Hapten Design and Development of an ELISA (Enzyme-Linked Immunosorbent Assay) for the Detection of the Mercapturic Acid Conjugates of Naphthalene

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Measurement of urinary metabolites constitutes a noninvasive method to assess toxic exposure. Naphthalene is a common environmental contaminant showing selective pulmonary toxicity in mice and presumably is involved in development of lung disease in man (Buckpitt, A.R.; Franklin, R.B. *Pharmacol. Ther.* **1989**, *41*, 339). A glutathione-based detoxification pathway leads to the formation of the mercapturic acid conjugates [NaphMA 1, (*R*)-*N*-acetyl-*S*-[(1*R**,2*R**)-1,2-dihydro-1-hydroxy-2-naphthyl]cysteine and (*R*)-*N*-acetyl-*S*-[(1*R**,2*R**)-1,2-dihydro-2-hydroxy-1-naphthyl]cysteine] which are excreted in urine. Herein we report the development of an immunoassay for the specific detection of these urinary metabolites. This study confirms the importance of appropriate hapten design and synthesis in controlling the specificity and sensitivity of the immunoassay. Our strategy was to prepare haptens that allow covalent attachment to a carrier protein at a site opposite to the *N*-acetylcysteine moiety. The antibodies obtained by immunizing six rabbits with these NaphMA derivatives (haptens 13 and 14) have been used for the development of an ELISA (enzyme-linked immunosorbent assay) which detects NaphMA 1 in the range between 100 and 5 pg/mL with an I_{50} of 29 pg/mL. Its ability to detect these important naphthalene metabolites in human urine is demonstrated.

Introduction

Environmental and biological monitoring requires the development of precise, specific, and sensitive techniques for the rapid detection and identification of the contaminant and/or toxicant. Analytical methods based on immunochemistry are widely used in biochemistry, endocrinology, medical chemistry,[1] and in the last years their use has also been extended to environmental and toxicological areas.[2] Measurement of urinary mercapturic acids (MA) as biomarkers of exposure to potentially reactive electrophiles allows assessment in a noninvasive manner. Gas chromatography (GC), high performance liquid chromatography (HPLC), and mass spectrometry (MS) are often employed for the detection of these compounds.[3] However interference from biological matrices, requiring prior cleanup and in some cases lack of specificity and/or sensitivity make these techniques intricate and time consuming. The initial step in the development of an immunoassay is production of specific antibodies, but since small molecules seldom stimulate an immune response, it is necessary to design appropriate chemical structures which can be covalently coupled to a carrier protein and presented to the animal's immune system. While there is insufficient information in the literature on hapten design, it seems that antibody specificity is generally highest for the part of the molecule furthest from the carrier.[4]

Naphthalene is a common environmental contaminant of water, soil, and air. Its derivatives constitute the major portion[5] of the polycyclic aromatic hydrocarbon (PAH) fraction in ambient air, and one of the primary mutagenic components of the ambient particulate fraction.[6] Main sources of contamination are petroleum refinery streams, industrial waste during the production of important organic compounds like carbaryl, phthalic anhydride, and surfactants, and combustion processes such as automobile exhaust gases, coal combustion procedures, and cigarette smoke.[7] In this context, cigarette smoking, of all environmental factors, is believed to contribute most to the development of lung diseases. Experimentally, naphthalene selectively produces necrosis of the Clara cells in the

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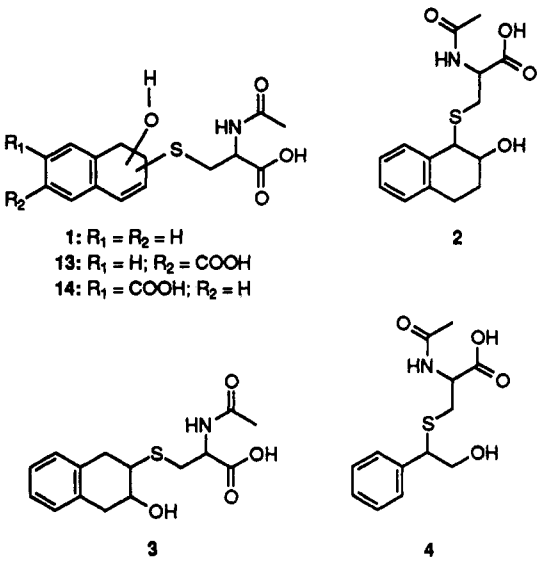
Scheme I



bronchiolar epithelium of mice.[8] However variations in tissue selectivity have been reported according to both dose and species.[9] Other toxicological information can be found in recent reviews.[10] Epoxidation of carbon double bonds by cytochrome P-450 constitutes the activation step for naphthalene.[11] Thus the electrophile, naphthalene-1,2-epoxide, is a reactive intermediate responsible for the alkylation of biomacromolecules that finally induces bronchiolar necrosis. It is well known that conjugation with the tripeptide glutathione (GSH, γ-Glu-Cys-Gly) catalyzed by glutathione S-transferase is an effective detoxification pathway.[12] Subsequently, enzymatic steps lead to the formation of mercapturic acid conjugates (S-substituted *N*-acetyl-L-cysteine derivatives) which are the major urinary metabolites following exposure to naphthalene.

Previously we obtained antibodies for the mercapturic acid conjugate of naphthalene (NaphMA 1, *N*-acetyl-*S*-(1,2-dihydro-1-hydroxy-2-naphthyl)cysteine, Scheme I) using the carboxylic acid of the mercapturate moiety for covalent attachment to a carrier protein.[13] In this paper we reaffirm that appropriate synthetic strategy and hapten design can dictate the specificity and sensitivity of immunoassays. Haptens designed for conjugation to a carrier protein at a site distal to the *N*-acetylcysteine (NAcCys) residue have been synthesized for use as immunogens (see Figure 1, haptens 13 and 14). With the antisera obtained, an ELISA (enzyme-linked immunosorbent assay) has been developed which exhibited a high degree of recognition for the mercapturic acid conjugate of naphthalene (NaphMA 1). Studies on matrix effects of human urine during the performance of this and the previously reported assay are described.

Results and Discussion

Preparation of the Haptens. Several authors have described the synthesis of the mercapturic acid conjugates of naphthalene (NaphMA, 1) and other PAHs.[14] Nucleophilic attack of the thiolate of *N*-acetylcysteine on to the corresponding epoxide is a commonly used strategy. A general method for the preparation of non-K region arene epoxides was reported by Yagi and Jerina.[15] Adopting these procedures, we have already reported the synthesis of our target molecule NaphMA 1 using 1,2-dihydronaphthalene as starting material.[13] In the present work, the concept was to apply the same strategy to syntheses of naphthalene mercapturate 2-carboxylic acid derivatives **13** and **14** (Scheme I). The corresponding 5,6- and 7,8-dihydronaphthalene-2-carboxylic acid are not commercially available; however, several methods have been published for the preparation of the 5,6,7,8-tetrahydro-2-carboxylic acid **6**.[16] Subsequent introduction of a double bond can be accomplished by acetoxylation of one of the benzylic positions followed by elimination.[15-17] Therefore compound **6** was prepared as previously described[16a] by Michael addition of the ketone enolate of cyclohexanone to dimethyl methoxymethylene)malonate to give 3-carbomethoxy-2-pyrone **5**. Subsequent Diels–Alder reaction with *N*-vinyl-2-pyrrolidinone, an electron-rich olefin (Scheme II), with concomitant loss of carbon dioxide gave **6**. The dihydronaphthalene carboxylic acid derivatives required for the synthesis of **13** and **14** were obtained by free radical acetoxylation of **6** with lead tetraacetate (Scheme III). This reaction, which occurred in low yield, led to a complex mixture of compounds from which the positional isomers **7a** and **7b** (~1:1) were separated after repeated chromatography. Elimination of acetic acid and subsequent hydrolysis of the methyl ester gave the necessary dihydroaromatic hydrocarbons **8c** and **8d**. Dibromo esters **10c** and **10d** were then prepared by treatment with *N*-bromoacetamide and bromination of the unsubstituted benzylic position with *N*-bromosuccinimide (NBS). Since the half life of naphthalene oxide has been estimated to be only 165 s in PBS (phosphate buffered saline solution, 0.1 M, pH 7.4) at 37 °C,[18] and is even shorter at acidic pH, no isolation of the corresponding epoxides **11** and **12** was attempted. Hence, the dibromo esters were used for the preparation of the corresponding mercapturic acid conjugates **13** and **14**, in a two step reaction monitored by HPLC. The appearance of an HPLC peak at 13.09 min (Spheri-5 RP-18 250 × 4.6 mm, mobile phase phosphoric acid–triethylamine buffer 0.05 M, pH 3.1/methanol 6/4) indicated the formation of the epoxide **11**. Subsequently, the reaction mixture was added

