.2 Hz, 1H), 4.31 (t, J = 6.0 Hz, 1H), 3.73 (s, 3H), 3.24 (s, 6H), 2.93 (dd, J = 5.2 Hz, 2H), 2.49 (t, J = 6.8 Hz, 2H), 2.00 (s, 3H), 1.68−1.52 (m, 4H); ^{13}C NMR δ 172.3, 170.7, 103.9, 52.8, 52.6 (2×), 51.7, 34.0, 32.8, 31.3, 24.5, 23.0.

Scheme 1. Synthesis of the Butyraldehyde Conjugate (Conjugate 5, Figure 1)[a]



[a] Exact reaction conditions (A−E) are given under Materials and Methods. Protein = CONA; Me = methyl.

To a solution of 191 mg (0.65 mmol) of acetal **C** in H_2O (2 mL)/acetone (2 mL) was added a catalytic amount of *p*-toluenesulfonic acid. The resulting mixture was stirred until TLC (ethyl acetate/hexane, 5:1) indicated complete hydrolysis. After the acetone had been evaporated, the residue was partitioned between H_2O (made slightly basic with 0.1 M NaOH) and ethyl acetate. The organic solvent was evaporated, and the crude aldehyde was chromatographed on a small amount (10−12 g) of silica gel (hexane/ethyl acetate, 1:5; TLC R_f 0.28). Methyl 2-(acetylamino)-3-(4-oxobutylthio)propanoate **D** was isolated as a slightly yellowish oil (77 mg, 0.31 mmol, 48%): ^{1}H NMR δ 9.74 (s, 1H), 6.46 (d br, J = 7.3 Hz, NH), 4.76 (ddd, J = 9.8, 7.3, 4.4 Hz, 1H), 3.71 (s, 3H), 2.91 (dd, J = 9.8, 4.4 Hz, 2H), 2.56−2.46 (m, 4H), 2.02 (s, 3H), 1.85 (tt, J = 7.3, Hz, 2H); ^{13}C NMR δ 201.3, 171.2, 169.8, 52.5, 51.7, 42.2, 33.8, 31.7, 22.9, 21.5.

Synthesis of Mercapturic Acids (MAs). *S*-Benzyl-, *S*-*n*-hexyl- and *S*-(1-hydroxybenzyl)mercapturic acids were synthesized by reacting respective electrophiles with *N*-acetyl-L-cysteine in water/acetonitrile (1:1) and NaOH (Kaye et al., 1972). Following the incubation, the acetonitrile was evaporated, the aqueous phase was made basic and starting materials were extracted into hexane/ethyl acetate (4:1). The aqueous phase was then acidified, and MAs were extracted into ethyl acetate. Products were characterized by TLC and ESI-MS as follows: *S*-Benzyl-MA (BzMA) was one dense spot on TLC by UV and one spot with iodine vapor, R_f 0.55 (system 1) and R_f 0.49 (system 2). The molecular ion $(M - H)^-$ using ESI-MS in negative mode appeared at 252 Da mass, and the thioether bond cleaved fragment $(M - H - 129)^-$ appeared at 123 Da mass, mp 143−145 °C. *S*-Hexyl-MA was one dense spot on TLC by UV, R_f 0.7 (system 1) and R_f 0.54 (system 2). The molecular ion $(M - H)^-$ by ESI-MS in negative mode was at *m*/*z* 246 Da, and the thioether bond cleaved fragment $(M - H - 129)^-$ was at *m*/*z* 117 Da, mp 83−85 °C. *S*-(1-Hydroxybenzyl)-MA was one dense spot on TLC by UV, R_f 0.53 (system 1) and R_f 0.43 (system 2). The molecular ion $(M - H)^-$ by ESI-MS in negative mode was at *m*/*z* 282 $(M - H)^-$, and the thioether bond cleaved fragment was at *m*/*z* 153 $(M - H - 129)^-$.

S-Methyl-MA, *S*-allyl-MA, and *S*-cyclohexylmethyl-MA were prepared by treatment of *N*-acetyl-L-cysteine with 2 equiv of sodium ethoxide in ethanol and then with the appropriate alkyl halide. After neutralization, the solid was collected and recrystallized. The structures were confirmed by ESI-MS by solid probe introduction.

rac-*S*-Phenyl-MA was prepared by treatment of 2-acetamidoacrylic acid with benzenethiol in the presence of a catalytic amount piperidine in dioxane according to the method of Hanzlick et al. (1990).

Protein Conjugate Preparation. BSA, KLH, and CONA were used to prepare hapten−protein conjugates. All conjugates were extensively dialyzed in PBS, pH 7.5 (8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl), and centrifuged or purified by gel permeation chromatography (Presto desalting columns, Pierce, Rockford, IL) prior to storage at −20 or −80 °C. To prepare conjugates **1**-BSA, **1**-KLH, and **1**-CONA, iodoacetamide was used to block free cysteine residues in the protein (Foster and Harrison, 1974). *p*-Bromomethylphenyl acetic acid was converted to the *N*-hydroxysuccinimide active ester (Staros et al., 1986; Bekheit et al., 1993). Aliquots of the active ester solution were added to the carboxyamidated proteins, the pH of the solutions was adjusted to 8.0, and the solutions were stirred at 4 °C for 30 min and then for 5 h at AT. Following dialysis against PBS, pH 6, protein solutions were degassed with nitrogen and cooled to 4 °C. *N*-Acetyl-L-cysteine (20 mg, 123 μmol) was added to each stirred protein solution, and the pH was adjusted to 8.5.

