Enzyme-Linked Immunosorbent Assay for the Specific Detection of the Mercapturic Acid Metabolites of Naphthalene

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The measurement of metabolites constitutes a useful tool for detection of exposure and in pharmacokinetic studies. Epoxidation with subsequent glutathione conjugation and mercapturic acid formation is an important deactivation pathway for naphthalene, a toxin which presumably causes lung disease. The mercapturic acid conjugates of naphthalene [NaphMA (1), N-acetyl-S-(1,2-dihydro-1-hydroxy-2-naphthyl)cysteine (1a), and N-acetyl-S-(1,2-dihydro-2-hydroxy-1-naphthyl)cysteine (1b)], its most important urinary metabolites, and other structurally related derivatives, such as N-acetyl-S-(1,2,3,4-tetrahydro-2-hydroxy-1-naphthyl)cysteine (2), N-acetyl-S-(3-hydroxy-1,2,3,4-tetrahydro-2-naphthyl)cysteine (3), and N-acetyl-S-(2-hydroxy-1-phenylethyl)cysteine (4a) and N-acetyl-S-(2-hydroxy-2-phenylethyl)cysteine (4b) as an isomeric mixture, were synthesized to develop an ELISA (enzyme-linked immunosorbent assay) for the specific detection of NaphMA (1). Compound 1, as an isomeric mixture, was used to raise antibodies by immunizing six rabbits with the corresponding KLH (keyhole limpet hemocyanin) and BSA (bovine serum albumin) derivatives (1KLH and 1BSA). The remaining compounds were covalently attached to BSA, conalbumin, and ovalbumin to be used as coating antigens. The best assay was obtained in a homologous system combining serum Ab2357 (1KLH) and 1BSA as coating antigen. The immunoassay has an I_{50} of 4-6 ng/mL and a detection limit of 1-2 ng/mL. Because of the known instability of the mercapturic acid conjugate of naphthalene 1, leading to the fully aromatic compound 20, a system involving HPLC is described to check the stability of the NaphMA stock solutions used in the assay. Cross-reactivity studies show high specificity toward the NaphMA. Other related compounds as well as the dehydrated derivative 20 are not recognized by the antibody in this ELISA system.

Introduction

Naphthalene is a polycyclic aromatic hydrocarbon, commonly found as an environmental contaminant. Hundreds of millions of pounds are produced every year from coal tar feedstocks (coal tar naphthalene) and from aromatic petroleum refinery streams (petroleum naphthalene) (1). Naphthalene is employed in some pesticide formulations, but is mainly a commercially important precursor of 2-naphthol, carbaryl, phthalic anhydride, surfactants, and other organic intermediates. Additionally, various combustion processes involve the formation of naphthalene, including cigarette smoke (2), automobile exhaust gases (3), and coal-combustion procedures. Naphthalene derivatives constitute the major portion of polyaromatics in ambient air (4).

Naphthalene derivatives selectively produce lesions in the lungs of mice and at higher doses also in the kidneys (5-7). At low naphthalene doses, necrosis of the Clara cells in the bronchiolar epithelium is produced; however, different effects have been reported depending on the concentration and frequency of the doses (8, 9) as well as tissue selectivity variations between species (10, 11).

Additional toxicological information can be found in recent reviews (12, 13). Cigarette smoking, together with other environmental factors, is believed to contribute substantially to lung diseases. Naphthalene is considered one of the specific environmental factors leading to lung diseases. Additionally, naphthalene has recently been identified as one of the major mutagenic components of the ambient particulate fraction (14).

The 1,2-epoxide of naphthalene is the main reactive intermediate responsible for the toxicity of naphthalene. Apparently, this electrophile, formed by oxidation under the action of the P-450 monooxygenase system, alkylates biomacromolecules, inducing bronchiolar necrosis. Its conjugation with the tripeptide GSH (γ-Glu-Cys-Gly) acts as a protective process (7). The mercapturic acids (MA;[1] S-substituted N-acetyl-L-cysteine derivatives) are the metabolic end products of the GSH conjugation pathway and the major urinary products. The measurement of MA conjugates allows assessment of exposure in a noninvasive manner (15). MAs have typically been detected using spectrophotometric techniques, GC (16, 17) and

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[1] Abbreviations: 1,2-diol, 1,2-dihydroxynaphthalene; BSA, bovine serum albumin; CONA, conalbumin; DCC, N,N'-dicyclohexylcarbodiimide; DMF, dimethylformamide; ELISA, enzyme-linked immunosorbent assay; FAB/MS, fast atom bombardment mass spectrum; HETCOR, heterocorrelation ¹H–¹³C NMR experiments; HOMCOR, homocorrelation ¹H–¹H NMR experiments; KLH, keyhole limpet hemocyanin; MA, mercapturic acid; NaphMA, mercapturic acid conjugates of naphthalene; NHS, N-hydroxysuccinimide; OVA, ovalbumin; PBS, phosphate-buffered saline solution; PBST, phosphate-buffered saline + Tween 20 solution.

Scheme I[a]

5 R= H
6 R= Br

R₁= SCH₂CH(NHCOCH₃)COOH;   R₂= OH

2 R₁= SCH₂CH(NHCOCH₃)COOH;   R₂= OH
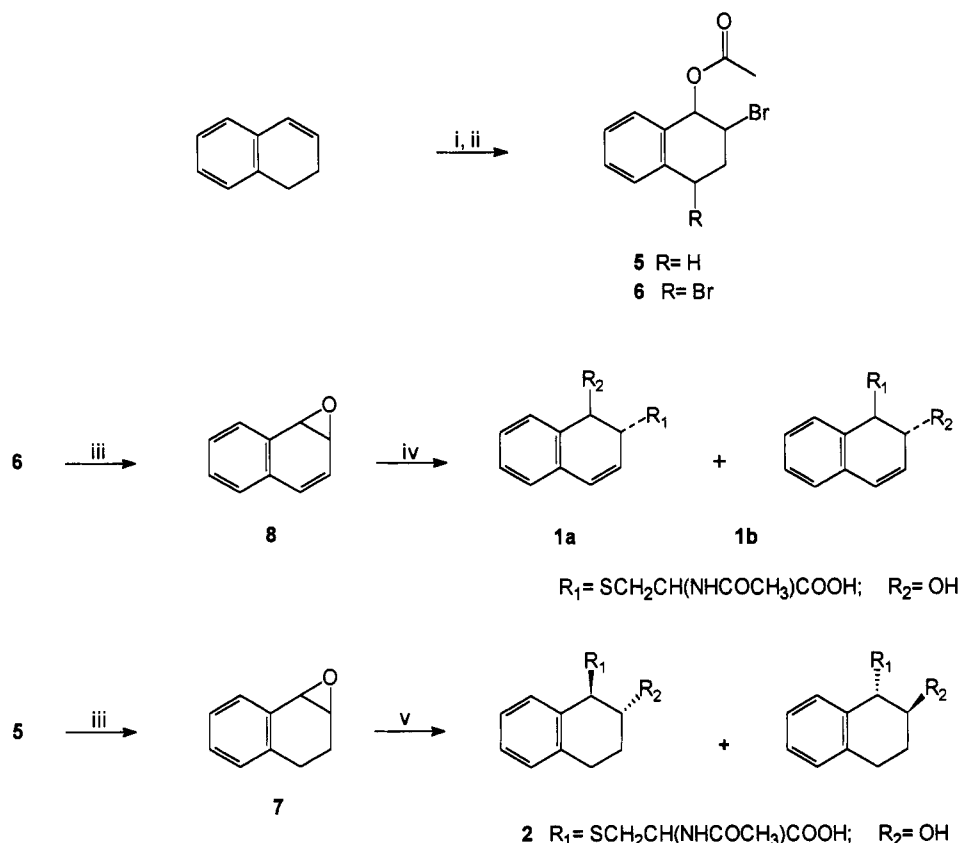
[a] Haptens 1 and 2 were obtained from 1,2-dihydronaphthalene according to the synthetic procedure shown. Compound 1 (isomeric mixture) was used for immunizing rabbits after covalent attachment to BSA and KLH. i, BrCH₂CONH₂/acetic acid, 3 h, room temperature; ii, N-bromosuccinimide/CCl₂, 30 min, 80 °C; iii, CH₃ONa/THF, overnight, 4 °C; iv, HSCH₂CH(NHCOCH₃)COOH, 1 N NaOH (2 eq)/THF, 1 h, room temperature; v, HSCH₂CH(NHCOCH₃)COOH, 1 N NaOH (2 eq)/THF, overnight, room temperature.