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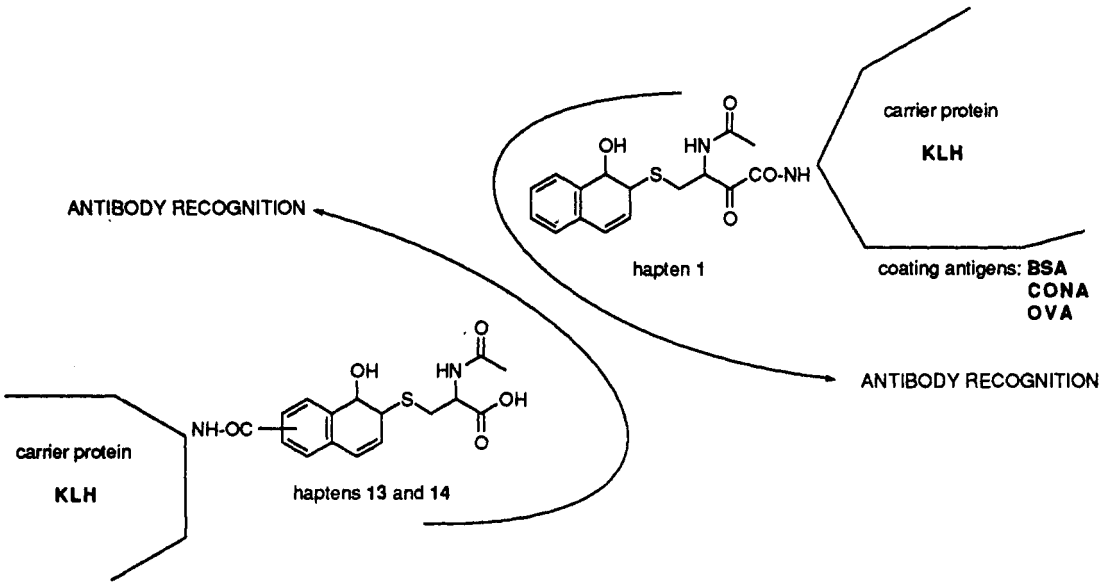
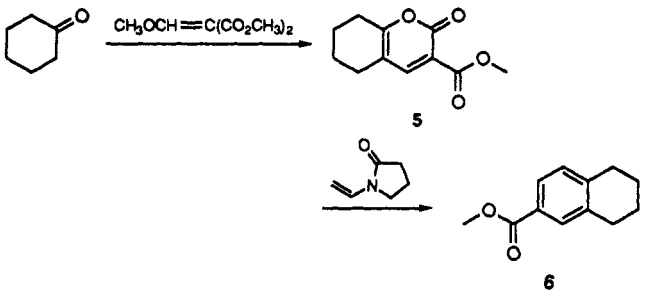
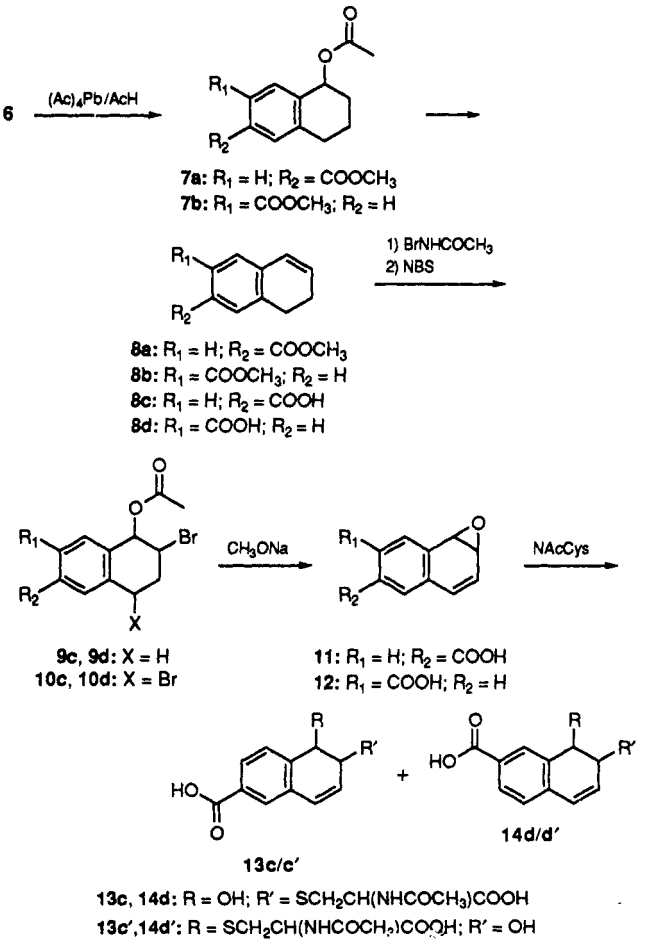


Figure 1. Antibody recognition is higher for the part of the molecule distal to the protein. The antibodies raised against 1KLH recognized NaphMA 1 at concentrations of a few ng/mL when 1BSA was used as coating antigen (1KLH/1BSA system).[18] Haptens 13 and 14 were designed to improve the antibody recognition of the mercapturate moiety of the NaphMA. Consequently recognition of other mercapturates is possible (see Table III for cross reactivity data). These antibodies detect the target molecule in the picogram range when used in a heterologous ELISA (13KLH/1CONA). In this case, more efficient competition is produced since the NAcCys residue is free in the analyte, but partially shielded by the protein on the coating antigen.

Scheme II



Scheme III



to the freshly prepared thiolate of NAcCys. HPLC indicated the disappearance of the epoxide and the appearance of a new peak at 4.57 min attributed to the mercapturic acid derivatives **13c/c'**. The same strategy was followed for the preparation of mercapturic acid **14d/d'**. Although epoxide opening could occur at both carbons of the oxirane ring of **11** and **12**, NMR data of the crude reaction mixture suggested selective nucleophilic attack at C-2 (**13c/14d**) rather than C-1 (**13c'/14d'**). Benzylic protons appear at δ 4.95 (MA **13**) and 4.97 (MA **14**) while the homobenzylic protons appear at δ 3.90 (MA **13**) and 3.91 (MA **14**) indicating that C-1 supports the hydroxyl group. This assignment is supported by 2D-NMR data for NaphMA 1.[13] Similar observations have been reported for the reaction of several other nucleophiles with 1,2-naphthalene oxide.[19] Purification of the immunogens **13** and **14** was accomplished by preparative C_{18} chromatography column; however, dehydration leading to the complete aromatic compound can occur during this process.[13] Consequently, even though a spacer between the target molecule and the protein would have been desirable since it decreases the steric shielding effect caused by the protein, MA **13** and MA **14** were immediately used for protein conjugation after spectroscopic identification.

