0026-895X/88/060829-08$02.00/0

MOLECULAR PHARMACOLOGY, 34:829-836

Sequential Oxidation and Glutathione Addition to 1,4-Benzoquinone: Correlation of Toxicity with Increased Glutathione Substitution

SERRINE S. LAU, BARBARA A. HILL, ROBERT J. HIGHET, and TERRENCE J. MONKS

Division of Pharmacology and Toxicology, College of Pharmacy, The University of Texas at Austin, Austin, Texas 78712 (S.S.L., B.A.H.), Laboratory of Chemistry, National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, Maryland 20892 (R.J.H.), and The University of Texas System Cancer Center, Science Park-Research Division, Smithville, Texas 78957 (T.J.M.)

Received May 18, 1988; Accepted September 8, 1988

SUMMARY

The chemical reaction of 1,4-benzoquinone with glutathione results in the formation of adducts that exhibit increasing degrees of glutathione substitution. Purification of these adducts and analysis by ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy revealed the products of the reaction to be 2-(glutathion-*S*-yl)hydroquinone; 2,3-(diglutathion-*S*-yl)hydroquinone; 2,5-(diglutathion-*S*-yl)hydroquinone;2,6(diglutathion-*S*-yl)hydroquinone; 2,3,5-(triglutathion-*S*-yl)hydroquinone; and 2,3,5,6-(tetraglutatathion-*S*-yl)hydroquinone. The initial conjugation of 1,4-benzoquinone with glutathione did not significantly affect the oxidation potential of the compound. However, subsequent oxidation and glutathione addition resulted in the formation of conjugates that, dependent upon the position of addition, become increasingly more difficult to oxidize. Increased glutathione substitutions, which resulted in an increase in oxidation potentials, paradoxically resulted in enhanced nephrotoxicity. The triglutathion-*S*-yl conjugate was the most potent nephrotoxicant; the diglutathion-*S*-yl conjugates exhibited similar degrees of nephrotoxicity; the mono- and tetraglutathion-*S*-yl conjugates were not toxic. Thus, with the exception of the fully substituted isomer, the severity of renal necrosis correlated with the extent of glutathione substitution. The lack of toxicity of the fully substituted isomer is probably a consequence of its inability to alkylate tissue components. Thus, the conjugation of glutathione with quinones does not necessarily result in detoxification, even when the resulting conjugates are more stable to oxidation. The inhibition of γ-glutamyl transpeptidase by AT-125 protected against 2,3,5-(triglutathion-*S*-yl)hydroquinone-mediated nephrotoxicity. It is suggested that other extra-renal sites expressing relatively high levels of γ-glutamyl transpeptidase might therefore also be susceptible to hydroquinone-linked glutathione conjugate toxicity. This pathway might also contribute to the carcinogenicity and mutagenicity of certain quinones.

Quinones represent an important class of naturally occurring compounds that are found in plants, fungi, and bacteria, primarily as important components of the electron transport chains involved in cellular respiration and photosynthesis (1). The quinones of polycyclic aromatic hydrocarbons are prevalent as environmental contaminants and are present in automobile exhaust, cigarette smoke, and air particulates (2, 3). In addition, many foodstuffs contain quinones (1, 4) and many anticancer drugs of clinical and research interest possess the quinone nucleus. Quinones may also be mutagenic and carcinogenic (5). Thus, quinones clearly exhibit diverse biological activity. The reactivity of quinones resides in their ability to undergo redox cycling and create an oxidative stress (6) and/or react directly with cellular nucleophiles, in particular, protein and non-protein sulfhydryls (7). GSH is the major non-protein sulfhydryl present in cells, the nucleophilicity of which affords cells protection against electrophilic insult (8). However, there are relatively few studies on the addition of sulfur nucleophiles to quinones and on the biological consequences of such reactions (9).

We have recently shown that oxidation of 2-bromohydroquinone in the presence of GSH gives rise to a mixture of mono- and disubstituted GSH conjugates (10). Administration of the disubstituted conjugate to rats (30 μmol/kg, intravenously) caused extensive necrosis of renal proximal tubular cells. In contrast, administration of either of the three monosubstituted GSH conjugates resulted in significantly less toxicity than that caused by the disubstituted conjugate (10, 11). The finding that a diglutathionyl conjugate can be formed raised an important point. It demonstrated that the precursor monosubstituted

This work was supported in part by United States Public Health Service Grants GM 39338 (S.S.L.) and ES 04662 (T.J.M.) awarded by the National Institutes of General Medicine and Environmental Health Sciences. S.S.L. is a recipient of a PMA Foundation Faculty Development Award.