Conjugates **2**-BSA and **2**-CONA were prepared using an *N*-hydroxysuccinimide active ester to couple BzMA to protein through the carboxylic acid group (Staros et al., 1986; Bekheit et al., 1993). Conjugates **3**-BSA and **3**-CONA were prepared with a maleimidohexanoyl *N*-hydroxysuccinimide ester (MHS) to couple *N*-acetyl-L-cysteine to protein (Peeters et al., 1989). For conjugates **4**-BSA and **4**-CONA *p*-benzoquinone was used to couple *N*-acetyl-L-cysteine to protein (Ternynck and Avrameas, 1977; Tijssen, 1985).

Conjugate **5**-CONA (**F** in Scheme 1) was prepared by reaction of the free aldehyde **D** to the carrier protein CONA

Table 1. Zero Charge State MS Data Extract Giving Detection Accuracy and Qualitative Proof of Basic Hydrolysis of Myoglobin Loaded with Hapten (D, Scheme 1)[a]

no. of ligands bound (n)	degree of hydrolysis (x)	mass (Da)		
		theor	obsd[b]	diff
3	3	17560.8	17560.6	0.2
3	2	17574.8	17575.4	0.6
3	1	17588.8	17590.2	0.4
4	4	17763.9	17762.5	1.4
4	3	17777.9	17777.8	0.1
4	2	17791.9	17792.3	0.4
5	5	17967.0	17965.0	2.0
5	4	17981.0	17980.6	0.4
5	3	17995.0	17995.2	0.2
6	6	18169.1	not observed	
6	5	18183.1	18183.3	0.2
6	4	18197.1	18198.2	1.1
6	3	18211.1	18212.9	1.8

[a] $P(L\text{-}COOMe)_n \rightarrow P(L\text{-}COOMe)_{n-x}(L\text{-}COOH)_x$. P = myoglobin, (L-COOMe) = *S*-(4-oxobutyl)-*N*-acetyl-L-cysteine methyl ester **D** (Scheme 1), $n = 1, 2, ...; x = 0, 1, ..., n$. See also **E** → **F** (Scheme 1; protein = myoglobin). [b] Mass range scanned: 600−2000 Da; resolution $R = 5000$; the spectra were the results of the average of two to five consecutive scans.

following a protocol by Hermanson (1996). To a stirred solution of 25 mg of CONA in 5 mL of coating buffer (1.70 g of Na_2CO_3 plus 2.86 g of $NaHCO_3$/L solution, pH 9.6) was added at AT a solution of 6 mg (24 μmol) of hapten **D** in 120 μL of DMSO (hapten-to-protein ratio of 1:4 w/w). A freshly prepared solution of 5 M $NaCNBH_3$ in 1 N NaOH was added dropwise, and the resulting mixture was stirred under N_2 for 2.5 h. To remove small molecules, the solution of the protein conjugate was exhaustively dialyzed against Nanopure water. In a final step the protein conjugate was lyophilized.

To hydrolyze the distal oriented methyl ester to free the mercapturate, the protein conjugate **E** described above was treated under basic hydrolytic conditions (ammonium carbonate buffer, 20 g/L solution, pH 8.9) for 18 h at AT. After a second lyophilization step, this sequence of hydrolysis and lyophilization was repeated once more to achieve a high rate of ester hydrolysis. The final lyophilized product **F** (Scheme 1) was taken up in 0.9% NaCl and stored at −20 °C. Product **F** is referred to as **5**-CONA.

The efficiency of the conjugation procedure and the hydrolysis of the methyl ester were qualitatively verified using hapten **D** (Scheme 1) coupled to the model protein myoglobin (16951.4 Da). ESI mass spectra of myoglobin loaded with hapten **D** (Scheme 1) after basic hydrolysis were generated using a standard nanospray interface (Table 1).

Antiserum Generation. Six female New Zealand white rabbits (2.5−3.0 kg) were housed in an NIH-approved rabbitry with temperature and light control, and all protocols were approved by the campus animal health committee. Rabbits were immunized every 30 days to generate polyclonal antisera 994, 995, 996, 2118, 2119, and 2120 (Table 2). Approximately 400 μg of each immunogen was diluted to 1.5 mL with PBS, pH 7.5, and emulsified with Freund's complete adjuvant (1.5 mL). One-third of the emulsion was injected into each rabbit subdermally in multiple locations on the back. Freund's incomplete adjuvant was used in all subsequent immunizations. Test bleeds (10 mL) were collected from the ear vein 7−10 days after each immunization. Rabbits were exsanguinated after the eighth boost.

All blood samples were collected into evacuated test tubes and coagulated overnight at 4 °C. Blood clots were removed from the antisera, and residual cells were separated by centrifugation. Antisera were preserved with sodium azide (0.02%) and stored at −20 °C.

The increase in each antiserum titer was monitored by two-dimensional titration noncompetitive ELISA (Gee et al., 1994) using hapten−protein conjugates heterologous in protein and homologous in spacer arm and hapten. Gradual increase in maximum absorbance with subsequent bleeds was observed until the fifth bleed, after which the titer remained constant. Serum from the eighth bleed was used for assay development.

Table 2. Screening by Two-Dimensional Titration Noncompetitive ELISA[a]

coating antigen[b]	rabbit polyclonal antiserum					
	immunogen **1**-BSA[b]			immunogen **1**-KLH[b]		
	994	995	996	2118	2119	2120
2-BSA	*c*	*c*	*c*	+++	+	+
2-CONA	+	+	+	++	+	+
3-BSA	*c*	*c*	*c*	+++	+++	++
3-CONA	++	++	++	+++	+++	++
4-BSA	*c*	*c*	*c*	+++	++	+
4-CONA	++	++	+	+	+++	++
5-CONA	nt	nt	nt	nt	nt	++

[a] Antisera and coating antigen combinations tested by checkerboard titration using coating antigens heterologous in protein and spacer arm and homologous in hapten (*N*-acetyl-L-cysteine). [b] Structure of immunizing and coating conjugates shown in Figure 1. [c] Combinations not tested because the coating antigen was homologous in protein. nt, not tested; +++, maximum absorbance >1 and good curve shape (exponential curve and steep slope); ++, maximum absorbance 0.5−1 and good curve shape; +, maximum absorbance <0.5 and poor curve shape.

