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-[((R*),2R*)-1,2-dihydro-1-hydroxy-2-naphthyl]cysteine and (R)-N-acetyl-S-[(1R*,2R*)-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 IgG of 20 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, and in the last years their application to environmental and toxicological areas. Measurement of urinary mercapturic acids (MA) as biomarkers of exposure to potentially mutagenic components of the ambient particulate fraction. 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. 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


bronchiolar epithelium of mice. However variations in tissue selectivity have been reported according to both dose and species. Other toxicological information can be found in recent reviews. Epoxidation of carbon double bonds by cytochrome P-450 constitutes the activation step for naphthalene. 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 (GS~Glu-Cys-Gly) catalyzed by glutathione S-transferase is an effective detoxification pathway. 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. 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). 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. Nuclearophilic 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. Adopting these procedures, we have already reported the synthesis of our target molecule NaphMA 1 using 1,2-dihyronaphthalene as starting material. 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-dihyronaphthalene-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. Subsequent introduction of a double bond can be accomplished by acetylation of one of the benzylic positions followed by elimination. Therefore compound 6 was prepared as previously described by Michael addition of the ketone enolate of cyclohexanone to dimethyl methoxymethyl)malonate to give 3-carboxymethoxy-2-pyrene 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 dihyronaphthalene carboxylic acid derivatives required for the synthesis of 13 and 14 were obtained by free radical acetylation 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, 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–triyethylamine buffer 0.05 M, pH 3.1/methanol 6/4) indicated the formation of the epoxy 11. Subsequently, the reaction mixture was added


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). 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.

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'). Benzyllic 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. Similar observations have been reported for the reaction of several other nucleophiles with 1,2-naphthalene oxide. Purification of the immunogens 13 and 14 was accomplished by preparative C18 chromatography column; however, dehydration leading to the complete aromatic compound can occur during this process. 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

<table>
<thead>
<tr>
<th>immunogen</th>
<th>antisera</th>
<th>1BSA</th>
<th>1CONA</th>
<th>1OVA</th>
<th>2BSA</th>
<th>2CONA</th>
<th>2OVA</th>
<th>3BSA</th>
<th>3CONA</th>
<th>3OVA</th>
<th>4BSA</th>
<th>4CONA</th>
<th>4OVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>13KLH</td>
<td>2642</td>
<td>L</td>
<td>H</td>
<td>*</td>
<td>L</td>
<td>L</td>
<td>*</td>
<td>L</td>
<td>*</td>
<td>L</td>
<td>L</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2698</td>
<td>*</td>
<td>*</td>
<td></td>
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<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td></td>
<td>2671</td>
<td>L</td>
<td>H</td>
<td>L</td>
<td>L</td>
<td>L</td>
<td>*</td>
<td>L</td>
<td>*</td>
<td>L</td>
<td>L</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>14KLH</td>
<td>2673</td>
<td>*</td>
<td>L</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>2677</td>
<td>L</td>
<td>L</td>
<td>L</td>
<td>L</td>
<td>L</td>
<td>*</td>
<td>L</td>
<td>*</td>
<td>L</td>
<td>L</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2676</td>
<td>L</td>
<td>H</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

* 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.

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

<table>
<thead>
<tr>
<th>coating antigen</th>
<th>antisera</th>
<th>AD</th>
<th>slope</th>
<th>I(50) ng/mL</th>
<th>r</th>
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</thead>
<tbody>
<tr>
<td>1CONA</td>
<td>2642</td>
<td>3.3</td>
<td>1.17</td>
<td>28.8</td>
<td>0.97</td>
</tr>
<tr>
<td></td>
<td>2671</td>
<td>27.6</td>
<td>0.9</td>
<td>0.027</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td>2673</td>
<td>3.4</td>
<td>0.72</td>
<td>3.5</td>
<td>0.98</td>
</tr>
<tr>
<td></td>
<td>2676</td>
<td>8.6</td>
<td>0.57</td>
<td>8.6</td>
<td>0.99</td>
</tr>
<tr>
<td>1BSA</td>
<td>2671</td>
<td>4.6</td>
<td>1.19</td>
<td>0.054</td>
<td>0.98</td>
</tr>
<tr>
<td></td>
<td>2676</td>
<td>4.2</td>
<td>0.8</td>
<td>16.5</td>
<td>0.96</td>
</tr>
</tbody>
</table>

* 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.