ABBREVIATIONS: AT-125, L-(αS-5*S*)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid; BUN, blood urea nitrogen; $E_{1/2}$, half-wave oxidation potentials; SGPT, serum glutamate pyruvate transaminase; HPLC, high performance liquid chromatography.

conjugate(s) can undergo further oxidation and react with a second molecule of GSH. Moreover, the addition of the second GSH molecule to 2-bromohydroquinone resulted in a product that was 30 times more potent than the unsubstituted hydroquinone. Thus, the initial conjugation of the quinone with GSH is not one of detoxication. In this respect, Nickerson *et al.* (12) have shown that both mono- and disubstituted GSH conjugates are formed during the chemical reaction between 1,4-naphthoquinones and GSH. Moreover, Blackhall and Thomson (13), in a study of the addition of thioglycolic acid to a series of simple quinones, found that sequential oxidation and addition took place readily, with only the completely substituted hydroquinone formed as the end product.

The observation that 2-bromo-(diglutathion-*S*-yl)hydroquinone caused nephrotoxicity was the first example of GSH conjugation to quinones mediating toxicity. In view of the ubiquitous nature of quinones, it seemed important to determine whether the conjugation of GSH with quinones might represent a general mechanism of toxicity of such compounds. To examine this possibility we have studied the reaction of a simple quinone, 1,4-benzoquinone, with GSH. The products formed include mono- di-, tri-, and tetrasubstituted GSH adducts. These compounds exhibited varying degrees of nephrotoxicity. With the exception of the completely substituted conjugate, the extent of toxicity was related to the extent of GSH substitution. The implication of the results are discussed with respect to the important role played by γ-glutamyl transpeptidase in facilitating the target-organ toxicity of these compounds. Moreover, this pathway might play a role in the carcinogenicity and mutagenicity of certain quinones.

Materials and Methods

Chemicals. Glutathione, aminooxyacetic acid, and probenecid were obtained from Sigma Chemical Co (St. Louis, MO). 1,4-Benzoquinone was a product of Fluka AG (Buchs SG, Switzerland). AT-125 (Acivicin; NSC 163501) was generously provided by the National Cancer Institute. All other chemicals were of the highest grade commercially available.

Animals. Male Sprague Dawley rats (Harlan Sprague-Dawley, Houston, TX; 140–170 g) were used for all experiments and were allowed food and water *ad libitum* before the experiments.

Synthesis of hydroquinone GSH conjugates. Glutathione (167 mM; 2 ml of distilled water) was added dropwise to 1,4-benzoquinone (167 mM; 2 ml of distilled water; dissolution was achieved by sonication) and the mixture was stirred at room temperature for 2 hr and allowed to stand at 4° for an additional 46 hr. The mixture was extracted twice with 3 volumes of ethyl acetate to remove residual 1,4-benzoquinone and hydroquinone formed by reduction. The aqueous phase was then lyophilized. The resulting product was purified by HPLC (Shimadzu LC-6A) by dissolving it in distilled water (100 mg/ml) and injecting aliquots (200 μl) onto a Whatman Magnum 9 ODS-3 reverse phase semipreparative column. The sample was eluted with a linear gradient of methanol/water/acetic acid (9:90:1 to 10:89:1), at a flow rate of 3 ml/min, over 30 min and monitored at 280 nm. Several UV-absorbing peaks were eluted from the column (Fig. 1). Individual peaks were collected from several injections of the lyophilized product and combined. The methanol content of each peak was allowed to evaporate at room temperature and the remaining aqueous fractions were then frozen in dry ice/acetone and lyophilized. Each of the resulting powders, when reanalyzed by HPLC, gave rise to a single UV-absorbing peak. The UV spectrum of each of the purified compounds was recorded on a Shimadzu UV-160 spectrophotometer.