ELISA. For titration experiments a noncompetitive ELISA was used in which microtiter plates were coated with hapten−protein conjugates (100 μL/well) diluted in coating buffer (0.05 M carbonate−bicarbonate, pH 9.6). Coated plates were incubated overnight at 4 °C and were washed five times with wash buffer (PBS/water, 1:1, plus 0.05% Tween 20, pH 7.5). Antisera diluted in assay buffer (PBS plus 0.05% Tween 20, pH 7.5) were added to the coated plates (50 μL/well). Plates were incubated for 1 h at room temperature and then washed. Second antibody conjugate, goat anti-rabbit IgG-HRP diluted in assay buffer (1:2500 to 1:10000) (50 μL/well) was added to the coated plates. Plates were incubated for 1 h at AT. After five washings with wash buffer, 100 μL/well substrate solution (25 mL of 0.05 M citrate−acetate buffer, pH 5.5, 100 μL of 1% H_2O_2, and 400 μL of a 0.6% solution of TMB in DMSO) was added. After 20 or 30 min, 2 M sulfuric acid (50 μL/well) was added, and absorbances were read at 450 and 650 nm.

Competitive ELISAs were run as described above except that in place of the addition of antibody, standard solutions of BzMA, other inhibitors, or samples were mixed with antibody prior to addition to the coated plate. Standard (or inhibitor or sample) solutions were added to an equal volume of antiserum solution in the wells of an uncoated (mixing) plate. Solutions were mixed thoroughly and transferred to the coated plates (50 μL/well). Standard solutions of BzMA (10000, 910, 83, 7.5, 0.68, 0.062, 0.0056, and 0 ng/mL) were prepared in assay buffer or 5−20% methanol as described for each experiment. A four-parameter algorithm (Rodbard, 1981) was used for curve fitting and interpolation.

SPE. SPE cartridges were conditioned with 2 mL each of ethyl acetate, methanol, Nanopure water, and 0.1 M phosphate buffer, pH 2.2 (PB). Urines (20 mL) were mixed with an equal volume of PB and loaded onto the column with 2−4 mL/min flow. Columns were washed with PB (10 mL) and then air-dried under high vacuum for 5 min. Columns were eluted with 100% methanol (3 mL). Eluates were diluted with PBST to a concentration of 20% methanol or less or evaporated to dryness and redissolved in PBST (2 mL) prior to analysis.

Analysis of Urine. Twelve urine samples were collected from laboratory volunteers with no known occupational exposure to high levels of electrophilic chemicals. Urines were stored at 4 °C for 2 weeks and then at −20 °C. Urine samples 1−8 (150−250 mL) were individual samples, and samples 9−12 were pooled from 25 first urine of the day samples (50 mL). BzMA equivalent concentrations (micromoles per liter) were determined for the whole urine by analysis at three different dilutions (1:40, 1:80, and 1:160). The 12 urine samples