HPLC (18-20). While assessment of exposure to toxic substances requires specific, accurate, and sensitive methods, some of the mentioned techniques are nonspecific and often suffer from interferences with the biological matrices. Mass spectrometry techniques provide specificity, and in the last years have made possible the characterization of several MA conjugates (21-23). However, it requires very expensive equipment, and it is time consuming when the number of samples to be analyzed is high, since prior cleanup of the samples is necessary (24). Enzyme-linked immunosorbent assays (ELISAs) for determination of small molecules have proven to be fast, specific, sensitive, accurate, and inexpensive methods. Immunoassays have been developed during the last years for the detection of pesticides (25) and for measuring alkaloids (26, 27), drugs (28), and metabolites (29, 30). In this paper we present the development of an immunoassay for the specific detection of the MA conjugate of naphthalene [NaphMA (1)] as a urinary biomarker of naphthalene exposure.

Experimental Procedures

Chemicals and Instruments. Chemical reagents were purchased from Aldrich Chemical Co. (Milwaukee, WI). Immunochemicals were obtained from Sigma Chemical Co. (St. Louis, MO). TLC was performed on 0.25-mm, precoated silica gel 60 F254 aluminum sheets from Merck (Gibbstown, NJ). ¹H and ¹³C NMR spectra were obtained with a QE-300 spectrometer (General Electric, 300 MHz for ¹H and 75 MHz for ¹³C nuclei). Chemical shifts (δ) are given in ppm relative to TMS as the internal reference. The following descriptions are used: br = broad, ca = complex absorption, d = doublet, m = multiplet, q

Scheme II[a]

20
R= SCH₂CH(NHCOCH₃)COOH
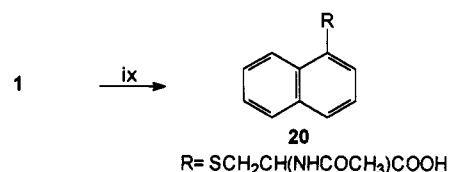
[a] Compound 1, the major isomer of the reaction mixture, gave 20 under acidic conditions by dehydration and intramolecular transposition (37). ix, 0.1 N HCl/methanol, overnight, room temperature.

= quartet, s = singlet, and t = triplet. Infrared spectra were measured on an IR/32 FTIR spectrophotometer (IBM Instruments Inc.) Electron impact mass spectra were recorded on a TRIO-2 (VG Masslab, Altricham, U.K.) apparatus, and data are reported as m/z (relative intensity). FAB/MS (fast atom bombardment mass spectrum) was obtained on a ZAB-HS-2F instrument (VG Analytical, Wythensahwe, U.K.). HPLC analyses were done using a Varian-9010 solvent delivery system equipped with a Rheodyne injector and a Varian-9050 variable-wavelength detector set at 260 nm. The chromatography column was Spheri-5 RP-18 (250 × 4.6 mm) from Applied Biosystems, and the mobile phase is described below for every case. Polystyrene microtiter plates were purchased from Nunc (Maxisorb, Roskilde, Denmark). The absorbances were read with a V_{max} microplate reader (Molecular Devices, Menlo Park, CA) in dual-wavelength mode (405-560). The inhibition curves were analyzed using a four-parameter logistic equation (Softmax, Molecular Devices). All the data presented correspond to the average of three well replicates.

Synthesis of Haptens. Haptens used for the preparation of immunizing and coating antigens have been numbered as 1-4 and were synthesized as follows (Schemes I and III). The intermediates for the synthesis of the mentioned immunochem-

Scheme III[a]

9   10   3 R= SCH₂CH(NHCOCH₃)COOH

R₁   R₂

4a   4b

R₁= SCH₂CH(NHCOCH₃)COOH; R₂= OH
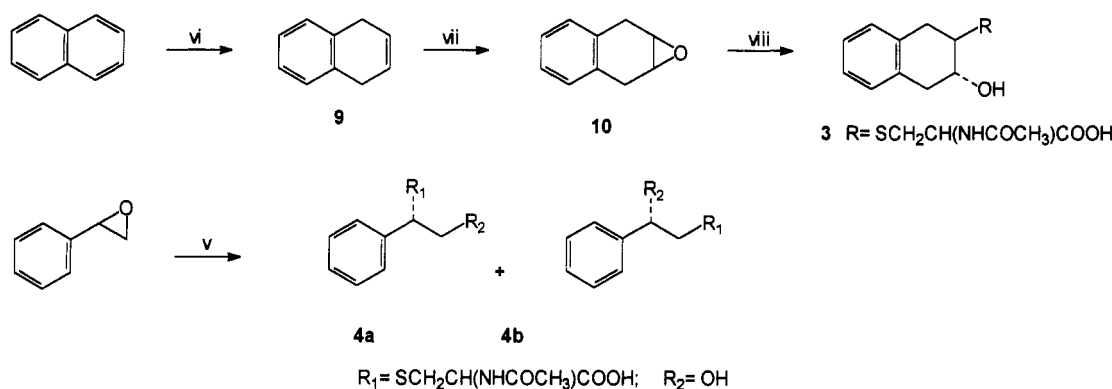
[a] Compounds 2a, isolated by crystallization, and 3 and 4 were used as coating antigens in the ELISA. v, HSCH₂CH(NHCOCH₃)COOH, 1 N NaOH (2 equiv)/THF, overnight, room temperature; vi, Na/ethanol, 1 h, room temperature; vii, *m*-chloroperbenzoic acid/chloroform, 30 min, room temperature; viii, HSCH₂CH(NHCOCH₃)COOH, 1 N NaOH (2 equiv)/THF, overnight, 40 °C.

icals are numbered consecutively. The preparation of fully aromatic MA conjugate of naphthalene 20 (Scheme II) is described.