NMR spectrometry. The spectra were determined on solutions of 5–10 mg of compound in 0.5 ml of D_2O. The ^1H and ^{13}C NMR spectra were recorded with a Varian XL200 Spectrometer at 200 MHz and 50 MHz, respectively, under conditions previously described (14).

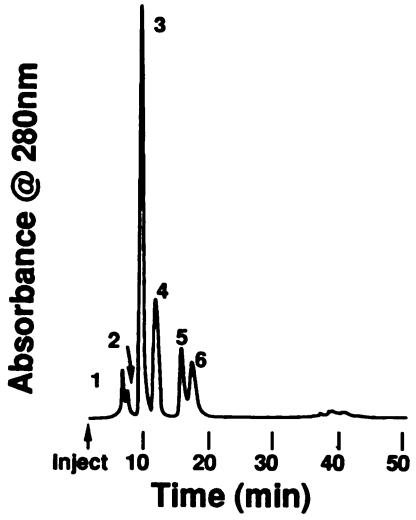

Fig. 1. HPLC elution profile of the products of the reaction of 1,4-benzoquinone with GSH. Chromatographic conditions consisted of a linear gradient of methanol/water/acetic acid (9:90:1 to 10:89:1) at a flow rate of 3 ml/min over 30 min. Peak 1, 2,3,5,6-(tetraglutathion-*S*-yl)hydroquinone, 6.9 min; peak 2, 2,3-(diglutathion-*S*-yl)hydroquinone, 7.3 min; peak 3, 2,3,5-(triglutathion-*S*-yl)hydroquinone, 9.4 min; peak 4,2-(glutathion-*S*-yl)-hydroquinone, 11.8 min; peak 5, 2,5-(diglutathion-*S*-yl)hydroquinone, 15.9 min; peak 6, 2,6-(diglutathion-*S*-yl)hydroquinone, 17.5 min.

Determination of oxidation potentials. Oxidation potentials were determined using HPLC with electrochemical detection (Shimadzu L-ECD-6A). The detector was equipped with a glassy carbon electrode and a silver/silver chloride reference electrode. The applied potential was varied over a range of +0.2 to +1.2V. Samples were dissolved in the mobile phase at a concentration of 10 μg/ml and 0.1 μg was injected onto a Partisil 5 ODS-3 reverse phase analytical column (Whatman, Clifton, NJ) and eluted with a mixture of 0.05 M potassium phosphate, methanol, and acetic acid (69:30:1), pH 4.0, at a flow rate of 1.5 ml/min. Peak areas were measured at each potential and variation between samples was less than 1%.

Toxicity studies. The various GSH conjugates of hydroquinone were dissolved in 200 μl of 0.85% phosphate-buffered saline and injected intravenously into rats at doses between 5 and 250 μmol/kg. Phosphate-buffered saline was injected into control rats. Some rats were pretreated with intraperitoneal injections of either AT-125 (10 mg/kg), aminooxyacetic acid (0.62 mmol/kg), or probenecid (0.88 mmol/kg) 1 hr before the administration of 2,3,5-(triglutathion-*S*-yl)hydroquinone (20 μmol/kg; intravenously). Probenecid was initially dissolved in 1 N NaOH and diluted with phosphate-buffered saline to a final pH of 6.8–7.0; AT-125 and aminooxyacetic acid were dissolved in phosphate-buffered saline. After 24 hr, a sample of blood (400 μl) was obtained via the retroorbital sinus of each rat. Plasma was separated by centrifugation and the degree of renal and hepatic damage was assessed by measuring BUN and SGPT concentrations, respectively. Livers and kidneys were removed and sections were prepared and immediately placed in buffered formalin. Histology slides were prepared and stained with hematoxylin and eosin.