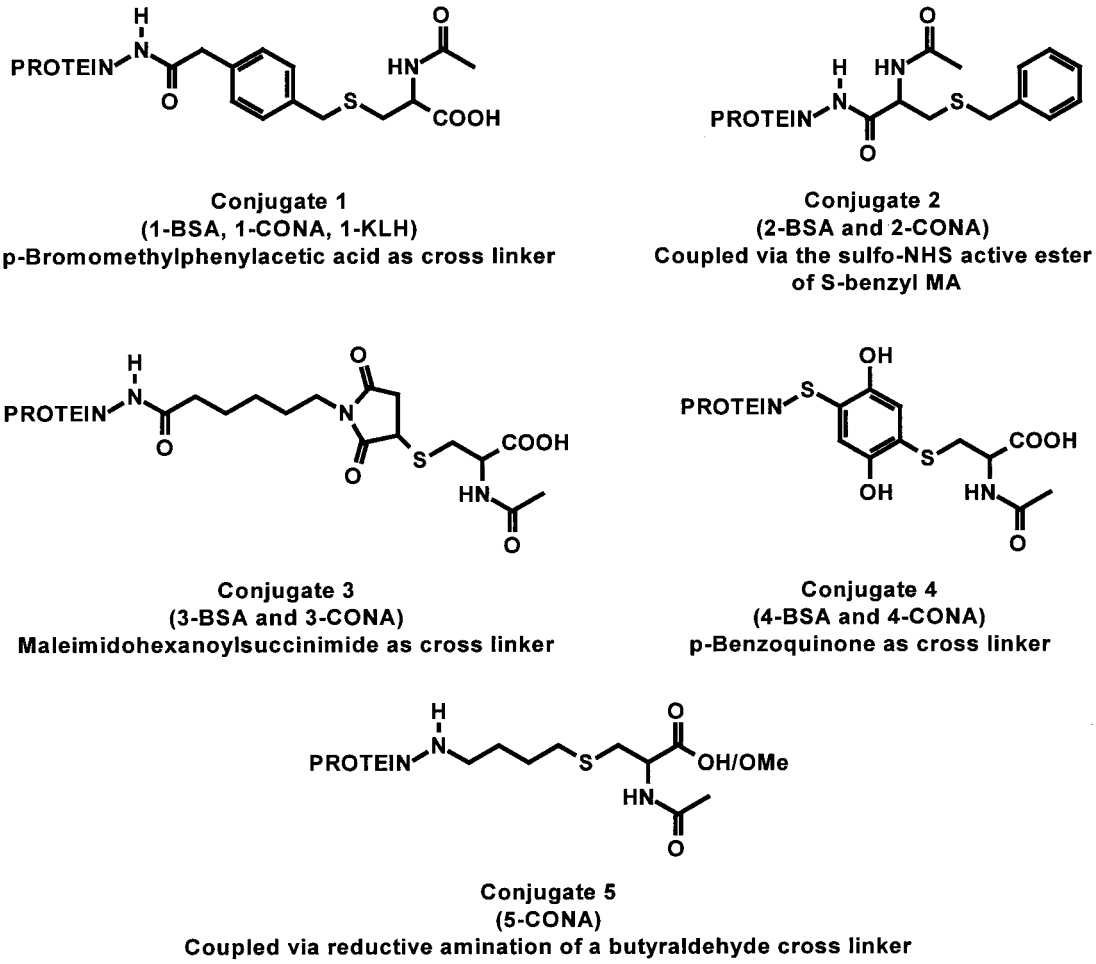


Figure 1. Structures of hapten−protein conjugates used as the immunogen (conjugate **1**) and as coating antigens (conjugates **2**−**5**). Conjugates **3**-CONA and **5**-CONA were selected for the final coating antigens.

and 1 buffer spiked with 0.2 μmol/L BzMA were also extracted by SPE, and BzMA equivalent concentrations were compared for whole and extracted urine. STA was measured before and after SPE, and creatinine concentration was determined according to the manufacturer's instructions (kit 555-A, Sigma).

RESULTS

Screening by Noncompetitive ELISA. Combinations of antisera and coating antigens (Figure 1; Table 2) were tested by two-dimensional titration noncompetitive ELISA to determine whether any of the antisera recognized *N*-acetyl-L-cysteine coupled to protein via different spacer arms. Characteristic families of exponential curves were generated for all combinations tested, showing an increase in absorbance with increasing antiserum and coating antigen concentrations. Fifteen combinations of antiserum and coating antigen produced exponential curves with relatively steep slopes and maximum absorbances >1 and were thus considered for subsequent competitive ELISA experiments (Table 2). Optimal concentrations of antisera and coating antigen were derived from these experiments and used in subsequent competitive ELISAs.

Screening by Competitive ELISA. Fifteen combinations of antiserum and coating antigen were tested by competitive ELISA with BzMA as the inhibitor. Fourteen of the antiserum/coating antigen combinations recognized BzMA (Table 3). Ten combinations produced curves with nearly equivalent IC_{50} values, maximum absorbances >0.8, minimum absorbances <0.2, and slopes >0.4. Five combinations were not suited for further ELISA development because they did not meet the above criteria. Multiple combinations with high sensitivity for BzMA were evaluated for cross-reactivity with various MAs and common urinary components listed in Table 4. Two assay combinations, antiserum 2120 with coating antigen **3**-CONA (2120/**3**-CONA) and antiserum 2120 with coating antigen **5**-CONA (2120/**5**-

Table 3. Competitive ELISA Using *S*-Benzyl-MA as the Inhibitor

antiserum	coating antigen[a]	*A*[b]	*B*[c]	*C*[d]	*D*[e]
994	**3**-CONA[f]	1.1	0.20	1.2	0.10
995	**3**-CONA	0.75	0.70	0.011	0.20
996	**3**-CONA[f]	0.95	1.2	0.22	0.45
2118	**3**-BSA[f]	0.71	0.92	4.0	0.10
2118	**3**-CONA[f]	0.70	0.62	5.9	0.02
2118	**4**-BSA[f]	0.47	NA[g]	NA	0.50
2119	**3**-BSA	1.2	0.98	0.028	0.10
2119	**3**-CONA	0.98	0.88	0.016	0.060
2119	**4**-BSA	1.4	0.42	0.071	0.02
2119	**4**-CONA	0.96	0.52	0.055	0.11
2120	**3**-CONA	1.0	1.0	0.033	0.070
2120	**3**-BSA	1.2	0.96	0.047	0.10
2120	**4**-BSA	1.2	0.80	0.055	0.15
2120	**4**-CONA	0.84	0.91	0.020	0.25
2120	**5**-CONA	0.86	1.26	0.017	0.13

[a] Structure of immunizing and coating conjugates shown in Figure 1. [b] *A*, maximum absorbance. [c] *B*, slope. [d] *C*, IC_{50} value (μmol/L). [e] *D*, minimum absorbance. [f] Combinations were eliminated because BzMA did not inhibit the combination or because curves had high IC_{50}, high minimum absorbance, or shallow slope. [g] NA, not applicable.

Table 4. Cross-Reactivity of Ab 2120 with MAs and *N*-Acetylated Amino Acids

mercapturic acid (MA)	Ab 2120/**3**-CONA IC_{50} (μmol/L)[a]	cross-reactivity (%)	Ab 2120/**5**-CONA IC_{50} (μmol/L)[a]	cross-reactivity (%)
S-benzyl-MA	0.018	100	0.018	100
S-cyclohexylmethyl-MA	0.046	39	0.042	40
S-*n*-hexyl-MA	0.26	7	0.024	75
S-phenyl-MA	9.7	0.2	0.021	86
S-allyl-MA	1.6	1	1.7	1
S-(1-hydroxynaphthal-2-yl)-MA	1.1	1.6	1.1	1.6
2-*N*-acetylaminophenyl-MA	6.4	0.3	nt[e]	
S-(1-hydroxybenzyl)-MA	3.5	0.5	nt	
4-hydroxynonenal-1-yl-MA	13	0.1	nt	
S-2,4-dinitrophenyl-MA	30	0.06	nt	
S-methyl-MA	no inhibition at 56 μmol/L[b]		no inhibition at 56 μmol/L[b]	
S-atrazinyl-MA	no inhibition at 30 μmol/L[b]		nt	