Hapten Conjugation to Carrier Proteins and Antisera Evaluation. Covalent attachment of these haptens to KLH (keyhole limpet hemocyanin) was accomplished by preparation of an acid anhydride and subsequent reaction with the amino groups of the protein. In our previous work, we found that the mixed anhydride method yielded better coating antigens and immunogens than the

Table I. Titer of the Antisera Raised to Two Different Immunogens Using a Checker Board Titration with 12 Different Coating Antigens[a]

immunogen	antisera	1BSA	1CONA	1OVA	2BSA	2CONA	2OVA	3BSA	3CONA	3OVA	4BSA	4CONA	4OVA
13KLH	2642	L	H	*	L	L	*	L	L	*	L	L	*
	2698	*	*	*	*	*	*	*	*	*	*	*	*
	2671	L	H	*	*	*	*	*	*	*	*	*	*
14KLH	2673	*	L	*	*	*	*	*	*	*	*	*	*
	2677	L	L	*	L	L	*	*	L	*	*	L	*
	2676	L	H	*	L	L	*	*	L	*	*	L	*

[a] H, L, and * indicate the serum dilution factor range which produces absorbances of 0.5 after 1 h. H, more than 1/4000; L, between 1/1000 and 1/4000; * less than 1/1000.

N-hydroxysuccinimide ester method.[13] Mixed anhydride formation occurs by nucleophilic attack of the carboxylate, generated with tributylamine, on isobutyl chloroformate. Although two carboxylic acids are available for protein conjugation, selectivity can be anticipated considering the more-hindered position of the aliphatic carboxy group and differences in pK_a. Conjugated carboxylic acids have a higher pK_a (i.e. benzoic acid, 4.19; 2-naphthoic acid, 4.17) compared with those of amino acids (i.e. *N*-acetylglycine, 3.66; glutathione, 3.59; hippuric acid, 3.80). Since stronger bases (stronger nucleophiles) derive from weaker conjugated acids, amide formation through the conjugated carboxylic group was expected. Therefore, 2 equiv of base were used in order to convert both carboxylic groups to the corresponding conjugated bases, whereas isobutyl chloroformate and hapten were reacted at equimolecular amounts. If these expected differences in reactivity would not be manifested, a mixture of antibodies with different degrees of recognition for each half of the NaphMA molecule would be obtained (see Figure 1). After immunoassay development the selectivity of coupling was demonstrated by the cross-reactivity data (see below). Immunizations were carried out with 13KLH (rabbits 2642, 2698, and 2671) and 14KLH (rabbits 2673, 2677 and 2676) and the sera of each of the animals were tested 10 days after each boosting by checkerboard titration against the coating antigens [haptens 1–4 coupled to BSA (bovine serum albumin), CONA (conalbumin), and OVA (ovalbumin), see Figure 1]. Boostings and bleeding were continued until no increase in titer was observed. Table I shows, in a relative scale (H, high; L, low; or *, very low), the final titer obtained for each rabbit antiserum against each coating antigen. As expected, protein conjugates of hapten 1 were the coating antigens best recognized by the antibodies whereas low or very low titers were obtained with the rest of the protein–hapten conjugates. From all the proteins used as coating antigens, CONA conjugates gave the highest titers. In contrast, very low absorbances were measured during antibody titrations using the corresponding OVA conjugates. Similar observations have been reported in other assays.[20] No significant differences in antibody titer were observed with regard to the immunogen employed (13KLH and 14KLH), and the diversity of response was more likely due to animal variability. Of all rabbits, only 2698 had very low antibody titers demonstrated by the absorbance measured for each coating antigen.

Competition Experiments. Those coating antigen/antibody combinations showing reasonable titers (H and L) were screened for inhibition by NaphMA 1 in competition experiments. Valuable assays were obtained only when 1BSA and 1CONA were used as coating antigens. Table II shows the characteristics of those assays according to their maximal absorbance (A) versus noise (D), slope (B), I_{50} (C) and regression coefficient (r).[21] The accuracy and reproducibility of immunoassays are strongly dependent on the slope and shape of the sigmoid curves obtained from the inhibition experiments. High slope values indicating significant antibody affinity and good sensitivity are common properties of these assays, especially those combinations where antisera 2671 was employed (B = 0.9 and 0.57; I_{50} = 27 and 54 pg/mL, 1CONA and 1BSA respectively). Additionally, antisera 2671 gave high signal versus noise (A/D = 27.6) when used with 1CONA. In contrast, an I_{50} of 4–6 ng/mL and an A/D ratio around 10 was obtained as the best case when 1KLH was used as the immunogen.[13] Therefore the combination of antibody 2671 with coating antigen 1CONA was chosen for assay optimization giving a usable ELISA for the determination of NaphMA 1 which is operative in the range between 100 and 5 pg/mL with an I_{50} of 29.1 ± 3.2 pg/mL (assay 13KLH/1CONA, immunogen/coating antigen). Figure 2 shows the calibration standard curve in comparison to that previously reported by us when system 1KLH/1BSA (immunogen/coating antigen)[22] was used. This large increase in the sensitivity of the assay is likely due to improved hapten design. Since the *N*-acetylcysteine residue of the molecule offers more immunogenic recognition points than the polyaromatic ring, a higher recog-

Table II. Characteristics of the Assays Obtained Using Different Antiserum/Coating Antigen Combinations[a]

coating antigen	antisera	A/D	slope	$I(50)$ ng/mL	r
1CONA	2642	3.3	1.17	28.8	0.97
	2671	27.6	0.9	0.027	0.99
	2673	3.4	0.72	3.5	0.98
	2676	8.6	0.57	8.6	0.99
1BSA	2671	4.6	1.19	0.054	0.98
	2676	4.2	0.6	16.5	0.96

[a] Only those combinations giving reasonable assays are shown above. Maximal absorbance (A), slope (B), $I(50)$ (C) and minimal absorbance (D) are values from the four parameter equation calculated for each assay: $y = (A - D)/[1 + (x/C)^B] + D$. Three replicates were used for every concentration of NaphMA 1 in the standard curves.

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(21) Rodbard, D. In *Ligand Assay: Analysis of International Developments on Isotopic and Nonisotopic Immunoassay*; Langan, J., Clapp, J. J., Eds.; Masson Publishing: New York, 1981; pp 45. The relative binding is determined by plotting the percent inhibition vs the log of the concentration. The resulting sigmoidal curve is defined by a four parameter equation: $y = (A - D)/[1+(x/C)^B] + D$ where A is the upper asymptote, D is the lower asymptote, C (or I_{50}) indicates the concentration of NaphMA which inhibits 50% of the binding of the antibodies to the coating antigen and B is the slope.

(22) We will refer to the immunoassay described in this paper as assay 13KLH/1CONA and that previously described as assay 1KLH/1BSA, making reference to the combination of immunogen and coating antigen used for optimization of the respective ELISAs.

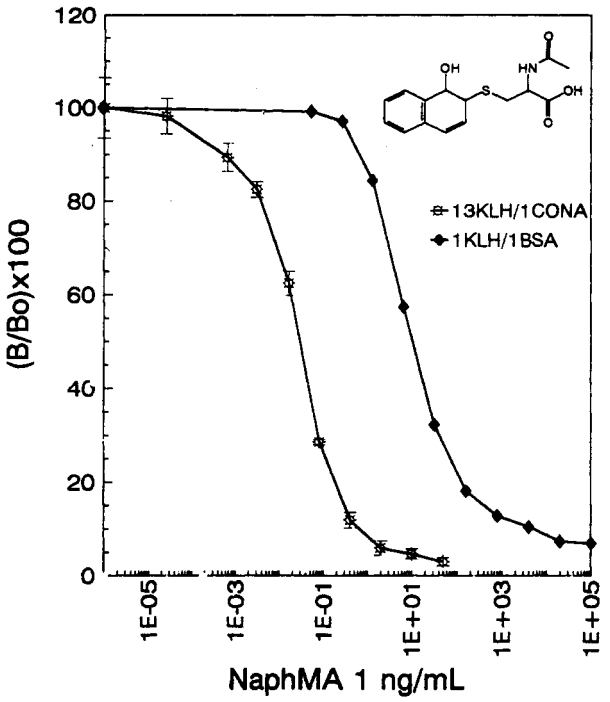


Figure 2. Standard curves obtained for the detection of NaphMA 1 using homologous (1KLH/1BSA) and heterologous (13KLH/1CONA) systems. The heterologous system developed by exposing the NAcCys residue for immune recognition resulted in an increase in sensitivity of >200 times over the homologous assay. The I_{50} is 29 pg/mL and the detection limit 5 pg/mL, whereas antibodies raised to 1KLH gave an assay with an I_{50} of 6 ng/mL and a 0.3 ng/mL detection limit.[13] The values presented for each point correspond to the average of experiments performed in two plates where three points were employed for every concentration. The coefficients of variation averaged 2.89 ± 1.02. B = Average absorbance measured for every concentration of the NaphMA standard curve; B_0 = average absorbance measured with a zero concentration of NaphMA.

nition for NaphMA 1 is expected when attachment to the protein is made through the aromatic ring instead of through the amino acid moiety of the mercapturate (see Figure 1). As other authors have reported,[23] heterology[24] between coating antigen and immunogen could be a second reason for the improvement in quality of the assay since the analyte is a more effective competitor for the antibody binding site than is the coating antigen. Here, we report an assay which is based upon hapten and coupling position heterology.