Results

Characterization of hydroquinone-glutathione conjugates. The chemical reaction of 1,4-benzoquinone with GSH gave rise to several UV-absorbing peaks upon analysis by HPLC (Fig. 1). Purification of each of these peaks and analysis by ^1H NMR (Fig. 2) and ^{13}C NMR revealed the formation of

several isomeric hydroquinone-GSH conjugates. Structural identification was assigned by the following criteria. The presence of GSH in each of the compounds was shown by the characteristic aliphatic groupings at high field (10, 14–17). The ^{1}H spectrum of 2-(glutathion-*S*-yl)-hydroquinone, HPLC peak 4, showed the characteristic pattern of a 1,2,4-substituted benzene: H-3,6.78 ppm, d (J = 2 Hz); H-5, 6.94, dd (7 and 2 Hz); and H-6, 6.83, d (7Hz); cysteine α, 4.44 ppm, dd; β, 3.32 and 3.20 ppm, dd; Glu α, 3.73 dd; β, 2.10, dd; γ, 2.45, t; Gly α, 3.75, s. ^{13}C: carbonyls, 175.5, 174.8, 174.6, 173.0 ppm; C-1, 4, 150.4, 149.9; C-3,-5,-6, 121.2, 118.0, 117.6; C-2, 120.8; Cys α, 55.0; β, 35.4; Glu α, 54.78; β, 27.0; γ, 32.3; Gly α, 42.8.

Each of the bis-glutathionyl derivatives possess an element of symmetry rendering the proton spectra degenerate. 2,6-