(A) 1-Acetoxy-2-bromo-1,2,3,4-tetrahydronaphthalene (5). Lithium acetate (8.2 g, 80 mmol) and *N*-bromoacetamide (3.0 g, 21.8 mmol) were added as solids to a solution of 1,2-dihydronaphthalene (2.6 g, 20 mmol) in glacial acetic acid (200 mL) under a nitrogen atmosphere. The mixture was stirred 3 h at room temperature protected from light until the complete disappearance of starting material as determined by TLC (hexane/ethyl ether, 8/2). The reaction mixture was poured into water (500 mL), and a colorless solid precipitated. After filtration, the solid was redissolved in ether (80 mL), washed with saturated sodium bicarbonate (2 × 50 mL) and saturated sodium chloride (50 mL), dried with potassium carbonate, and evaporated under reduced pressure to give 4.41 g (82%) of a white solid which was recrystallized from petroleum ether; mp 94.5–96 °C (uncorrected). The spectral data of 5 are similar to those reported (31).

(B) 1-Acetoxy-2,4-dibromotetralin (6). *N*-Bromosuccinimide (NBS, 1.3 g, 7.0 mmol) and α,α'-azoisobutyrodinitrile (10 mg, catalytic amount) were added to a solution of 5 (1.6 g, 5.9 mmol) in CCl₄ (40 mL) and heated at reflux at 80 °C for 30 min. The mixture was filtered and the solvent was removed under reduced pressure to leave 1.92 g (79%) of a solid which was recrystallized from chloroform–petroleum ether to give white prisms; mp 115–116 °C (uncorrected). The ¹H NMR data are similar to those reported (31).

(C) Naphthalene 1,2-Oxide (8). A solution of sodium methoxide in methanol (1.7 mL, 7.4 mmol, 25% w/w) was added to an ice-cold solution of 6 (400 mg, 1.15 mmol) in dry THF (8 mL). The reaction mixture was stirred in the dark at 4 °C overnight, poured into cold water (25 mL), and extracted with ether (3 × 25 mL). The combined organic layers were dried with potassium carbonate, and the solvent was removed under reduced pressure. The product was recrystallized from petroleum ether to obtain 8 as pale yellow crystals (129 mg, 60%). The ¹H NMR data are similar to those previously reported (32).

(D) *N*-Acetyl-*S*-(1,2-dihydro-1-hydroxy-2-naphthyl)cysteine (1a) and *N*-Acetyl-*S*-(1,2-dihydro-2-hydroxy-1-naphthyl)cysteine (1b). A solution of 8 (150 mg, 1.04 mmol) in THF (6 mL) was added dropwise to a cold solution of *N*-acetylcysteine (204 mg, 1.2 mmol) in degassed 1 N sodium hydroxide (2 mequiv, 2.4 mL). The reaction was stirred for approximately 1 h, until the complete disappearance of the starting material as 1-naphthol[2] on the HPLC (mobile phase H₂O–1% acetic acid/methanol, 6/4) was observed. The reaction mixture was diluted with water (25 mL), and the organic solvent was evaporated under reduced

pressure. The aqueous layer was acidified to pH 3.1 and purified on a reverse-phase C-18 flash chromatography column (30 g, particle size 55–105 μm). Activation of the stationary phase was performed with acetonitrile (200 mL), water (200 mL), and phosphoric acid–triethylamine buffer (0.05 M, pH 3.1, 200 mL). Salts and excess *N*-acetylcysteine were eliminated by washing the column with water (200 mL), and a mixture of 1a and 1b was eluted with acetonitrile (100 mL). The organic solvent was evaporated under reduced pressure, and the water was removed by lyophilization to obtain 223 mg (68%) of a stereoisomeric mixture of 1a (>95%; ~1:1 mixture of *R*(*R**,*R**) isomers) and 1b (<5%)[3] as a white powder. The position of the *N*-acetylcysteine residue was confirmed by two-dimensional homocorrelation (HOMCOR, ¹H–¹H) and heterocorrelation (HETCOR, ¹H–¹³C) NMR experiments. Data for 1a: ¹H NMR (D₂O) δ 1.80 (s, 3 H, CH_3CONH) 2.56 and 2.59 (dd, *J* = 14.1 and 7.2 Hz, 1 H, SCH_aH$_b$CH, diastereomers), 2.76 and 2.79 (dd, *J* = 14.1 and 4.5 Hz, 1 H, SCH$_a$$H_b$CH, diastereomers), 3.63 (dd, *J* = 6.0 and 1.7 Hz, 1 H, H-2), 4.18 and 4.21 (dd, *J* = 7.2 and 4.5 Hz, 1 H, SCH₂C*H*, diastereomers), 4.64 (dd, *J* = 6.0 and 1.7 Hz, 1 H, H-1), 5.82 and 5.84 (ddd, *J* = 9.6, 6.0, and 1.2 Hz, 1 H, H-3, diastereomers), 6.52 and 6.53 (d, *J* = 9.6 Hz, 1 H, H-4, diastereomers), 7.05 (d, *J* = 7.2 Hz, 1 H, H-8), 7.10–7.25 (ca, 3 H, H-5, H-6, H-7); ¹³C NMR (D₂O) δ 23.2 (*C*H₃CONH), 33.7 (S*C*H₂CH), 45.3 and 46.7 (C-2, diastereomers), 55.5 and 55.8 (SCH₂*C*H, diastereomers), 71.3 and 71.7 (C-1, diastereomers), 126.4 (C-3), 127.7, 128.7, 129.1, and 129.7 (C-6, C-7, C-8, and C-9), 132.1 (C-5), 134.0 (C-9), 173.8 (CH₃*C*ONH), 174.3 (*C*HCOOH); FAB/MS (*m/z*) 306 (M – 1, base peak), 288 (M – 1 – H₂O), 247 and 162.

(E) 3,4-Dihydronaphthalene 1,2-Oxide (7). Sodium methoxide powder (203 mg, 3.76 mmol) was added to an ice-cooled solution of the bromoacetate 5 (203 mg, 0.75 mmol) in THF (20 mL), under nitrogen atmosphere. The reaction mixture was stirred overnight at 4 °C and treated as described for the preparation of epoxide 8, to obtain 7 (90 mg, 82%): ¹H NMR (CDCl₃) δ 1.72 (ddd, *J* = 16.2, 16.2, and 6.0 Hz, 1 H, H-3), 2.34–2.42 (ddd, *J* = 15, 5.8, and 1.2 Hz, 1 H, H-3'), 2.48–2.55 (dd, *J* = 18 and 4.5 Hz, 1 H, H-4), 2.71–2.77 (ddd, *J* = 17.5, 17.5, and 6.1 Hz, 1 H, H-4'), 3.70 (m, 1 H, H-2), 3.81 (d, *J* = 6 Hz, 1 H, H-1), 7.05–7.37 (m, 4 H, H aromatic); ¹³C NMR (CDCl₃) δ 21.6 (C-3), 24.1 (C-4), 52.3 (C-2), 54.6 (C-1), 125.7 (C-5), 128.0 (C-8), 128.0 (C-7), 129.1 (C-6) 132.4 (C-9), 136.3 (C-10).

(F) *N*-Acetyl-*S*-(1,2,3,4-tetrahydro-2-hydroxy-1-naphthyl)cysteine (2). The epoxide 7 (90 mg, 0.61 mmol) in THF (2 mL) was added to a freshly prepared solution of *N*-acetylcysteine (99.5 mg, 0.61 mmol) in degassed 1 N sodium hydroxide (2 mequiv, 1.2 mL). The reaction was stirred overnight at room temperature, protected from light. The organic solvent was

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[2] 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 into 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 1-naphthol.