N-acetylated amino acids	Ab 2120/**3**-CONA IC_{50} (μmol/L)[a]
N-acetylmethionine	52
N-acetylcysteine (25−35 μmol/L)[c] (Hannestad and Sorbo, 1979)	80
N-acetylphenylalanine	>48[d]
N-acetylvaline (<0.1 μmol/L)[c] (Lehnert and Werle, 1988)	150
N-acetyl-leucine (0.3 μmol/L)[c] (Lehnert and Werle, 1988)	870
N-acetylarginine (15−150 μmol/L)[c] (Marescau et al., 1990)	930
N-acetyltyrosine	>450[d]
N-acetyl-lysine (30−42 μmol/L)[c] (Armstrong et al., 1967)	>530[d]
N-acetylhistidine (29 μmol/L)[c] (Wadman et al., 1971)	>510[d]

[a] The assay used polyclonal antiserum 2120, raised against conjugate **1**-KLH (Figure 1) with conjugate **3**-CONA or **5**-CONA (Figure 1) as the coating antigen. [b] No inhibition indicated that no reduction in maximum absorbance occurred at the concentration shown. [c] Typical urinary concentrations reported by indicated author. [d] Significant inhibition at the indicated concentration, but <25%. [e] nt, not tested.

CONA), were ultimately selected for further development because the inhibition curve shape was good, sensitivity was high, and background and cross-reactivity with other unspecified urinary components were low. In addition, coating antigens **3**-CONA and **5**-CONA represent two structurally different approaches to coating antigens potentially suitable in class-selective ELISAs. The MHS-derived coating antigen (**3**-CONA) was favored above the benzoquinone-derived coating antigen (**4**-CONA) because less of the reagent was required for the assay. For alternate applications of the antibody library, including assays more generally selective for a variety of mercapturate structures or for affinity purification prior to mass spectrometric analysis, combinations giving lower slopes (*B*) and lower maximum absorbance values (*A*, Table 3) could be useful.

Assay Optimization. Assay reagents were optimized to achieve the lowest IC_{50} value for BzMA and to maintain a maximum absorbance >0.6 and a minimum absorbance <0.1. Hereafter assays are referred to by antiserum number (i.e., 2120) and the coating antigen (i.e., **5**-CONA). Optimal dilution factors for the assay 2120/**3**-CONA were established as follows: antiserum 2120 (1/10000), coating antigen **3**-CONA (1/500000), and goat anti-rabbit IgG-HRP (1/4000). For the assay 2120/**5**-CONA, antiserum 2120 (1/2000), coating antigen **5**-CONA (1/18800), and goat anti-rabbit IgG-HRP (1/3000) provided results that met all criteria. The assay buffer, buffer additives (NaCl, KCl, and Tween 20), and buffer pH (7.5) were not optimized. Minor effects on the assays were seen with methanol up to 20% (data not shown). Thus, in some experiments methanol eluates of SPE columns were diluted to 10 or 20% methanol and the standard curves run in 10 or 20% methanol to correct for these effects.

Cross-Reactivity. The cross-reactivities of selected MAs, urinary compounds, *N*-acetylamino acids, and benzyl compounds were determined (Table 4). Solutions were prepared to yield sigmoidally shaped standard curves with asymptotes at both high and low concentrations, when possible. Standard solutions of endogenous urinary compounds were prepared to encompass concentrations normally found in urine.

Assay 2120/**3**-CONA recognized all tested MAs with the exceptions of *S*-methyl-MA and *S*-atrazinyl-MA. The inhibition slopes of the different cross-reacting MAs varied from 0.66 to 1.3. Several of the *N*-acetylamino acids were recognized by the assay 2120/**3**-CONA but at relatively high concentrations (IC_{50} = 50−930 μmol/L) (Table 4). None of the individual urinary compounds (ascorbic acid, citric acid, D-glucuronic acid, guanidinoacetic acid, L-cysteine, L-methionine, taurine, hippuric acid, urea, and *p*-aminobenzoic acid) were recognized at normal urinary concentrations (Lentner, 1981). Additionally, none of the compounds with a benzyl moiety (*S*-benzyl-L-cysteine, benzyl alcohol, benzaldehyde, benzoic acid, benzylamine, benzyl bromide, *N*,*N*-dimethylbenzylamine, *N*-benzylmethylamine, or benzyl disulfide) showed any inhibition at 10 mg/L.

Similar to assay 2120/**3**-CONA, assay 2120/**5**-CONA strongly recognized *S*-benzyl-MA (IC_{50} = 0.018 μmol/L) and *S*-cyclohexylmethyl-MA (IC_{50} = 0.042 μmol/L) but did not recognize *S*-methyl-MA. However, recognition by assay 2120/**5**-CONA of *S*-*n*-hexyl-MA and *S*-phenyl-MA was 10 and 400 times greater, respectively, than by assay 2120/**3**-CONA (Figure 2). Recognition for *S*-allyl-MA (IC_{50} = 1.7 μmol/L) and *S*-(1-hydroxynaphthal-2-yl)-MA (IC_{50} = 1.0 μmol/L), although weak, was similar for both assays.