For the immunoassay previously reported,[13] the antibodies showed no recognition for the fully aromatized compound Naph MA obtained after dehydration under acidic conditions. Those data and the high recognition of antibodies, herein described, for the analyte NaphMA 1 could suggest that the dehydration process does not occur to a significant extent after protein conjugation or during the immunization protocol.

Cross-Reactivity Studies. The specificity of this immunoassay was in large part determined by hapten design. Therefore several mercapturic acids (**1–4**, **13–17**, and **30**), naphthalene derivatives (**18–28**), and *S*-methyl (**29**) and glutathione (**31**) conjugates of paracetamol were chosen to be tested in the cross reactivity experiments. Table III, shows the structures and cross reactivity data of the compounds tested. None of the naphthalene derivatives **18–28** tested were recognized by the antibodies at concentrations less than 500 ng/mL (5×10^3 times higher than the highest measurable NaphMA **1** concentration with this assay). The fact that none of the naphthalene derivatives possessing carboxyl groups cross-react competitively indicates that protein coupling occurred selectively through the conjugated acid (see Figure 1). Cross-reactivity also was not observed for the paracetamol metabolites **29–31**. Only those compounds possessing the NAcCys residue could compete for the coating antigen in the ELISA, and then only if some other similarities to the parent compound **13** were also present. In contrast to the NaphMA immunoassay (1KLH/1BSA) previously reported[13] which recognized only naphthalene metabolites, mercapturates in general are recognized somewhat by this assay as expected from the hapten design (see Figure 1). In support of this statement, I_{50} values obtained for these compounds in this ELISA, as well as the cross-reactivity data obtained after measuring them in both systems, are reported in Table III.

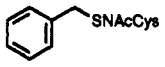
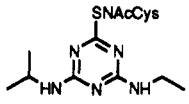
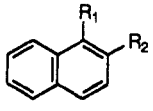
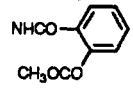
Studies of Matrix Effects. Both the immunoassays developed from the coupling of the mercapturate[13] and the coupling of the conjugated acid were subject to a comparative study to evaluate their utility as a tool for the measurement of NaphMA 1 in urine samples. Human urine was collected and used at different concentrations to prepare standard curves of NaphMA 1. To avoid variability in buffer concentrations (PBST), urine was diluted with water instead of buffer, and 2 × PBST was employed for diluting the antisera to yield identical salt concentration in all cases. This procedure did not affect the characteristics of the immunoassay (see Figure 3C). The sigmoid curves obtained for each urine dilution after their measurement with both ELISA systems are also shown in Figure 3 (A, assay 13KLH/1CONA and B, assay 1KLH/1BSA) compared with those prepared in water. In both assays a decrease of the absorbance and a slight improvement of the sensitivity are the main effects observed at increasing urine concentrations, but a parallelism is maintained between the different curves. Components of human urine gave a greater interference with the more-sensitive assay (13KLH/1CONA), and the urine had to be diluted at least 100× to allow color development. In contrast, our first assay (1KLH/1BSA) yielded a good assay after a 1:1 dilution of the urine.

In an attempt to determine the nature of factors responsible for interference of urine on the assay (13KLH/1CONA), a fast cleanup procedure was applied to another fraction of the same urine. By filtration through a Sep-Pak cartridge at pH 7 the more nonpolar components are retained on the solid phase, whereas salts and other more polar compounds present in urine (urea, uric acid, NaCl, phosphates, sulfates, etc.) elute with aqueous fractions. However the collected colorless solution gave the same interference with the assay as the original urine sample. As a result of this experiment we could conclude that dilution factors of 1/1000 (Figure 3A) and 1/100 (Figure 3B) must be applied to the urine in order to have accurate measurements of NaphMA if no other purification method is utilized. This fact implied a decrease in the sensitivity of the analytical method proposed in this paper. Nevertheless since the slope is not significantly affected by

(23) Harrison, R. O.; Goodrow, M. H.; Hammock, B. D. *J. Agric. Food Chem.* **1991**, *39*, 122.

(24) An heterologous ELISA system uses as a coating antigen a different hapten or a different coupling position or procedure from that employed in the immunization protocols to generate antibodies.

Table III. Cross-Reactivities for Several Mercapturic Acid Conjugates and Naphthalene Derivatives[a]

compd	R	R^1	R^2	13KLH/1CONA $I(50)$ pmol/mL	13KLH/1CONA cross-reactivity (%)	1KLH/1BSA: cross-reactivity (%)
1				0.09 ± 0.04	100	100
2				1.15	8	1.1
3				0.21	42.5	5.5
4				2.4	3	nc
13				0.12	95	nc
14				0.24	49	0.2
NAcCys 15				nd	nc	nc
 16				38.1	23	nc
 17				nd	nc	nt
 18–28						
18		H	H	nd	nc	nc
19		OH	H	nd	nc	nc
20		H	OH	nd	nc	nc
21		OH	OH	nd	nc	nc
22		COOH	H	nd	nc	nt
23		H	COOH	nd	nc	nt
24		OCH_2COOH	H	nd	nc	nt
25		H	OCH_2COOH	nd	nc	nt
26		CH_2COOH	H	nd	nc	nt
27		$OCONHCH_3$	H	nd	nc	nc
28			H	nd	nc	nc
 29–31						
29	CH_3			nd	nc	nt
30	NAcCys			nd	nc	nt
31	glutathione			nd	nc	nt

[a] Standard curves for every compound were prepared in PBST and measured in the ELISA using triplicate wells. The cross-reactivity values were calculated according to the equation: [$I(50)$ NaphMA 1/$I(50)$ compound] × 100. In the table there are also shown the cross-reactivity values, obtained for some of the compounds, on the ELISA system 1KLH/1BSA[13] previously developed. The $I(50)$ is reported for those compounds that inhibited the bonding of the antibodies to the plate in the ELISA system 13KLH/1CONA. nd, not detected and nc, no cross-reactivity at the assayed concentrations, nt, not tested.

the presence of urine (Figure 3, parts A and B) an alternative approach which would solve this problem would be to run the standard curve in urine or in the presence of other factors that mimic the behavior of the matrix. The high-salt concentration present in urine led us to perform the inhibition experiments under different phosphate buffer concentrations. As a result of these experiments we found that a slight increase of the buffer concentration produced a decrease in absorbance and a slight improvement in sensitivity as compared to urine. For example, 0.3 M PBS in the immunoassay 1KLH/1BSA, leads to an assay with similar characteristics to the one performed with urine diluted 1/2 (see Figure 3C). Consequently, accurate analyses of the mercapturic acid metabolites of naphthalene without prior purification of the samples could be performed with both mentioned assays by just applying a moderate dilution of the matrix.