[3] Approximate ratio according to ¹H NMR integration. Signals attributed to 1b in the ¹H NMR (D₂O): δ 4.00 (dd, *J* = 6.1 and 1.6 Hz, 1 H, H-1) and 4.25 (dd, *J* = 6.1 and 1.7 Hz, 1 H, H-2).

evaporated and the mixture diluted with water and washed with ethyl acetate, acidified to pH 2 and extracted with ethyl ether, dried with magnesium sulfate, and evaporated to dryness to obtain a diastereomeric mixture (1:1, according to NMR integration) of **2'** and **2''** (170 mg, 90%): ^{1}H NMR (CDCl$_3$/CD$_3$OD) δ 1.85 (m, 1 H, H-3ax), 2.04 and 2.07 (s, 3 H, C*H*$_3$CON, diastereomers), 2.26 (m, 1 H, H-3eq), 2.70–2.92 (m, 2 H, H-4), 3.02–3.14 (m, 2 H, SC*H*$_2$CH), 3.95 and 3.97 (dd, J = 6.5 Hz, 1 H, H-1, diastereomers), 4.10 (m, 1 H, H-2), 4.75 (dd, J = 7.1 and 5.0 Hz, 1 H, SCH$_2$C*H*), 7.02–7.60 (ca, 4 H, H-aromatic); ^{13}C NMR (CDCl$_3$/CD$_3$OD) δ 21.8 (*C*H$_3$CONH), 24.8 (S*C*H$_2$CH), 26.4 (C-3), 32.4 and 32.9 (C-4 diastereomers), 50.7 and 51.2 (*C*SCH$_2$, diasteromers), 51.8 and 52.1 (SCH$_2$*C*H, diastereomers), 69.1 and 69.5 (*C*OH, diastereomers), 125.6 (C-5), 126.6 (C-8), 128.1 (C-7), 130.3 (C-6), 133.4 (C-9), 136.3 (C-10), 171.3 (NH*C*OCH$_3$), 172.2 (CH*C*OOH); FAB/MS (m/z) 310 (M + 1, base peak), 292 (M + 1 − H$_2$O), 164, 130. Compound **2'** $R(R^*,R^*)$ was isolated from the mixture after crystallization from CHCl$_3$/CH$_3$OH (9.5/0.5) and the position of the N-acetylcysteine residue determined by 2D-HOMCOR and HETCOR NMR experiments. ^{1}H NMR data are similar to those described for the mixture except for the signal at δ 3.96 that appears like a d, J = 6.7 Hz: mp 174.8–175.9 °C; ^{13}C NMR (CDCl$_3$/CD$_3$OD) δ 21.5 (*C*H$_3$CONH), 24.5 (S*C*H$_2$CH), 26.1 (C-3), 32.3 (C-4), 50.2 (C-1, *C*SCH$_2$), 51.9 (SCH$_2$*C*H), 69.0 (C-2, *C*OH), 125.39 (C-5), 126.3 (C-8), 128.0 (C-7), 130.3 (C-6), 133.1 (C-9), 136.2 (C-10), 171.4 (NH*C*OCH$_3$), 171.9 (CH*C*OOH); FAB/MS [m/z (relative intensity)] 310 (98), 292 (50), 164 (base). Evaporation of the solvent gave the second enantiomer **2''** as an oil. ^{1}H NMR data are consistent with those described for the mixture: ^{13}C NMR (CDCl$_3$/CD$_3$OD) δ 22.0 (*C*H$_3$CONH), 25.1 (S*C*H$_2$CH), 26.7 (C-3), 33.0 (C-4). 51.3 (C-2, *C*SCH$_2$), 52.3 (SCH$_2$*C*H), 69.4 (C-1, *C*-OH), 125.6 (C-5), 126.8 (C-8), 128.2 (C-7), 130.3 (C-6) 133.7 (C-9), 136.4 (C-10), 171.2 (NH*C*OCH$_3$), 172.6 (−CH*C*OOH).

(G) 1,4-Dihydronaphthalene (9). To a solution of naphthalene (6.4 g, 50 mmol) in ethanol (300 mL) was added sodium (4.6 g, 200 mmol) in pieces. The resulting mixture was heated at reflux for 1 h until the TLC (hexane/ethyl acetate, 9/1) indicated complete disappearance of the starting material. The cooled reaction mixture was poured into 10% cold hydrochloric acid (160 mL) and extracted with ethyl ether (2 × 200 mL). The combined organic extracts were washed with saturated sodium chloride (50 mL) and saturated sodium bicarbonate (2 × 50 mL) solutions and added to aqueous 0.53 M mercuric acetate (100 mL). After 24 h at room temperature, the mercuric adduct was filtered and crystallized from toluene. To remove the mercuric acetate, a solution of the adduct in ethyl ether (100 mL) was poured into 30% cold hydrochloric acid (15 mL) and stirred at room temperature for 1 h. The organic layer was separated and the aqueous layer was extracted with ethyl ether (2 × 25 mL). The combined organic extracts were finally washed with saturated sodium chloride (25 mL) and saturated sodium bicarbonate (50 mL) solutions and dried with potassium carbonate, and the solvent was evaporated under reduced pressure to give 3.2 g (69%) of a clear oil identified as **9**. NMR data agree with those previously reported (*33*).

(H) 1,4-Dihydronaphthalene 2,3-Oxide (10). Compound **9** (2.64 g, 20 mmol) in methylene chloride (10 mL) was added dropwise to a solution of m-chloroperbenzoic acid (MCPBA) (3.8 g, 22 mmol) in the same solvent (50 mL). The mixture was stirred at room temperature for 30 min until analysis by TLC (hexane/ethyl acetate, 9/1) indicated complete disappearance of the starting material. The cooled reaction was added to a mixture of saturated sodium thiosulfate solution (25 mL) and methylene chloride (25 mL). The organic fraction was separated and the aqueous layer was extracted again with methylene chloride (2 × 25 mL). The combined organic extracts were washed with saturated sodium thiosulfate (25 mL) and saturated sodium bicarbonate (25 mL) solutions and dried with potassium bicarbonate. Evaporation of the organic solvent under reduced pressure gave **10** (2.3 g, 79%) as a clear oil: IR (CHCl$_3$) 3426, 3019, 1216; ^{1}H NMR (CDCl$_3$) δ 3.14 (d, J = 17.7 Hz, 2 H, benzylic), 3.27 (d, J = 17.7 Hz, 2 H, benzylic), 3.42 (s, 2 H, C*H*OC), 7.01

(d, J = 3.12, 2 H, H-6 and H-7 Ar), 7.09 (d, J = 2.7 Hz, 2 H, H-5 and H-8); ^{13}C NMR (CDCl$_3$) δ 29.5 (C-1 and C-4), 51.7 (C-2 and C-3), 126.3 (C-5 and C-8), 129.0 (C-6 and C-7), 131.3 (C-9 and C-10). Anal. Calcd for C$_{10}$H$_{10}$O: C, 82.16; H, 6.89. Found: C, 82.26; H, 6.89.