Analysis of Urine. All 12 urine samples tested gave a strong positive response in the ELISA. Serially diluted solutions of the whole urines resulted in slopes varying from 0.63 to 1.1, suggesting that different combinations of analytes were contributing to inhibition. SPE indicated that the immunoreactive materials were neutral or acidic with medium to low polarity. Nearly all of the immunoreactive materials remained on the columns during the washing step. For samples 1 and 9, some of

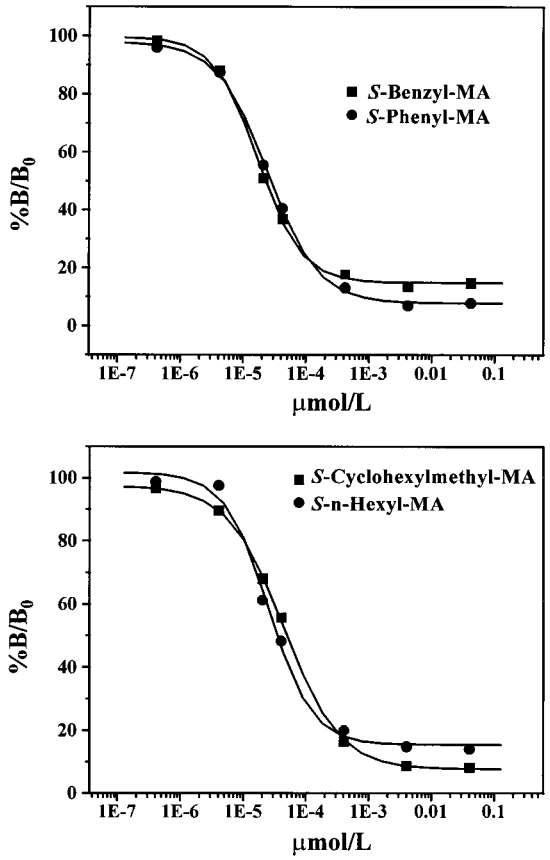


Figure 2. Typical standard curves of four MAs using polyclonal antiserum 2120 (1:2000) and **5**-CONA (1:18800) as the coating antigen. The curves are the average of three replicates each, performed on separate microtiter plates. The average IC_{50} values are *S*-benzyl-MA, 0.018 ± 0.002 μmol/L; *S*-phenyl-MA, 0.021 ± 0.002 μmol/L; *S*-*n*-hexyl-MA, 0.024 ± 0.003 μmol/L; and *S*-cyclohexylmethyl-MA, 0.040 ± 0.005 μmol/L. B = average absorbance measured for every concentration of the MA standard curve. B_0 = average absorbance measured with a zero concentration of the MA.

the immunoreactive materials (possibly MAs with considerable polar character) eluted off the column during the washing step, but losses of recognized compounds during washing were <10% for all other urines. Spike recovery results (98% recovery) demonstrated that BzMA remained bound to the column during the washing step but was recovered in high yield with methanol, indicating that other less polar MAs would also remain bound.

The urine samples were also subjected to the STA (Figure 3). To separate the nonpolar thioether conjugates from the polar disulfides (known interferents) (Henderson et al., 1984), samples were subjected to SPE and the eluate was analyzed. The thioether values were comparable to reported ranges for both whole and SPE-enriched urines (Table 5), indicating samples contained normal concentrations of thiol-forming materials such as disulfides, thiols, thioesters, and thioethers (including MAs). However, SPE removed a significant portion of some thiol-forming materials (such as cystine) from the urine (Figure 3).

The thioether values and the ELISA values for the SPE-enriched urines were compared but did not correlate well (r^2 = 0.1). This was expected because the selectivities of the two assays are different. MAs present in the urine samples contributed to the positive response of both the ELISA and the STA. However, other not further characterized urinary compounds contributed to the STA response that did not contribute to the ELISA response.

DISCUSSION

The first objective was to generate antisera that would recognize the *N*-acetyl-L-cysteine moiety of MAs and to use the antiserum in a competitive ELISA. Previous ELISAs for MAs coupled the MA hapten to protein by the reaction of free proteinogenic amino groups with the activated carboxylic group of the *N*-acetyl-L-cysteine moiety (Lucas et al., 1993; Marco et al., 1993a; Feng et al., 1994). This coupling strategy resulted in the generation of antisera selective for the *S*-substituent of the MA. However, the goal of this project was to generate antisera selective for the *N*-acetyl-L-cysteine moiety of MAs. An ideal immunogen would present *N*-acetyl-L-cysteine distal to the surface of the immunizing protein via an inert spacer arm (Goodrow et al., 1995). Immunogens (conjugate **1**, Figure 1) were synthesized by coupling *N*-acetyl-L-cysteine to protein, via the thiolate anion, using *p*-bromomethylphenylacetic acid as a cross-linking agent. This hapten was designed to orient *N*-acetyl-L-cysteine distal to the protein and also to resemble BzMA, a known biomarker of exposure to

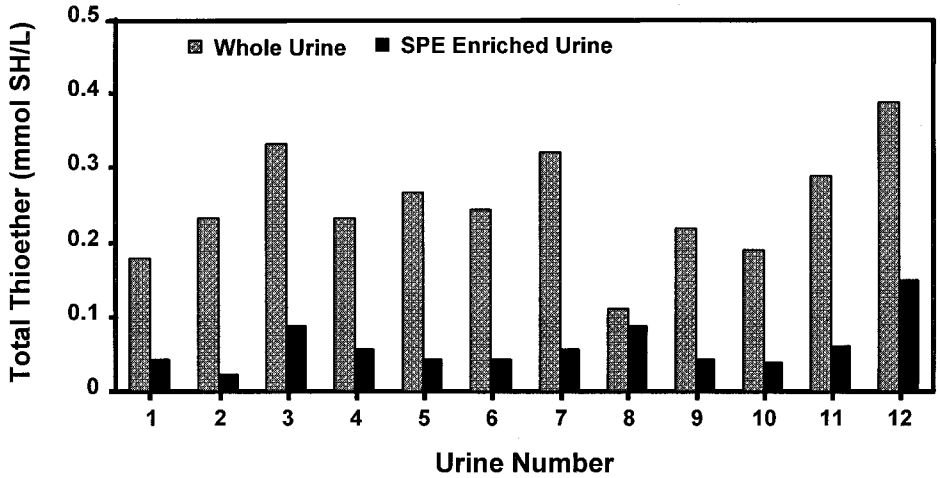


Figure 3. Results of the STA, expressed in millimoles of SH per liter, of human urine samples (both whole urine and SPE C18 enriched urine) from persons with no known occupational exposure to electrophilic xenobiotics. Samples 1−8 were individual urine samples. Samples 9−12 were pooled from 25 first urine of the day urines.