N'-hydroxy succinimide ester method. 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 acid was expected. Therefore, 2 equiv of base were used in order to convert both conjugated 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 were the final titer obtained for each rabbit antiserum against 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. 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 J.) 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), slope (B), I(50) (C) and regression coefficient (r). 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. 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 1](image-url)
the analyte is a more effective competitor for the antibody
reason for the improvement in quality of the assay since
design. Therefore several mercapturic acids (1-4,13-17,
and 30), naphthalene derivatives (18-28), and 5'-methyl
the immunization protocol.

hapten or a different coupling position or procedure from that employed
between coating antigen and immunogen could be a second
phosphates, sulfates, etc.) elute with aqueous fractions.
As a result of this experiment we could conclude that
retained on the solid phase, whereas salts and other more
interference with the assay
However the collected colorless solution gave the same
consistent 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\(^\text{13}\) 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 X 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 100X 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 (uric acid, NaCl, NaAc,
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 1/100 (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. Never-
theless since the slope is not significantly affected by

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\(^{13}\) Harrison, R. O.; Goodrow, M. H.; Hammock, B. D. J. Agric. Food

\(^{23}\) 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.

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

<table>
<thead>
<tr>
<th>compd</th>
<th>R</th>
<th>R¹</th>
<th>R²</th>
<th>13KLH/ICONA</th>
<th>1KLH/BSA:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I(50) pmol/mL</td>
<td>cross-reactivity (%)</td>
<td></td>
<td>cross-reactivity (%)</td>
<td>nd</td>
</tr>
<tr>
<td>1</td>
<td>0.09 ± 0.04</td>
<td>100</td>
<td></td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1.15</td>
<td>8</td>
<td></td>
<td></td>
<td>1.1</td>
</tr>
<tr>
<td>3</td>
<td>0.21</td>
<td>42.5</td>
<td></td>
<td>6.5</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>2.4</td>
<td>3</td>
<td></td>
<td></td>
<td>nc</td>
</tr>
<tr>
<td>13</td>
<td>0.12</td>
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<td></td>
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<td>nc</td>
</tr>
<tr>
<td>14</td>
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<td>49</td>
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<tr>
<td>15</td>
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<td>nc</td>
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<td>16</td>
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<td>nd</td>
<td>nc</td>
<td></td>
<td></td>
<td>nc</td>
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</table>

* 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: \( [I(50) \text{ NaphMA}/1(50) \text{ compound}] \times 100. \) In the table there are also shown the cross-reactivity values, obtained for some of the compounds, on the ELISA system 1KLH/BSA 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/ICONA. 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.
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. 

**H and lac**

Mercapturic acids 16 and 17 were obtained as 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.

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was quenched with l NHCl to pH 6 and the methanol evaporated. The aqueous residue was diluted with water and extracted with ethyl acetate, dried over MgSO4, filtered, and concentrated to give the crude product (6.7 mg, 0.2 mmol). 1H NMR (CDCl3) δ 2.27 (t, J = 8.2, 2.8 Hz, 2H, H-7), 2.86 (t, J = 8.2, 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); 13C NMR (CDCl3) δ 23.05, 27.03, 125.63, 129.61, 131.22, 134.43, 152.35, 152.35, 199.67; IR (KBr) 3020, 1742, 1699, 1498, 1214, 753; ESI-MS (m/z) 224.1 (M+H+, 100%), 206.1 (M+Na+, 41%), 169.1 (M+K+, 7%); Anal. Calc. for C11H9NO2 (224.21): C, 70.00; H, 5.37; N, 3.60. [22] N-Acetyl-S-[6R,8R]-2-carboxy-5,6-dihydro-6-hydroxy-6-naphthyl]cysteine (13c'). The crude residue 13c (105 mg, 0.4 mmol) in anhydrous THF (1 mL) was added to a suspension of NaOCH3 (320 mg, 5.5 mmol) in the same solvent (1 mL). The mixture was stirred overnight at 4 °C at which time HPLC analysis (Spheri-5 RP-18 250×4.6 mm, mobile phase: methanol/triethylamine buffer 0.05 M, pH 3.1) 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) (fa compared to that of 1-naphthol, tR for 10.42 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, 1.5 mL) and stirred for 1 h at room temperature. HPLC analysis revealed the appearance of a new peak at 4.47 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 C18 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 washing the column with water (100 mL) and the desired compound 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*) isomers based on NMR data. Spectroscopic data for 13c: 1H NMR δ (D2O-C2D2OD) 2.03 (s, 3H, CH3CO), 2.31 (m, 1H, H-4'), 2.48 (ddd, J = 16.5, 5.5, 5.6 Hz, 1H, H-3'), 3.31 (d, J = 7.8 Hz, 2H, H-1'), 3.62 (dq, J = 7.8, 1.1 Hz, 1H, H-4'), 4.48 (m, 1H, H-5'), 6.71 (d, J = 4.6 Hz, 1H, H-5), 7.22 (d, J = 8.5 Hz, 1H, H-4), 7.89 (s, 1H, H-1), 7.88 (d, J = 8.5 Hz, 1H, H-3'); 13C NMR (CDCl3-C2D2OD) δ 20.94, 25.98, 25.98, 27.67, 47.95, 72.72, 120.88, 120.40, 130.14, 130.80, 133.86, 133.77, 170.03, 171.73; IR (KBr) 3620, 3019, 1735, 1735, 1700, 1217 cm⁻¹; FABMS (m/z) 312, 312, 174; HRMS (m/z) calcd for M–H, C12H12BrO3S 310.9918; obsd 310.9886. Anal. Calc. for C20H18BrO4S: C, 49.8; H, 4.18; Br, 25.51. Found: C, 49.04; H, 4.18; Br, 25.51.