(I) N-Acetyl-S-(3-hydroxy-1,2,3,4-tetrahydro-2-naphthyl)cysteine (3). The mercapturic acid conjugate **3** was prepared from **10** (500 mg, 3.4 mmol), as described before for compound **2**, but in this case the reaction was stirred overnight at 40 °C. After acidification to pH 2, the mixture was extracted with ethyl acetate (3 × 30 mL), washed with saturated sodium chloride (25 mL), and dried with magnesium sulfate and the solvent evaporated under reduced pressure to give, after recrystallization from ethyl acetate, 492 mg (52%) of the desired compound as a colorless prisms: mp 165–167 °C; ^{1}H NMR (CDCl$_3$) δ 2.00 (s, 3 H, C*H*$_3$CONH), 2.73–3.34 (ca, 6 H, H-1, H-4, and SC*H*$_2$CH), 3.93 (m, 1 H, C*H*OH), 4.64 (m, 1 H, C*H*SCH$_2$), 5.01 (dd, J = 7.0 and 4.8 Hz, 1 H, SCH$_2$C*H*), 7.02–7.09 (m, 1 H, H-aromatic); ^{13}C NMR (CDCl$_3$/CD$_3$OD) δ 23.0 (*C*H$_3$CONH), 34.5 (*C*OH), 35.6 (*C*SCH$_2$), 37.7 (S*C*H$_2$CH), 49.1 (S*C*H$_2$CH), 54.4 (*C*SCH$_2$), 71.8 (*C*OH), 127.2 (C-8 and C-5), 129.3 (C-6 and C-7), 130.2 (C-9 and C-10), 171.3 (NH*C*OCH$_3$), 172.7 (CH*C*OOH).

(J) N-Acetyl-S-(2-hydroxy-1-phenylethyl)cysteine (4a) and N-Acetyl-S-(2-hydroxy-2-phenylethyl)cysteine (4b). Styrene oxide (200 mg, 1.66 mmol) and N-acetylcysteine (271 mg, 1.66 mmol) were reacted according to the procedure described for compound **2**. Evaporation of the organic solvent gave 329 mg of **4** (70%) as an oil corresponding to the isomeric mixture. ^{1}H and ^{13}C NMR data are consistent with those previously reported (*34*). FAB/MS (m/z) 284 (M + 1, base peak), 266 (M + 1 − H$_2$O), 224, 185, 164, and 121.

(K) N-Acetyl-S-(1-naphthyl)cysteine (20). Approximately 5 mg of the isomeric mixture **1** was kept overnight in methanol (1 mL) and 0.1 N HCl (0.5 mL) at room temperature. The mixture was then diluted with water (3 mL) and extracted with chloroform. The organic layer was dried over magnesium sulfate, filtered, and evaporated. ^{1}H NMR (CDCl$_3$) δ 1.64 (s, 3 H, C*H*$_3$CONH), 3.39 (dd, J = 14.5 and 5.4 Hz, 1 H, SC*H*$_a$H$_b$CH), 3.57 (dd, J = 14.1 and 4.2 Hz, 1 H, SCH$_a$*H*$_b$CH), 4.78 (dd, J = 5.5 and 4.8 Hz, 1 H, SCH$_2$C*H*), 7.38–7.89 (ca, 7 H, Ar*H*), 8.41 (d, J = 8.7 Hz, 1 H, H-5); ^{13}C NMR (CDCl$_3$/CD$_3$OD) δ 22.4 (*C*H$_3$CONH), 36.6 (S*C*H$_2$CH), 52.6 (SCH$_2$*C*H), 125.0, 125.6, 126.3, 126.8, 128.4, 128.6, 130.8, 132.1, 133.2, 133.9 (Ar*C*H), 170.5 (*C*H$_3$CONH), 172.0 (CH*C*OOH).

Conjugation to Carrier Proteins. (A) Mixed Anhydride Method. Haptens **1** (**1a**:**1b**, ≅95:5, each containing a mixture 1:1 of diastereomers), **2'** (pure compound), **3** (diastereomeric mixture 1:1), and **4** (**4a**:**4b**, ≅2:1, each is a mixture 1:1 of diastereomers) were covalently attached through the carboxylic acids of the mercapturate moiety to the lysine groups of KLH (keyhole limpet hemocyanin), BSA (bovine serum albumin), conalbumin, and ovalbumin using the mixed anhydride method (*35*). Briefly tri-n-butylamine (0.055 mmol), followed by isobutyl chloroformate (0.06 mmol), was added to the carboxylic acid haptens (0.05 mmol) dissolved in ice bath-cooled anhydrous dimethylformamide (DMF). The resulting mixture was stirred for 30 min and then added dropwise to a solution of the corresponding protein (50 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) N-Hydroxysuccinimide Ester Method. Hapten **1** (0.05 mmol) was dissolved in 500 μL of anhydrous DMF and stirred with N,N'-dicyclohexylcarbodiimide (DCC, 0.25 mmol) and N-hydroxysuccinimide (NHS, 0.25 mmol) overnight under N$_2$ atmosphere. After centrifugation, the supernatant was added dropwise to a solution of KLH (50 mg) in 10 mL of borate buffer (0.2 M borate–boric, pH 8.7) and stirred for 7 h at room

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temperature. The protein conjugate was dialyzed as before and stored at −80 °C after lyophilization.

Immunization of the Rabbits. Haptens 1KLH and 1BSA were used to immunize female New Zealand White rabbits (Herbert's Rabbitry, Plymouth, CA) weighing 2–4 kg according to the following protocol. The immunizing antigen (100 μg) was dissolved in PBS (0.5 mL), emulsified with Freund's complete adjuvant (0.5 mL), and injected intradermally at multiples sites in the back. After 1 month the animals were boosted twice more, separated by 15–20 days, with an additional 100 μg of antigen emulsified with Freund's incomplete adjuvant. The first bleed was obtained 8–10 days later for antibody titer determination. Subsequent boostings were done intravenously in the ear vein, dissolving the immunogens in sterile saline solution. Boostings and bleedings continued every 3 weeks until no increase in the antibody titer was observed. Serum was obtained by centrifugation and stored at −80 °C. Rabbits 2355, 2357, 2491, and 2492 were immunized with 1KLH, and rabbits 2358 and 2359 were immunized with 1BSA.

Analysis of the Titer. The titer of the serum from each animal was determined by measuring the binding of serial dilutions (1/1000 to 1/64000) to microtiter plates coated with several concentrations of 1BSA, 1CONA, 1OVA, 2BSA, 2CONA, 2OVA, 3BSA, 3CONA, 3OVA, 4BSA, 4CONA, and 4OVA (5–0.3 μg/mL). Optimal concentrations for coating antigen and antisera dilution were determined.

Competitive Enzyme Immunosorbent Assay. Microtiter plates were coated with 1BSA at 1 μg/mL (100 ng/well) in coating buffer (0.1 M carbonate–bicarbonate, pH 9.6) overnight at 4 °C covered with adhesive plate sealers. The following day the plates were washed 5 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 50-μL volume to the coated plates followed by 50 μL of the antibody (Ab2357) previously diluted 1/4000 in the same buffer (final dilution in the well 1/8000). After 1 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 anti-rabbit IgG–alkaline phosphatase was added and incubated for 1 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 Figure 2 were prepared in DMF. Standard curves were prepared in PBST by serial dilutions (100 μg/mL −0.05 ng/mL), and each I_{50} was determined in the competitive experiment. The cross-reactivity values were calculated according to the following equation: $[I_{50}(\text{NaphMA})/I_{50}(\text{compound})] \times 100$.