Table 5. Total Thioether Values for Extracted Urines from Occupationally Unexposed Persons

sample preparation	mmol of SH/mol of creatinine	reference
SPE enriched urine (extracted)	7.1 ± 1.6 (n = 12)	current data[a]
	av = 3.8, P_{95}[b] = 5.9 (n = 196)	van Doorn et al. (1981)[c]
	4.7 ± 3.3 (n = 23) (city)	Malonova et al. (1983)[c]
	4.6 ± 6.6 (n = 11) (industrial town)	Malonova et al. (1983)[c]
	3.9 ± 4.2 (n = 17) (mountain village)	Malonova et al. (1983)[c]
	mean = 6.05 (n = 56)	van Welie et al. (1991)[c]
whole urine (not extracted)	39 ± 14 (n = 12)	current data
	54 ± 20 (n = 50)	Seutter Berlage et al. (1977)[d]
	30 ± 7 (n = 20 males)	Vainio et al. (1978)[d]
	32 ± 6 (n = 11 male smokers)	Vainio et al. (1978)[d]
	37 ± 9 (n = 16 females)	Vainio et al. (1978)[d]
	44 ± 19 (n = 13 female smokers)	Vainio et al. (1978)[d]
	37 ± 2 (n = 63 males)	Kilpikari (1981)[d]
	42 ± 2 (n = 48 females)	Kilpikari (1981)[d]
	79 ± 4 (84 males)	Kilpikari and Savolainen (1982)[d]

[a] Enriched by SPE. [b] Outer limit of percentile. [c] Enriched by liquid/liquid extraction with ethyl acetate. [d] Samples were deproteinized.

benzyl halides (Chasseaud, 1979) and other electrophilic xenobiotics such as benzyl acetate (Chidgey et al., 1986), 1-menaphthyl alcohol, and propionate esters (Clapp and Young, 1970; Laham and Potvin, 1987; Shuker et al., 1993). Three variations were synthesized using heterologous coupling chemistry, proteins, and spacer arms such as maleimidohexanoylsuccinimide and *p*-benzoquinone (Table 1; Figure 1, conjugates **2**−**4**).

In an alternative approach to a suitable hapten featuring a distal *N*-acetyl-L-cysteine subunit, *N*-acetyl-L-cysteine methyl ester was coupled with 4-bromo-1,1-dimethoxybutane, hydrolyzed under acidic conditions, and subsequently coupled to the carrier protein by reductive amination (Scheme 1). Using this method, the *N*-acetyl-L-cysteine methyl ester subunit was connected to the protein by a simple *n*-alkyl linker mimicking *n*-alkyl-MAs. Such a linker features no bulky or polar groups, which normally tend to negatively influence the binding of the antibody. The *N*-acetyl-L-cysteine methyl ester represents a protected form of the carboxylic group of *N*-acetyl-L-cysteine and in this form facilitates hapten synthesis and protein coupling. The *N*-acetyl-L-cysteine carboxylic group was recovered by basic hydrolysis (Scheme 1). Under the conditions applied, the carrier protein was loaded with both free distal *N*-acetyl-L-cysteine and unhydrolyzed *N*-acetyl-L-cysteine methyl ester. This protein conjugate proved to be suitable as coating antigen in combination with antibodies raised in rabbit 2120 (Figure 1, conjugate **5**). Subsequent competitive ELISA screening experiments using BzMA as an inhibitor identified several potential ELISAs selective for a class of MAs (Table 2). These data show that the immunogen design used produced antiserum that recognized the functional group [$-SCH_2CH(NHAc)$-COOH] of interest.

Cross-reactivities were evaluated to (1) determine the selectivity of the assays 2120/**3**-CONA and 2120/**5**-CONA for other MAs, (2) identify potential urinary interferences, and (3) determine the relative affinity of compounds with a benzyl moiety. In both assays (2120/**3**-CONA and 2120/**5**-CONA) recognition appears to be a function of the polarity and size of the analyte. *S*-methyl-MA and *S*-allyl-MA seem to be too small to produce high-affinity binding between the antigen and Ab 2120. The MA of styrene oxide cross-reacted much less than BzMA, indicating that a hydroxy group alpha to the thioether bond reduced binding in the assay 2120/**3**-CONA. In case of the MA of 1,2-naphthalene oxide, the extended aromatic system seems to compensate in part for the polar character of the hydroxy group. The presence of *o*- and *p*-nitro groups in *S*-2,4-dinitrophenyl-MA caused a 3-fold increase in IC_{50} (30 μmol/L) compared to *S*-phenyl-MA. The MAs showing highest cross-reactivity (in combination 2120/**5**-CONA) are structurally closely related. Three of them (*S*-benzyl-MA, *S*-*n*-hexyl-MA, and *S*-cyclohexylmethyl-MA) feature a methylene unit adjacent to the sulfur. Additionally, they all have a highly lipophilic (or aromatic) character. They are also comparable in size. The use of the coating antigen **5**-CONA proved to have a strong positive impact on broadening the range of recognized MAs in the competitive assay format. The hapten and the linker loaded on coating antigen **3**-CONA closely resemble BzMA. These structural characteristics are strongly recognized by Ab 2120, limiting the suitability of **3**-CONA for class-selective ELISA development (Table 4). The specific characteristics of the structurally different coating antigens **3**-CONA and **5**-CONA dramatically influence the selectivity and specificity of a particular assay. Even though assay 2120/**5**-CONA does not represent a fully optimized assay (solvent and urine matrix effects, pH influence, salt effects are still under investigation), the presented data show how selectivity (and/or specificity) of an ELISA can be tuned by the choice of the suitable hapten (and linker) loaded onto the coating antigen. The data also indicate that the antibody may bind still a broader range of mercapturates when used in procedures such as affinity chromatography that do not involve competitive binding.