8-Acetyl-7-bromo-5,6,7,8-tetrahydronaphthalene-2-carboxylic acid (9d). Following the procedure previously described, from the acid 8d (143 mg, 0.62 mmol), 9d was obtained as a white solid (270 mg, 55%), purified by preparative TLC (hexane/ether 8/2, 1% acetic acid). 9d was obtained as a white solid (270 mg, 55%) and purified by preparative TLC (hexane/ether 8/2, 1% acetic acid). 1H NMR (CDCl3) δ 2.90 (s, 3H, CH3CO), 2.10 (m, 1H, H-6), 2.17 (m, 1H, H-7), 2.84 (ddd, J = 17.5, 5.8, 5.5 Hz, 1H, H-3), 3.05 (ddd, J = 17.8, 5.8, 5.4 Hz, 1H, H-1), 4.06 (d, J = 4.2 Hz, 1H, H-4), 7.15, 12, 13, (d, J = 7.8 Hz, 1H, H-4'), 7.87 (s, J = 7.8 Hz, 1H, H-3'); 10.65 (brs, COOH); 13C NMR (CDCl3-C2D2OD) δ 20.68, 25.99, 26.68, 48.04, 72.39, 123.79, 123.04, 130.08, 131.68, 132.23, 134.44, 169.99, 172.01; IR (KBr) 3671, 2934, 1738, 1869, 1285 cm⁻¹; HRMS (m/z) calcd for M–H, C12H12BrO3S 310.9918; obsd 310.9886. Anal. Calc. for C20H18BrO4S: C, 49.8; H, 4.18; Br, 25.51. Found: C, 49.78; H, 4.21; Br, 25.80.

(26) Sample aliquots were taken from the reaction mixture, diluted with 1% acetic acid aqueous solution to a final concentration of 0.1 mg/μL and injected in the HPLC system (10 μL). Under these conditions, no nucophile 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: [H NMR δ (D$_2$O-CD$_3$OD) 2.01 (s, 3H, CH$_3$CONH), 2.92-3.28 (m, 1H, -SCH$_2$CH-), 3.91 (dd, $J = 7.3, 4.2$ Hz, 1H, -SCH$_2$CH-), 4.97 (dd, $J = 5.8, 1.3$ Hz, 1H, H-6), 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$_8$H$_7$SNO$_2$ 350.0698, obsd 350.0688.

Conjugation to Carrier Proteins. (A) Immunogens. Hapten 13 and 14 were covalently attached through their carboxylic acids to the lysine groups of KLH (keyhole limpet hemocyanin) using the mixed anhydrides method. Briefly tri-n-butylamine (0.072 mM) followed by isobutyl chloroformate (0.036 mM) were added to the carboxylic acid hapten (0.036 mM) 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 at pH 8.5) 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 -20 °C.

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

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 PBS (phosphate-buffered saline solution, 0.8% NaCl and 0.05% Tween 20, pH 7.4) to microtiter plates coated with several concentrations (5-0.3 μg/mL) of lBSA, CONA (conalbumin), and OVA (ovalbumin) as previously described.

Immunogen 1KLH/Coating Antigen LCONA. 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 (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 2X 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$_8$ 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.

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 by adding the standards to the precoated plates (1BSA, 1μg/mL) followed by the antisera (Ab2357), diluted 1/4000 in 2 X PBST.

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Supplementary Material Available: $^1$H-NMR data for 7a, 8c, 10c, 10d, 13e/c', and 14d/d' (6 pages). This material is available 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.