Stability Studies. (A) Preparation of the Calibration Curve. Several solutions of NaphMA (1) (200, 150, 100, 80, 60, 40, 20, 10, and 5 ng/μL) were prepared in DMF containing 12.5 ng/mL 1,2-dihydroxynaphthalene (1,2-diol) as an internal standard on the basis of peak area. Every concentration was prepared separately and in triplicate from three different stock solutions of 1 μg/μL. Ten microliters was injected into the HPLC system equilibrated with a mixture of phosphoric acid–triethylamine buffer, 0.05 M, pH 3.1/methanol (6/4) as a mobile phase at a flow rate of 1.0 mL/min, and the peak areas were measured. The area ratio [area NaphMA (1)/area 1,2-diol] was used for calculation of linear regression versus the ratio of the injected amounts of both components in the mixtures [ng of NaphMA (1)/ng of 1,2-diol].

(B) Effect of the pH on the Stability of NaphMA. Solutions of NaphMA (1) (100 ng/μL) were prepared in triplicate in DMF/PBS (1/1) at pH 2.00, 3.44, 7.43, and 8.60 and stored at 4 °C. Daily aliquots of 25 μL were taken, mixed with 1,2-diol, injected into the HPLC system, and quantified according to the equation obtained from the standard curve. The concentration of the internal standard was checked periodically, measuring the absorbance in the UV spectra at λ = 233 nm.

Results and Discussion

Haptens. The key step in developing immunoassays for small molecules is the synthesis of appropriate haptens providing functional groups for their covalent coupling to carrier proteins. The MA conjugate of naphthalene possesses a free carboxylic group in its N-acetylcysteine moiety. On the basis of previous experience (25, 27, 36) a spacer between the target molecule and the protein which decreases the steric shielding effect caused by the protein is desirable. However, in this case, our target molecule [NaphMA (1)] shows a tendency to lose a water molecule to give the completely aromatized compound 20 (37), especially under acidic conditions [see below, effect of pH on the stability of the MA conjugate of naphthalene, NaphMA (1)]. Therefore, the idea to chemically modify the MA conjugate in later steps seemed unworkable. Consequently, the racemic mixture of 1,2-naphthalene epoxide was synthesized according to described procedures (31). Subsequently the diastereomeric mixture of the MA conjugates of naphthalene was obtained by nucleophilic attack of the epoxide by the thiolate of the N-acetylcysteine generated in a sodium hydroxide solution (see Scheme I). This reaction occurs at room temperature and is complete after 1 h. The main difficulties encountered in this reaction derived from the high solubility of NaphMA in water and its instability at acidic pH (see pH effect below). This can be the reason for the low reaction yield since, according to the HPLC analysis, the reaction is complete after a short period of time. The extraction of our compound from the aqueous reaction mixture with organic solvent was difficult and required acidification of the mixture below the pK_a of the amino acid [i.e., glutathione pK_a = 3.59]. The use of small-scale solid extraction procedures for the purification of this and other related compounds was already reported (38, 39). Additionally, other investigators (40, 41) have mentioned the preparative-scale advantages of using reverse phase on the flash chromatography technique introduced by Clark-Still and co-workers (42), for the accurate separation of isomeric mixtures, for desalting, or for concentration purposes. In this case, only a slight excess of N-acetylcysteine was used during the synthesis of 1. Therefore, NaphMA (1), small amounts of N-acetylcysteine, salts, and on some occasions 1-naphthol[4] were found in the reaction mixture analyzed by HPLC. Thus, the purification was performed by placing the mixture (pH 3.1) on a flash chromatography column filled with reverse-phase C18 and equilibrated by washing the column with methanol, water, and phosphoric acid–triethylamine buffer (0.05 M, pH 3.1) in this order. After a water washing step to remove the salts and the excess N-acetylcysteine, the NaphMA was eluted with methanol/water (1:1) or with methanol when no appreciable amounts of naphthol were observed in the reaction mixture. Additionally, a fraction of NaphMA (1) was eluted during the water washing step, due to its high polarity; therefore, it is necessary to monitor the aqueous fractions.

Presumably four isomers are possible (see Scheme I); however, analysis of the spectral data suggested that the diastereomers formed by nucleophilic addition at C-2 (1a, 1OH-2SR derivative) are the main compounds. In the ¹H NMR, proton H-1 appears downfield at δ 4.64 and H-2 at

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[4] Formed by nucleophilic attack of the hydroxide anion in less than 10% according to the HPLC analysis.

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Table I. Titer of the Antisera on Different Coating Antigens[a]

immunogen	antisera	1BSA	1CONA	1OVA	2'BSA	2'CONA	2'OVA	3BSA	3CONA	3OVA	4BSA	4CONA	4OVA
1KLH (method A)	2355	H	H	L	H	H	L	H	H	L	H	H	L
	2357	H	H	L	H	H	L	H	H	L	H	H	*
	2492	H	H	*	H	H	L	L	L	H	L	H	L
1KLH (method B)	2358	H	H	L	H	H	L	*	*	L	H	H	L
	2359	H	H	L	L	L	*	*	L	*	H	H	*
1BSA (method A)	2361	H	*	*	H	*	*	L	*	*	L	*	*
	2368	H	*	*	H	*	*	H	*	*	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. Method A, mixed anhydride method; method B, N-hydroxysuccinimide method.

δ 3.63, indicating that probably C-1 is supporting the hydroxyl and C-2 the sulfur atom of the N-acetylcysteine. The assignments were confirmed by the appearance of correlation signals in the HOMCOR spectra, indicating the coupling between H-1 and H-2, and by the correlation shown with the carbons at δ 71.3/71.7 (COH) and 45.3/46.7 (CS) respectively on the HETCOR spectra. Signals of low intensity were observed at δ 4.00 (H-1) and 4.25 (H-2) which correspond to the 1SR-2OH derivative, 1b. These results are in accordance to data previously reported for the corresponding glutathione (11) and thioethyl derivatives (43). The presence of two diastereomers is demonstrated by a doubling of the olefinic signals at δ 5.82 and 5.84 for H-3 and at δ 6.52 and 6.53 for H-4 as well as on the protons of the N-acetylcysteine residue. Analogously, a doubling of the ^{13}C NMR signals corresponding to C-1 (δ 71.3 and 71.7) and C-2 (δ 45.3 and 46.7) is observed. However, under longer reaction times or during purification procedures 20 is formed, as evidenced by disappearance of the olefinic signals and increasing complexity in the aromatic region of the ^{1}H NMR. To prove the structure of the degradation compound, 5 mg of 1 was kept under acidic conditions and the reaction mixture analyzed by HPLC and NMR techniques (see Scheme II). Some traces of 1-naphthol, present in the initial reaction mixture, were eliminated by washing the mixture with ether under slightly basic conditions. The obtained compound corresponds to (N-acetylcystein-S-yl)naphthalene (20), formed as a result of the migration of the N-acetylcysteine residue from C-2 to C-1 on the main compound 1a, as evidenced by the appearance of a doublet at δ 8.41 corresponding to H-5. This behavior has been reported by Jeffery and Jerina (37) for C-2 derivatives which are capable of stabilizing the positive charge formed on the cyclic intermediate implicated in the rearrangement. Supposedly, compound 1b dehydrates without transposition. Finally two sets of conjugates were obtained by covalent coupling of NaphMA 1 (1a:1b, ≅95:5) to BSA, CONA, OVA, and KLH using the mixed anhydride and the NHS ester methods.