Conclusions

Measurement of mercapturic acids provides an excellent method to assess exposure to electrophilic xenobiotics in general and to naphthalene in particular. The ELISA presented constitutes a simple and useful tool for better understanding pharmacokinetic processes, biomonitoring and toxicological studies. This paper confirms the great influence hapten design has in determining the specificity and sensitivity of an immunoassay. By appropriate hapten design, an exceptionally sensitive ELISA, which can measure the mercapturic acid metabolites of naphthalene at the pmol level, has been developed. The synthesis of a derivative possessing a carboxylic group distal to the most characteristic part of the molecule determines the recognition and directs the specificity of the assay. Thus the immunoassay we have developed detects other mercapturic acid conjugates at concentrations of pmol/mL

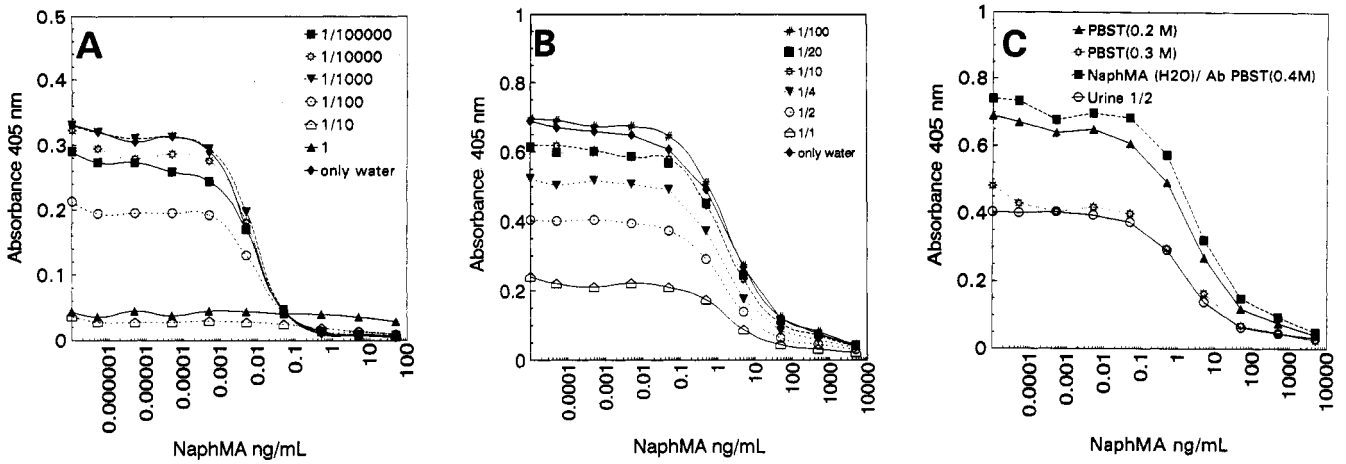


Figure 3. Evaluation of parallelism and sensitivity of standard curves for NaphMA performed in the presence of urine at different concentrations: A and B, assays 13KLH/1CONA and 1KLH/1BSA, respectively. A greater interference of the components of the urine is observed with the most-sensitive assay; however, the parallelism is maintained since no significant variations on the slope are observed. C: An increase in PBS concentration to 0.3 M produces a similar effect to urine diluted 1/2 for assay with 1KLH/1BSA. Consequently urine could be analyzed without prior purification by just applying a moderate dilution factor to the matrix and increasing the salt concentration in the buffer. No significant difference is observed when the standards were diluted in water and the antibody in 2 × PBST.

and nmol/mL. These results are supported by data from the previous assay we developed in which attachment to the carrier protein was accomplished through the NAcCys residue. Additionally, synthesis of coating antigens which present only a partial view of the target molecule to the antibody allow one to tailor the assay to yield the desired sensitivity and specificity. Both the homologous and heterologous assays will be used to follow excretion of the NaphMA in mice treated with subtoxic doses of naphthalene and naphthalene oxide.

Experimental Section

Thin-layer chromatography (TLC) was performed on 0.25-mm, precoated silica gel 60 F254 aluminum sheets. ^1H and ^{13}C NMR spectra were recorded at 300 and 75 MHz, respectively. Infrared spectra were measured on an IR/32 FTIR spectrophotometer. Mass spectra were obtained using electron impact (EI) ionization and fast atom bombardment (FAB) and data are reported as *m/z* (rel inten). Calculated exact mass is reported as M − H or M + H, according to the mode (negative or positive) in which the spectrum was recorded. HPLC analyses were done using a solvent delivery system equipped with a variable wavelength UV-vis detector set at 260 nm. The chromatography column was Spheri-5 RP-18 (250 × 4.6 mm) and the mobile phase is described below for every case. Immunoassays were performed on 96-well polystyrene microtiter plates with high adsorption for proteins. Absorbances were read with a microplate reader in dual wavelength mode (405–560). The inhibition curves were analyzed using a four-parameter logistic equation. Data presented correspond to the average of at least three well replicates. Mercapturic acids 1–4 were synthesized as we previously reported.[13] Mercapturic acid derivatives 16 and 17 were obtained from Dr. A. D. Jones (Facility for Advanced Instrumentation, UC Davis, CA) and Dr. A. Lucas (Entomology Department, UC Davis, CA), respectively. Finally paracetamol metabolites 29–31 were obtained from Eric Dietze (Department of Medicinal Chemistry, University of Washington, Seattle, WA).

Methyl 5-Acetyl-5,6,7,8-tetrahydronaphthalene-2-carboxylate (7a) and Methyl 8-Acetyl-5,6,7,8-tetrahydronaphthalene-2-carboxylate (7b). Tetralone 6[16] (960 mg, 5.0 mmol) in acetic acid (30 mL) was added to a solution of lead tetraacetate (85%,[25] 2.4 g, 5.5 mmol) in the same solvent (20 mL). The resulting clear solution was kept for 12 h at 70 °C (internal temperature) until no changes by TLC (hexane/ether 7/3) were observed. After addition of ethylene glycol (4.4 mL) to consume excess oxidant and benzene (60 mL), the mixture was washed with water (2 × 50 mL), 10% ammonium hydroxide (50 mL), and water (50 mL). The organic layer was dried with K_2CO_3 and filtered, and the solvent was evaporated to give 1.1 g of an oil containing a mixture of starting material, **7a**, **7b**, and other more polar unidentified compounds. Column chromatography over silica gel using a mixture of hexane/ether 8/2 gave 225 mg of **7a** and 231 mg of **7b** (total yield 36%). **7a** (high R_f): ^1H NMR ($CDCl_3$) δ 1.85–2.09 (m, 4H, H-6 and H-7), 2.10 (s, 3H, CH_3COO), 2.77 (ddd, *J* = 17.3, 7.1, 7.1 Hz, 1H, H-8), 2.90 (ddd, *J* = 17.3, 7.1, 7.1 Hz, 1H, H-8′), 3.90 (s, 3H, CH_3OCO), 6.00 (t, *J* = 4.4 Hz, 1H, H-5), 7.33 (d, *J* = 8.5 Hz, 1H, H-4), 7.81 (s, 1H, H-1), 7.82 (d, *J* = 8.5 Hz, 1H, H-3); ^{13}C NMR ($CDCl_3$–CD_3OD) δ 18.57, 21.01, 28.61, 69.18, 69.29, 126.75, 128.77, 129.34, 130.05, 137.79, 139.30, 166.52, 170.24; IR ($CHCl_3$) 2871, 1718, 1577, 1616, 1285, 1174 cm^{-1}; EIMS (*m/z*) 248, 188, 157, 147, 129; HRMS (*m/z*) calcd for M − H $C_{14}H_{15}O_4$ 247.0970, obsd 247.0980. **7b** (low R_f): ^1H NMR ($CDCl_3$) δ 1.75–2.04 (m, 4H, H-6 and H-7), 2.10 (s, 3H, CH_3COO), 2.76 (ddd, *J* = 18.0, 7.1, 7.1 Hz, 1H, H-5) and 2.89 (ddd, *J* = 18.0, 7.1, 7.1 Hz, 1H, H-5′), 3.90 (s, 3H, CH_3OCO), 5.99 (t, *J* = 4.3 Hz, 1H, H-8), 7.19 (d, *J* = 8.0 Hz, 1H, H-4), 7.87 (dd, *J* = 8.0, 1.6 Hz, 1H, H-3), 7.93 (d, *J* = 1.6 Hz, 1H, H-1); ^{13}C NMR ($CDCl_3$–CD_3OD) δ 18.47, 21.31, 28.80, 29.15, 69.46, 69.57, 128.07, 128.90, 129.37, 130.76, 134.79, 143.35, 166.74, 170.46; IR ($CHCl_3$) 2868, 1718, 1616, 1577, 1245, 1198 cm^{-1}; FABMS (*m/z*) 248, 234, 174. Anal. Calcd for $C_{14}H_{16}O_4$: C, 67.73; H, 6.50. Found: C, 67.90; H, 6.48.