In a set of experiments the 2120/**3**-CONA ELISA response was compared with the STA. For the 2120/**3**-CONA ELISA, urine samples required large dilution factors (between 50- and 100-fold) to produce signals greater than the minimum absorbance. This may in part be due to cross-reacting urinary mercapturates excreted in high concentrations (milligrams per liter) (de Rooij et al., 1998) and simultaneously showing weak affinities for the applied antibodies. The cross-reactivity results (Table 4) revealed important structural information about the immunoreactive materials present in urine: (1) Because none of the individual urinary compounds (urea, hippuric acid, etc.) were immunoreactive, they did not cause a strong positive ELISA response. (2) None of the compounds with benzyl moieties were recognized by the assay, indicating that the benzyl functional group alone was not a strong antigenic determinant. (3) The assay 2120/**3**-CONA recognized *N*-acetylamino acids but at concentrations far greater than found in diluted urine

(Table 3). (4) Because *S*-benzyl-L-cysteine (lacking only the acetyl group) did not inhibit the assay at 47 μmol/L, nitrogen acetylation seems to be important for antiserum binding. (5) *N*-Acetylphenylalanine was recognized (IC_{50} > 48 μmol/L) but at a concentration 2400-fold greater than that of BzMA, indicating the thioether moiety was important for strong antibody binding. (6) *N*-Acetyl-L-cysteine (similar to an MA, but lacking an *S*-substituent) was recognized (IC_{50} = 80 μmol/L) but at a concentration 4000-fold greater than that of BzMA. Thus, an *S*-substituent is important for strong antibody binding. These observations suggest that the strong antibody response observed in the urine samples was due to MAs similar in size and polarity to BzMA present at low micromoles per liter concentrations.

This work illustrates the potential use of MA class-selective ELISAs for the rapid screening of large numbers of human urine samples to monitor electrophilic burden. The 2120/**5**-CONA assay, selective for MAs that resemble BzMA, represents one of many ELISAs that could be developed for different groups of MAs. Several MA class-selective assays could be used in an array to quickly assess different types of MAs excreted by agricultural or industrial workers exposed to electrophilic xenobiotics. The MA class-selective ELISAs could potentially be used in a manner analogous to the STA, by which increased levels of immunoreactivity would indicate exposure to certain types electrophilic food constituents, drugs, or environmental and industrial chemicals. In addition, these types of assays may provide indices of glutathione conjugation, which reflect xenobiotic burden and induction of glutathione-*S*-transferases.

ABBREVIATIONS USED

Ab, antibody; Ab/protein conjugate (e.g., 2120/**3**-CONA) refers to an immunoassay system consisting of antibody 2130 and coating antigen, **3**-CONA; AT, ambient temperature; BQ, *p*-benzoquinone; BSA, bovine serum albumin; br, broad; BzMA, *S*-benzylmercapturic acid; CONA, conalbumin; DCM, dichloromethane; DME, 1,2-dimethoxyethane; d, doublet; ELISA, enzyme-linked immunosorbent assay; EPA, U.S. Environmental Protection Agency; ESI-MS, electrospray ionization mass spectrometry; equiv, equivalent; FAB-MS, fast atom bombardment mass spectrometry; GC, gas chromatography; HPLC, high-pressure liquid chromatography; HRP, horseradish peroxidase; IC_{50}, median inhibitory concentration; IgG-HRP, immunoglobulin G−horseradish peroxidase conjugate; *J*, coupling constant (Hz); KLH, keyhole limpet hemocyanin; MA, mercapturic acid [*N*-acetyl-L-cysteine *S*-conjugate, R−S−CH_2CH(NHAcetyl)COOH]; MHS, maleimidohexanoyl *N*-hydroxysuccinimide; mp, melting point; o.d., outer diameter; PB, phosphate buffer; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline with Tween 20; Q-TOF MS, quadrupole time-of-flight mass spectrometer; R, residue, in case of MAs, an *S*-substituent; R_f, retention factor; s, singlet; SPE, solid phase extraction; STA, spectrophotometric thioether assay; TLC, thin layer chromatography; TMB, 3,3′,5,5′-tetramethylbenzidine; t, triplet; UV, ultraviolet (light).

ACKNOWLEDGMENT

We thank Bernd Bruenner, Dexter Morin, Dave Grant, and Pilar Marco for donating mercapturic acids; Ferenc Szurdoki for assistance with conjugate synthesis; U.C. Davis Lab Animal Health Clinic for animal handling and antiserum generation; Adam Harris for helpful consultation; and George Dunstan for laboratory assistance.

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Received for review May 18, 2000. Revised manuscript received October 13, 2000. Accepted October 16, 2000. This publication was made possible by Grants P42 ES04699 (National Institute of Environmental Health Sciences Superfund Basic Research Program) and P30 ES05707 (National Institute of Environmental Health Sciences Center). This work was also supported by Grant CR819658, Environmental Protection Agency Center for Ecological Health Research. Although the information in this document has been funded in part by the U.S. Environmental Protection Agency, it may not necessarily reflect the views of the Agency and no official endorsement should be inferred. C.L. was supported by a fellowship (81EZ 55210) from the Swiss National Science Foundation. L.L.J. was the recipient of two U.C. Davis Jastro Shields Research Scholarships and an NIEHS Center for Environmental Health Sciences Fellowship. B.D.H. was supported by a McMaster's Fellowship from CSIRO Australia and NSF Award INT-96-02893.

JF000621B