Previous studies have very often shown that heterologous ELISA systems[5] provide highly sensitive and selective assays (44–46). For this reason the MA derivatives 2–4 were synthesized (see Schemes I and III) and coupled to BSA, CONA, and OVA by the mixed anhydride method. To the extent that homobenzylic opening may compete with benzylic opening, four isomers are possible for 2 and 4; however, for the first of them, only the C-1 adduct is observed to be formed according to the spectroscopic data. Compound 2', as a pure enantiomer, was isolated by

crystallization, characterized using 2D HOMCOR and HETCOR NMR techniques, and used for the protein conjugation reaction. Mercapturic conjugate 4 has been previously described to be a 2:1 isomeric mixture resulting from C-1 versus C-2 addition (4a and 4b, respectively). Due to the symmetry of the epoxide 10 only a pair of diastereomers are possible for compound 3. MAs 3 and 4 were used as an isomeric mixture for the protein conjugation reaction.

Preparation of the Protein Conjugates and Screening of the Sera. Immunizations were carried out with compound 1 attached covalently to proteins. The isolation of NHS esters of NaphMA was troublesome due to the dehydration reaction described previously. To circumvent this, the mixed anhydride method was alternatively employed. No changes were observed in the UV spectra of the proteins before and after conjugation, and consequently the only evidence of conjugation was the screening of the sera. For this reason, both sets of protein conjugates were used during the immunization protocol. Additionally, two different proteins were used to raise antibodies, KLH and BSA.

Using a checkerboard titration, the sera of each animal was tested against 1CONA after every bleeding until no increase in the titer was observed. Table I shows the titer of the final bleed obtained for each different coating antigen. Evaluating the coupling procedures, the mixed anhydride method had a better efficiency for the KLH conjugates since the absorbances measured were often higher. However, this concept did not hold true for the BSA immunogen which only gave reasonable titers when measured against plates coated with the same protein. Nevertheless, variability among animals must be considered, and in this case too few were used in every case to establish a conclusion. Regarding the coating antigen proteins, OVA conjugates gave, in every case, the lowest titers, while the absorbances were always slightly higher for the CONA coating antigens. Similar results have already been reported[6] (47). For all sera, the highest titer was measured when the immunizing hapten was also used as the coating antigen, even if haptens 2' 3, and 4 showed similar or only moderately lower values. From these experiments suitable antisera dilutions and coating antigen concentrations for the ELISA were determined.

Competition Experiments. According to the titration results shown in Table I, only those combinations showing reasonable titers were used for screening NaphMA (1) in the competition experiments. Thus, antisera 2355, 2357, 2492, 2358, and 2359 were appropriately diluted and used for the ELISA in plates coated with 1, 2', 3, and 4 BSA

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[5] A heterologous ELISA system uses as a coating antigen a different hapten or a different coupling position or procedure, from that employed on the immunizing antigen used to generate antibodies.

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

antisera	coating antigen	A/D	slope	I_{50} (ng/mL)	r
2355	1BSA	1.6	0.37	4	0.95
2357	1BSA	11.8	0.76	42.9	0.99
	1CONA	5	0.79	57	0.99
	2′BSA	2	0.67	9	0.92
	2′CONA	2	0.64	3	0.96
	4BSA	2.7	0.41	26	0.96
	4CONA	1.7	0.6	18	0.95
2492	1BSA	8.2	0.59	16.5	0.97
	1CONA	7	0.49	20.5	0.97

[a] Only those combinations that had I_{50} values below 100 ng/mL are shown. A/D, slope, and I_{50} are values from the four-parameter equation calculated for each combination; A and D values represent the maximal absorbance and the background of the assay, respectively. Three replicates were used for every concentration of NaphMA in the standard curves.

Table III. Cross Reactivities for Some Structurally Related Compounds[a]

compound (no.)	cross reactivity (%)	compound (no.)	cross reactivity (%)
NaphMA (1)	100	2-naphthol (14)	nd
MA derivative (2)	1.1	1,2-diol (15)	nd
MA derivative (3)	5.5	naptalam (16)	nd
styrene MA (4)	0.4	carbaryl (17)	nd
benzyl MA (11)	nd	naphthaleneacetamide (18)	3.5
naphthalene (12)	nd	N-acetylcysteine (19)	nd
1-naphthol (13)	nd	MA naphthalene (20)	nd

[a] Standard curves for every compound (see Schemes I–III and Figure 2 for structures) were prepared in PBST and measured in the ELISA. MA, mercapturic acid. nd indicates that no decrease in absorance was observed with concentrations of at least 100 000 ng/mL. Standard curves for every compound were prepared using three well replicates. The cross-reactivity values are calculated according to the equation: $[I_{50}(NaphMA)/I_{50}(compound)] \times 100$.

and CONA conjugates. No competition was observed using the antisera generated against the immunogen prepared by the NHS method. In contrast, all the rabbits immunized using the mixed anhydride method for the coupling gave usable assays. When plotting measured absorbances (*y*) versus the logarithm of the different concentrations (*x*) of the analyte, a sigmoid curve is obtained which can be fitted by a four-parameter logistic equation. The characteristics of those assays regarding their maximal absorbance (A) versus noise (D), slope (B), I_{50} (C),[7] and regression coefficient (r) are summarized in Table II. Antiserum 2357 gave assays with all coating antigens except 3BSA and 3CONA. Hapten 3, of all haptens used as coating antigens, is the one which showed highest cross-reactivity probably due to the attachment of the N-acetylcysteine residue to C-2 (see Table III). Nevertheless, in this case the coating antigens possessing the same hapten as the one used for immunizing provided the best assays with all the antisera. The fact that a homologous system gave the best combination contrasts with the results previously reported in this laboratory (*45*, *46*). On the other hand, some authors indicate that heterologous assays minimize the problems associated with handle recognition; however, in this assay the N-acetylcysteine moiety is in fact the handle, and consequently the mentioned effect is desirable. Therefore, combination 2357/1BSA was chosen considering the high A/C ratio as well as the reasonable

[7] A, B, C, and D are values extracted from the four-parameter equation, $y = (A - D)/[1 + (x/C)^B] + D$, where *y* corresponds to the absorbance measured at dual wavelength (405–560 nm) and *x* is the concentration of analyte.
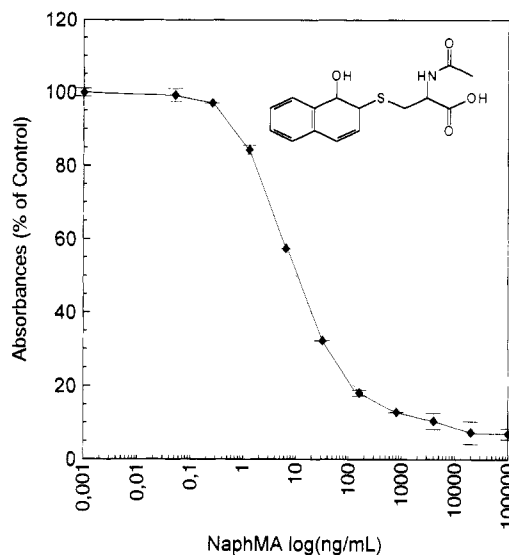

Figure 1. Standard curve obtained for the detection of NaphMA (1) (1a/1b ~95/5) in the assay using 1BSA as a coating antigen and Ab 2357 diluted 1/4000. The I_{50} is 4–6 ng/mL and the detection limit 1–2 ng/mL. The values presented for each point correspond to the average of experiments performed in two plates where three points were employed for every concentration. The coefficients of variation averaged $3.1 \pm 1.8\%$. Control: Averaged absorbances measured at zero concentration of NaphMA.
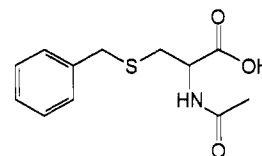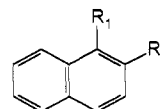
n	R1	R2
Naphthalene 12	H	H
1-Naphthol 13	OH	H
2-Naphthol 14	H	OH
1,2-Diol 15	OH	OH
Carbaryl 17	OCONHCH3	H
18	CH2CONH2	H
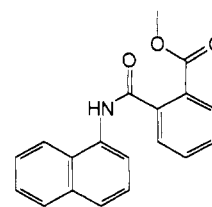
Figure 2. Chemical structures of some of the naphthalene derivatives tested for cross reactivity in the ELISA.

slope and I_{50}. Figure 1 shows the calibration curve with the standard deviations of the optimized assay which provides an accurate measurement of the MA conjugate of naphthalene between 1 and 100 ng/mL with an I_{50} of 4–6 ng/mL.