7,8-Dihydronaphthalene-2-carboxylic Acid (8c). A mixture of **7a** (208 mg, 0.83 mmol), acetic acid (21 mL), and concentrated HCl (5 drops) was heated at 110 °C, under N_2 atmosphere in the dark for 2 h, until complete disappearance of the starting material as monitored by TLC (hexane/ether 8/2). The mixture was diluted with water (50 mL), extracted with ether (2 × 30 mL), and washed extensively with 5% $NaHCO_3$ (3 × 50 mL). The organic fractions were dried over $MgSO_4$ and filtered, and the solvent was evaporated to obtain methyl 7,8-dihydronaphthalene-2-carboxylate (**8a**) as a red oil (153 mg, 98%): ^1H NMR ($CDCl_3$) δ 2.31 (td, *J* = 8.2, 2.9 Hz, 2H, H-7), 2.79 (t, *J* = 8.2, 2H, H-8), 3.86 (s, 3H, CH_3OCO), 6.13 (dt, *J* = 9.0, 2.9 Hz, 1H, H-6), 6.45 (d, *J* = 9.1 Hz, 1H, H-5), 7.01 (d, *J* = 9.1 Hz, 1H, H-4), 7.74 (s, 1H, H-1), 7.84 (d, *J* = 9.1 Hz, 1H, H-3); ^{13}C NMR ($CDCl_3$–CD_3OD) δ 22.87, 26.89, 125.62, 126.89, 127.15, 127.98, 128.77, 131.46, 135.04, 138.24, 166.85; IR ($CHCl_3$) 2930, 1717, 1439, 1289 cm^{-1}; EIMS (*m/z*) 188, 157, 129. Subsequently, methyl ester **8a** was dissolved in methanol (5 mL) and 1 N NaOH

(25) Freshly crystallized from acetic acid and titrated according to the procedure described in Fieser, L. F.; Fieser, M. In *Reagents for Organic Synthesis*; John Wiley & Sons: New York, 1967; Vol. 1, p 537.

(1 mL, 1.2 meq) was added. The mixture was refluxed for 2 h until the complete disappearance of starting material (TLC analysis, hexane/ether 8/2, 1% acetic acid). The reaction mixture was quenched with 1 N HCl to pH 6 and the methanol evaporated. The aqueous residue was diluted with water and extracted with ethyl acetate, dried over $MgSO_4$, filtered, and concentrated to give compound **8c** as a clear oil (130 mg, 83%): ^{1}H NMR ($CDCl_3$) δ 2.37 (td, J = 8.2, 2.8 Hz, 2H, H-7), 2.86 (t, J = 8.2 Hz, 2H, H-8), 6.20 (dt, J = 9.6, 2.8 Hz, 1H, H-6), 6.51 (d, J = 0.6 Hz, 1H, H-5), 7.08 (d, J = 7.8 Hz, 1H, H-4), 7.83 (s, 1H, H-1), 7.89 (d, J = 7.8 Hz, 1H, H-3); ^{13}C NMR ($CDCl_3$–CD_3OD) δ 23.05, 27.03, 125.63, 127.30, 128.91, 129.18, 132.15, 132.39, 135.32, 139.27, 172.31; IR ($CHCl_3$) 3416, 2952, 1716, 1439, 1289, 1204 cm^{-1}; HRMS (m/z) calcd for M + H, $C_{11}H_{11}O_2$ 175.0759; obsd 175.0727.

5,6-Dihydronaphthalene-2-carboxylic Acid (8d). Following the procedure for the preparation of **8c**, compound **7b** (209 mg, 0.84 mmol) gave methyl 5,6-dihydronaphthalene-2-carboxylate **8b** (147 mg, 93%): ^{1}H NMR ($CDCl_3$) δ 2.34 (td, J = 8.2, 2.8 Hz, 2H, H-6), 2.84 (t, J = 8.2 Hz, 2H, H-5), 3.90 (s, 3H, CH_3OCO), 6.08 (dt, J = 9.6, 2.8 Hz, 1H, H-7), 6.49 (d, J = 9.6, 1H, H-8), 7.15 (d, J = 7.7 Hz, 1H, H-4), 7.68 (s, 1H, H-1), 7.79 (d, J = 7.7 Hz, 1H, H-3); IR ($CHCl_3$) 2929, 1716, 1439, 1289 cm^{-1}; EIMS (m/z) 188, 157, 147, 129. Hydrolysis of the methyl ester was carried out as described before to obtain **8d** as clear oil (143 mg, 98%); ^{1}H NMR ($CDCl_3$) δ 2.34 (td, J = 8.3, 2.8 Hz, 2H, H-6), 2.85 (t, J = 8.3 Hz, 2H, H-5), 6.10 (dt, J = 9.5, 2.8 Hz, 1H, H-7), 6.52 (d, J = 9.5 Hz, 1H, H-8), 7.22 (d, J = 7.8 Hz, 1H, H-4), 7.71 (s, 1H, H-1), 7.88 (d, J = 7.8, 1H, H-3); ^{13}C NMR ($CDCl_3$–CD_3OD) δ 23.11, 28.04, 127.39, 127.61, 127.98, 129.23, 129.92, 130.03, 134.65, 142.24, 172.70; IR ($CHCl_3$) 3420, 2950, 1715, 1438, 1290, 1205 cm^{-1}; EIMS (m/z) 175, 167, 149, 129, 115. Anal. Calcd for $C_{11}H_{10}O_2 \cdot {}^1/_{16}CH_3COOH$; C, 75.09; H, 5.81. Found: C, 75.30; H, 5.76.

5-Acetyl-6-bromo-5,6,7,8-tetrahydronaphthalene-2-carboxylic Acid (9c). Lithium acetate (281 mg, 2.7 mmol) and N-bromoacetamide (96.6 mg, 0.7 mmol) were added as a solid powder to a solution of **8c** (130 mg, 0.69 mmol) in glacial acetic acid (20 mL) under N_2 atmosphere. The mixture was stirred 3 h at room temperature in the dark, until the complete disappearance of the starting material by TLC analysis (hexane/ether 8/2, 1% acetic acid). The reaction was poured into water (70 mL), extracted with ethyl acetate (2 × 50 mL), washed extensively with water and saturated NaCl solution, dried over $MgSO_4$, filtered, and evaporated to dryness to give **9c** as a solid (203 mg, 83%) which was recrystallized from Cl_3CH–CH_3OH: mp 155.8–156.9 °C; ^{1}H NMR ($CDCl_3$) δ 2.13 (s, 1H, CH_3COO), 2.28 (m, 1H, H-7), 2.47 (m, 1H, H-7′), 2.94 (ddd, J = 17.5, 5.8, 5.8 Hz, 1H, H-8), 3.10 (ddd, J = 17.5, 7.3, 5.4 Hz, 1H, H-8′), 4.48 (m, 1H, H-6), 6.17 (d, J = 4.6 Hz, 1H, H-5), 7.32 (d, J = 8.5 Hz, 1H, H-4), 7.89 (s, 1H, H-1), 7.88 (d, J = 8.5 Hz, 1H, H-3); ^{13}C NMR ($CDCl_3$–CD_3OD) δ 20.94, 25.98, 27.67, 47.95, 72.72, 128.08, 129.40, 130.14, 130.80, 136.38, 137.33, 170.03, 171.73; IR ($CHCl_3$), 3620, 3019, 1735, 1700, 1217 cm^{-1}; FABMS (m/z) 312, 352, 174; HRMS (m/z) calcd for M − H, $C_{13}H_{12}BrO_4$ 310.9918; obsd 310.9985. Anal. Calcd for $C_{13}H_{13}BrO_4$: C, 49.86; H, 4.18, Br, 25.51. Found: C, 50.04; H, 4.18, Br, 25.09.