Cross-Reactivity Studies. Figure 2 shows the series of compounds related to the target molecule which were tested to determine their interference in the ELISA. We were interested in the effect of other urinary metabolites, since this assay is directed mainly to applications on this

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body fluid. Therefore, 1,2-dihydroxynaphthalene (15), naphthalene (12), and some of its metabolites such as 1-naphthol (13) and 2-naphthol (14) were tested in the competition experiments but showed no cross reactivity. The glutathione pathway is accepted as one of the more usual ways of detoxification of xenobiotics; consequently, several MA conjugates are commonly present in urine. Compounds 2–4 and 11 were chosen for having the mercapturate moiety and chemical structures similar to our target. Additionally, N-acetylcysteine (19) was also tested. Table III shows the cross-reactivity values expressed as a percentage of the I_{50} of NaphMA. Only compound 3 showed a value slightly higher than 5%, demonstrating the high specificity of the assay. Of the compounds tested, 3 is the only which is substituted at C-2.[8] It is also worth mentioning that the fully dehydrated compound 20 did not cross-react in the assay. A substantial change in the conformation is produced during dehydration, and the additional rearrangement gives a compound which is not recognized by the antibody. Finally, other structurally related pesticides like naptalam (16) and carbaryl (17) did not show any significant interference, with the exception of naphthaleneacetamide (18) which cross-reacted at 3.5%.

Stability of the Assay. During performance of the immunoassay, a slight constant decrease in assay sensitivity was observed after long periods of storage of stock solutions containing NaphMA (1). This problem required repeated preparation of new stock solutions. As we indicated above, significant dehydration of 1 can occur, leading to the formation of 20 which is not recognized by the antibody. Since the preparation and purification of the standard NaphMA (1) is expensive, intricate, and time consuming, a system for periodic quantitative analysis of our stock solutions was desirable. HPLC has proven to be useful for the detection of the NaphMA (48). Consequently, we developed a quantification method based on the measurement of the area of the peak obtained with UV detection at 260 nm. 1,2-Diol was selected as an internal standard because it has a chromophore that shows absorption in the UV at 260 nm and a retention time close to that of NaphMA under the chromatographic conditions used (t_R of NaphMA 7.08 min, t_R of 1,2-diol 10.62 min). Figure 3 shows the calibration graph obtained by injecting different concentrations of our analyte together with the 1,2-diol. A reverse-phase C_{18} stationary phase was used with a 4/6 mixture of methanol/buffer (H_3PO_3–NEt$_3$, pH 3.1) as mobile phase. Using these conditions, the stability of different stock solutions of NaphMA (1) stored at four different pHs (2.00, 3.44, 7.43, and 8.60) and at 4 °C were monitored over a period of 1 week. A solution stored in aliquots at –20 °C in a mixture of DMF/PBS (pH = 7.40) was additionally injected daily and used as a control. These studies showed that the stability of the MA conjugate of napthalene, NaphMA (1), decreases significantly below pH 3. In contrast, no significant change was produced during the period used in these studies in the solution stored at –20 °C and at neutral pH. As a consequence of these studies subsequent stock solutions were stored in small aliquots at –20 °C in a mixture of DMF/PBS (1/1). The presence of water should reduce the rate of dehydration by shifting the equilibrium constant to the intact compound. DMF is a solvent with a high boiling point,
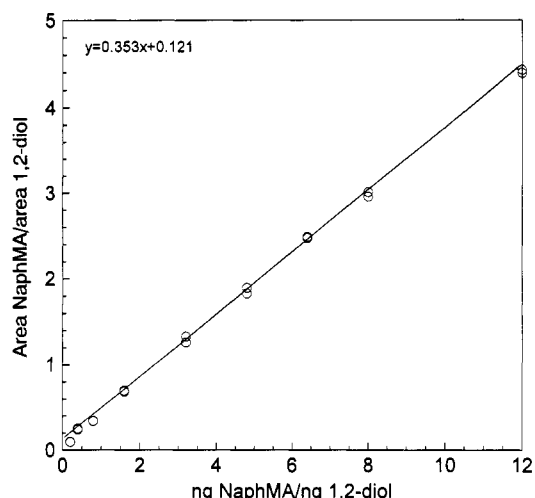

Figure 3. HPLC linear calibration graph obtained for the mercapturic acid conjugate of naphthalene using 1,2-dihydroxynaphthalene as an internal peak area standard. Stationary phase, Spheri-5-RP-18 (250 × 4.6 mm); mobile phase, H_3PO_3–NEt$_3$, 0.05 M, pH 3.1/methanol, 6/4, 1 mL/min. Three replicates were used for every concentration of NaphMA, and the average of the standard deviations is 0.01.

which ensures a constant concentration and guarantees a neutral or slightly basic pH since dimethylamine is produced after degradation. It is also worth mentioning that similar problems may occur for the coating antigen (1BSA). Since the ELISA presented is based on a homologous system, periodic 2D checkerboard titrations to determine the optimal concentrations for the antibody and the coating antigen are advisable.

Conclusions

An immunoassay has been developed for the specific detection of the MA metabolites of naphthalene. A homologous ELISA system provides an assay detecting this urinary metabolite at 1–2 ng/mL (lower detection limit). This immunoassay provides an easy and useful tool for the detection of NaphMA as an indicator of exposure. Antibodies have been obtained by direct attachment of the metabolite to a carrier protein, activating the carboxylic acid of the N-acetylcysteine moiety. Since the immunizing antigen was prepared using an isomeric mixture where the diastereomers 1a are the main component, a higher degree of recognition would be expected in urine toward the product resulting from the enzymatic conjugation at C-2. Nevertheless, additional studies would be required in order to assign the stereo- and regiospecificity of the enzymes involved in a particular biological system. Further studies, now in progress in this laboratory, will demonstrate the applicability of the method to pharmacological studies. The use of efficient techniques for the separation of the four possible isomers of 1 (HPLC, capillary electrophoresis, etc.) prior to the ELISA will provide information regarding the stereo- and regiospecificity of the antibody. An additional interest in the measurement of MAs stems from recent studies suggesting mutagenic activity for these compounds and other thioether conjugates (49). Thus the present immunoassay represents a useful tool for mechanistic, pharmacokinetic, biomonitoring, and toxicological studies.

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[8] Compound 4 is an isomeric mixture where the product from addition at C-1 constitutes the major compound.

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