8-Acetyl-7-bromo-5,6,7,8-tetrahydronaphthalene-2-carboxylic Acid (9d). Following the procedure previously described, from the acid **8d** (143 mg, 0.82 mmol), **9d** was obtained as a white solid (270 mg, 85%) and purified by preparative TLC (hexane/ether 8/2, 1% acetic acid). ^{1}H NMR ($CDCl_3$) δ 2.00 (s, 3H, CH_3COO), 2.10 (m, 1H, H-6), 2.17 (m, 1H, H-6′), 2.84 (ddd, J = 17.8, 5.2, 5.2 Hz, 1H, H-5), 3.05 (ddd, J = 17.8, 7.6, 5.4 Hz, 1H, H-5′), 4.41 (m, 1H, H-7), 6.04 (d, J = 4.2 Hz, 1H, H-8), 7.16, d, H = 7.9 Hz, 1H, H-4), 7.87 (d, J = 7.9 Hz, 1H, H-3), 7.88 (s, 1H, H-1), 10.65 (brs, COOH); ^{13}C NMR ($CDCl_3$–CD_3OD) δ 20.86, 25.99, 26.68, 48.04, 72.39, 127.73, 129.04, 130.08, 131.68, 132.23, 143.44, 169.99, 172.01; IR ($CHCl_3$) 3671, 2934, 1738, 1694, 1289 cm^{-1}; HRMS (m/z) calcd for M − H, $C_{13}H_{12}BrO_4$ 310.9918; obsd 311.0000. Anal. Calcd for $C_{13}H_{13}BrO_4$: C, 49.86; H, 4.18; Br, 25.51. Found: C, 49.78; H, 4.21; Br, 25.80.

5-Acetyl-6,8-dibromo-5,6,7,8-tetrahydronaphthalene-2-carboxylic Acid (10c). A mixture of bromo ester **9c** (200 mg, 0.61 mmol), N-bromosuccinimide (NBS, 130 mg, 0.73 mmol), and α,α'-azobis(isobutyronitrile) (1 mg, catalytic amount) in CCl_4 (5 mL) was stirred under N_2 atmosphere at 90 °C (external temperature) for 2 h until the reaction was complete as indicated by TLC analysis. The mixture was filtered and the solvent evaporated to give **10c** which was purified by preparative TLC (hexane/ether 8/2, 1% acetic acid) (213 mg, 89%): ^{1}H NMR ($CDCl_3$) δ 2.18 (s, 3H, CH_3COO), 2.78 (m, 1H, H-7), 2.90 (m, 1H, H-7′), 4.74 (m, 1H, H-6), 5.55 (t, J = 4.4 Hz, 1H, H-8), 6.32 (d, J = 8.2 Hz, 1H, H-5), 7.26 (d, J = 8.1 Hz, 1H- H-4), 7.97 (ss, J = 8.1, 1.2 Hz, 1H, H-3), 8.18 (s, J = 1.2 Hz, 1H, H-1), 10.50 (brs, COOH); ^{13}C NMR ($CDCl_3$–CD_3OD) δ 20.29, 40.08, 45.33, 46.46, 73.21, 127.10, 127.43, 129.25, 131.06, 135.65, 136.18, 166.39, 169.70; IR ($CHCl_3$) 3020, 1742, 1699, 1427, 1214, 753; FABMS (m/z) 391, 311, 275, 229, 183; HRMS calcd for $C_{13}H_{11}Br_2O_4$ 388.9024; obsd 388.8855.

8-Acetyl-5,7-dibromo-5,6,7,8-tetrahydronaphthalene-2-carboxylic Acid (10d). Compound **10d** was obtained (258 mg, 98%) as a solid from the corresponding bromo ester **9d** (220 mg, 0.67 mmol) as described above for **10c**: mp 160.7–161.2 °C; ^{1}H NMR ($CDCl_3$) δ 2.24 (s, 3H, CH_3COO), 2.81 (m, 1H, H-6), 2.95 (m, 1H, H-6′), 4.69 (m, 1H, H-7), 5.51 (t, J = 4.8 Hz, 1H, H-5), 6.28 (d, J = 7.64 Hz, 1H, H-8), 7.56 (d, J = 8.2 Hz, 1H, H-4), 7.87 (s, 1H, H-1), 8.00 (s, J = 8.2 Hz, 1H, H-3); ^{13}C NMR ($CDCl_3$–CD_3OD) δ 20.73, 40.10, 45.80, 45.96, 73.96, 129.62, 129.98, 130.44, 132.44, 140.48, 167.51, 170.71; IR ($CHCl_3$), 3019, 1735, 1616, 1425, 1217, 757 cm^{-1}, FABMS 391, 311, 275, 229, 183; HRMS (m/z) calcd for M − H, $C_{13}H_{11}Br_2O_4$ 388.9024; obsd 388.8974.

(*R*)-*N*-Acetyl-*S*-[(5*R,6*R**)-2-carboxy-5,6,-dihydro-5-hydroxy-6-naphthyl]cysteine (13c) and (*R*)-*N*-acetyl-*S*-[(5*R**,6*R**)-(2-carboxy-5,6-dihydro-6-hydroxy-5-naphthyl]-cysteine (13c′).** The dibromo ester **10c** (150 mg, 0.4 mmol) in anhydrous THF (1 mL) was added to a suspension of $NaOCH_3$ (320 mg, 5.9 mmol) in the same solvent (1 mL). The mixture was stirred overnight at 4 °C at which time HPLC analysis[26] (Spheri-5 RP-18 250 × 4.6 mm, mobile phase phosphoric acid–triethylamine buffer 0.05 M, pH 3.1/methanol 6/4) revealed the appearance of a peak at 13.09 min indicating the formation of the corresponding 5,6-epoxide-5,6-dihydronaphthalene-2-carboxylic acid (**11**) (t_R compared to that of 1-naphthol, t_R for **10c** 42.84 min). Subsequently, the reaction mixture was added to a degassed, freshly prepared solution of N-acetylcysteine (141.7 mg, 0.8 mmol) in 1 N NaOH (2 mequiv, 1.6 mL) and stirred for 1 h at room temperature. HPLC analysis revealed the appearance of a new peak at 4.57 min. Finally, the reaction mixture was diluted with water (5 mL), the organic solvent was removed by evaporation, and the aqueous layer was acidified to pH 2. The resulting solution was placed on top of a C-18 flash chromatography column (30 g, particle size 55–105 μm), previously activated with acetonitrile (200 mL), water (200 mL), and phosphoric acid–triethylamine buffer (200 mL). Salts and excess of N-acetylcysteine were eliminated by washing the column with water (100 mL), and the desired compound then was eluted with acetonitrile (100 mL). HPLC analyses of the column fractions showed that compounds **13c/13c′** (56 mg, 40%) eluted between the last aqueous and the first organic fractions. **13c**, >90%, ~1:1 mixture of *R*(*R**,*R**) isomers based on NMR data. Spectroscopic data for **13c**: ^{1}H NMR δ (D_2O–CD_3OD) 2.03 (s, 3H, CH_3CONH), 2.81–3.12 (m, 2H, $-SCH_2CH-$), 3.90 (dd, J = 6.0, 1.2 Hz, 1H, H-6), 4.35 (dd, J = 7.0, 4.1 Hz, 1H, $-SCH_2CH-$), 4.95 (d, J = 6.0 Hz, 1H, H-5), 6.14 (dd, J = 8.6, 1.2 Hz, 1H, H-7), 6.80 (d, J = 8.6 Hz, 1H, H-8) 7.52 (dd, J = 8.2, 1.2 Hz, 1H, H-4), 7.85 (d, J = 1.1 Hz, 1H, H-1), 7.95 (dd, J = 8.2, 1.1 Hz, 1H, H-3); ^{13}C NMR ($CDCl_3$–CD_3OD) δ 22.37, 32.50, 46.25, 47.40, 52.29, 71.3, 126.9, 127.7, 127.9, 129.4, 130.4, 131.7, 139.0, 139.1, 168.5, 171.4, 172.3; FABMS (m/z) 351, 333, 323, 307; HRMS (m/z) calcd for M − H, $C_{16}H_{17}SNO_8$ 350.0698, obsd 350.0702.

(*R*)-*N*-Acetyl-*S*-[7*R,8*R**)-2-carboxy-7,8-dihydro-8-hydroxy-7-naphthyl]cysteine (14d) and (*R*)-*N*-Acetyl-*S*-[7*R**,8*R**)-2-carboxy-7,8-dihydro-7-hydroxy-8-naphthyl]cysteine (14d′).** According to the procedure described for **13c/c′**, from **10d** (100 mg, 0.2 mmol), compounds **14d/14d′** (**14d**, >90%,

(26) Sample aliquots were taken from the reaction mixture, diluted with 1% acetic acid aqueous solution to a final concentration of 0.1 μg/μL, and injected in the HPLC system (10 μL). Under these conditions nucleophilic attack over the epoxide by the hydroxide anion is produced followed by dehydration under acidic catalysis to produce presumably 5-hydroxynaphthalene-2-carboxylic acid.

~1:1 mixture of $R(R^*,R^*)$ isomers according to NMR data) were obtained (35 mg, 38%). Spectroscopic data for **14d**: ^{1}H NMR δ (D_2O–CD_3OD) 2.01 (s, 3H, CH_3CONH), 2.82–3.26 (m, 1H, $-SCH_2CH-$), 3.91 (dd, J = 5.8, 1.3 Hz, 1H, H-7), 4.32 (dd, J = 7.3, 4.2 Hz, 1H, $-SCH_2CH-$), 4.97 (d, J = 5.8 Hz, 1H- H-8), 6.37 (dd, J = 8.5, 1.3 Hz, 1H, H-6), 6.80 (d, J = 8.5 Hz, 1H, H-5), 7.37 (d, J = 8.1 Hz, 1H, H-4), 8.00 (d, J = 8.1 Hz, 1H, H-3), 8.01 (s, 1H, H-1); FABMS (*m/z*), 350, 332, 254, 203; HRMS calcd for M – H, $C_{16}H_{17}SNO_6$ 350.0698, obsd 350.0688.

Conjugation to Carrier Proteins. (A) Immunogens. Haptens **13** and **14** were covalently attached through their carboxylic acids to the lysine groups of KLH (keyhole limpet hemocyanin) using the mixed anhydride method. Briefly tri-*n*-butylamine (0.072 mmol) followed by isobutyl chloroformate (0.036 mmol) were added to the carboxylic acid hapten (0.036 mmol) dissolved in ice-bath-cooled anhydrous DMF (1 mL). The resulting mixture was stirred for 30 min and then added dropwise to a solution of the corresponding protein (36 mg) in 5 mL of borate buffer (0.2 M borate–boric, pH 8.7) and stirred for 6 h at room temperature. The conjugates were extensively dialyzed in PBS (0.01 M phosphate-buffered saline solution, pH 7.4), and finally against water, lyophilized, and stored at –80 °C. Stock solutions of 1 mg/mL were prepared with PBS buffer and stored in aliquots at –20 °C.

(B) Coating Antigens. Haptens **1–4** were covalently attached through the carboxylic acid of the mercapturate moiety to BSA (bovine serum albumin), CONA (conalbumin), and OVA (ovalbumin) as previously described.[13]

Immunization. Six New Zealand white rabbits weighing 2–4 kg were immunized with the same protocol previously described.[13] Rabbits 2642, 2698, and 2671 were immunized with **13**KLH and rabbits 2673, 2677, and 2676 were immunized with **14**KLH.

Analysis of the Antibody Titer. The titer of the antibodies in the serum of each animal was determined by measuring the binding of serial dilutions (1/1000 to 1/64000) in PBST (phosphate-buffered saline solution containing Tween; 0.2 M phosphate buffer, 0.8% NaCl and 0.05% Tween 20, pH 7.5) to microtiter plates coated with several concentrations (5–0.3 μg/mL) of 1BSA, 1CONA, 1OVA, 2BSA, 2CONA, 2OVA, 3BSA, 3CONA, 3OVA, 4BSA, 4CONA, and 4OVA in coating buffer (0.1 M carbonate-bicarbonate, pH 9.6). Optimal concentrations for coating antigen and antisera dilution were determined.

Competitive Enzyme Immunosorbent Assay. Microtiter plates were coated with 1CONA at 1 μg/mL (100 μL/well) in coating buffer overnight at 4 °C covered with adhesive plate sealers. The following day the plates were washed five times with PBST buffer (0.2 M, phosphate-buffered saline solution containing 0.05% Tween 20, pH 7.4). Serial dilutions of the analyte were prepared in PBST and added in a 50-μL volume to the coated plates followed by 50 μL of the antibody (Ab2671) previously diluted 1/4000 in the same buffer (final dilution in the well 1/8000). After 2 h of incubation at room temperature the plates were washed as described before and 100 μL of a PBST solution of a 1/5000 diluted goat antirabbit IgG-alkaline phosphatase was added and incubated for 2 h more. Finally, the plates were washed and 100 μL of a 1 mg/mL solution of 4-nitrophenyl phosphate in DEA buffer (10% diethanolamine, pH 9.8) was added. Absorbances were read after 60 min.

Cross-Reactivity Determinations. Stock solutions of 1 mg/mL of the compounds shown in Table III were prepared in DMF. Standard curves were prepared by serial dilutions in PBST (500 ng/mL to 2.5×10^{-5} ng/mL) and each I_{50} was determined in the competitive experiment. The cross-reactivity values were calculated according to the following equation: (I_{50} naphthalene–mercapturic acid conjugate/I_{50} compound) × 100.

Matrix Effects Studies. Studies directed to determine the applicability of the assay to measure NaphMA1 in urine was performed by studying the parallelism of standard curves prepared in buffer with different concentrations of the matrix.

(A) Immunogen 13 KLH/Coating Antigen 1CONA. Human urine diluted in water (1/1, 1/10, 1/100, 1/1000, 1/10000, and 1/100000) was used to prepare standard curves of NaphMA 1 (1000 to 1E-5 ng/mL). The ELISA was performed as described before by adding the standards to the precoated plates followed by the antibody (Ab2671) diluted in 2 × PBST (0.4 M phosphate-buffered saline solution, 0.05% Tween 20, pH 7.4). A simple cleanup was performed with another portion of the same urine. The sample (5 mL of urine, pH 7.0) was placed on top of a conditioned C_{18} Sep-Pak cartridge. The eluate was collected in a volumetric flask, and the column was rinsed with water to complete the original urine volume. The purified urine was used to examine matrix effects as described before.

(B) Immunogen 1KLH/Coating Antigen 1BSA. Six different dilutions of human urine in water (1/1, 1/2, 1/4, 1/10, 1/20, and 1/100) were used to prepare standard curves of NaphMA 1 (100 to 0.001 ng/mL). The ELISA was performed as described previously[13] by adding the standards to the precoated plates (1BSA, 1μg/mL) followed by the antisera (Ab2357), diluted 1/4000 in 2 × PBST.

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Supplementary Material Available: ^{1}H-NMR data for **7a, 8c, 10c, 10d, 13c/c′**, and **14d/d′** (6 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

Abbreviations: *, very low antibody titer; Ab, antibody; BSA, bovine serum albumin; CONA, conalbumin; DEA, diethanolamine; ELISA, enzyme-linked immunosorbent assay; GSH, glutathione; H, high antibody titer; I_{50}, concentration of analyte which inhibits 50% color development; KLH, keyhole limpet hemocyanin; L, low antibody titer; MA, mercapturic acid; NAcCys, *N*-acetylcysteine; NaphMA, mercapturic acid conjugate of naphthalene; OVA, ovalbumin; PBS, phosphate-buffered saline solution; PBST, phosphate-buffered saline solution containing Tween 20; PAH, polycyclic aromatic hydrocarbon.