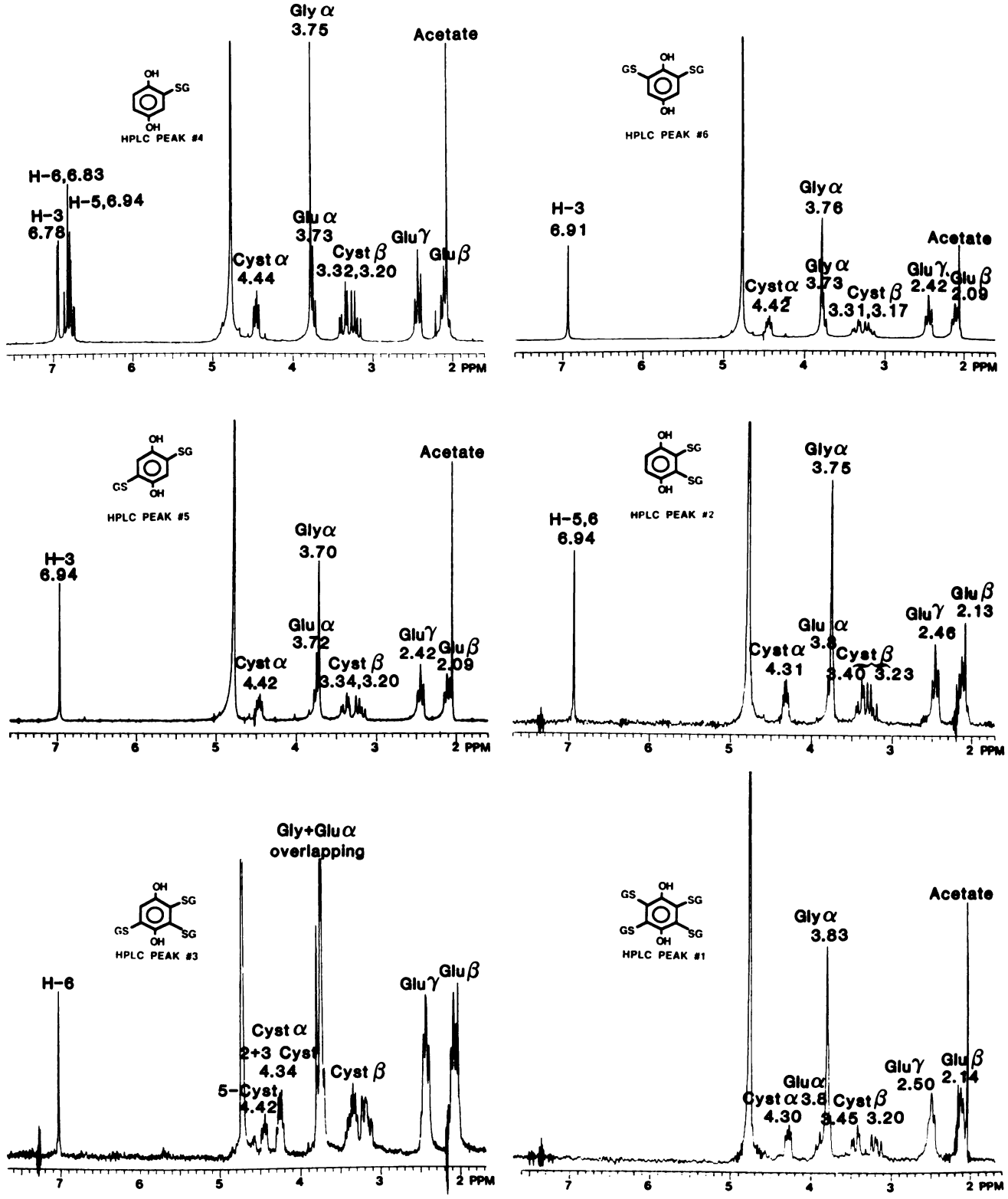

Fig. 2. ^{1}H NMR spectroscopy of hydroquinone-GSH conjugates.

(Diglutathion-*S*-yl)-hydroquinone, HPLC peak 6, exhibits an axis of symmetry through C-1 and C-4, which can be recognized by their different chemical shifts, C-1, 151.1; C-4, 150.7; carbonyls, 176.3, 175.4, 175.3, 173.8; C-3,5, 122.7; C-2,6, 122.2; Cys α, 54.9; β, 36.55; Glu α, 54.5; β, 27.9; γ, 33.1; Gly α, 44.0. The remaining two diglutathionyl isomers could not be differentiated in this way. 2,5-(Diglutathion-*S*-yl)hydroquinone, with a center of symmetry, and 2,3-(diglutathion-*S*-yl)hydroquinone, with a 2-fold axis or plane through the C-2, C-3 and C-5, C-6 bonds, possess only three types of aromatic carbon atoms. HPLC peak 5 was recognized as 2,5-(diglutathion-*S*-yl)hydroquinone by a difference nuclear Overhauser effect experiment. Irradiation of the aromatic proton produced an enhanced signal from the three protons of the cysteine residue. Chemical shifts were: carbonyls, 175.5, 172.7; C-1,4, 150.0; C-2, -5, 121.7; C-3, -6, 120.7; Cys α, 55.0; β, 35.2; Glu α, 54.1; β, 27.0; γ, 32.3; Gly α, 43.6. HPLC peak 2, the third material exhibiting 2-fold degeneracy, is therefore 2,3-(diglutathion-*S*-yl)hydroquinone, with chemical shifts supporting this assignment as follows: carbonyls, 175.5, 172.9; C-1, -4, 152.3; C-2, -3, 124.0; C-5, -6, 119.0; Cys α, 55.0; β, 36.6; Glu α, 54.4; β, 27.2; γ, 32.4; Gly α, 43.1.

HPLC peak 3 can be readily recognized as 2,3,5-(triglutathion-*S*-yl)hydroquinone. A single remaining proton appears in the aromatic region and two different multiplets attributable to Cys α protons appear at 4.34 and 4.42 ppm, with intensities of approximately 2 to 1. Chemical shifts were: carbonyls, 175.5, 174.8, 174.6, 173.0; C-1, -4, 150.4, 149.9; C-2, -3, -5, 124.8, 124.0, 123.9; C-6, 121.0; Cys α, Glu α, 54.9, 54.3, 54.2, 53.8; Cys β, 36.9, 36.6, 34.9; Glu β, 27.0; γ, 32.3; Gly α, 42.7.

The fully substituted character of HPLC peak 1, 2,3,5,6,-(tetraglutathion-*S*-yl)hydroquinone, is shown by the absence of aromatic protons and the extensive degeneracy of the carbon nuclei resulting from the three 2-fold rotation axes. Carbonyls, 174.6, 175.4, 173.0; C-1, -4, 153.6; C-2, -3, -5, -6, 127.0; Cys α, 54.9; β, 37.0; Glu α, 54.1; β, 27.0; γ, 32.4; Gly α, 42.8.

Throughout the series, glutathionyl groups without *ortho* protons show resonances from the α cysteinyl protons near 4.3 ppm (HPLC peaks 1, 2, and 3) and those with *ortho* protons show these resonances near 4.42 ppm (HPLC peaks 3, 4, 5, and 6). This observation provides further support for the assignment of the structure for HPLC peak 2. In all of the spectra, the carbon atom attributable to the β carbon of cysteine occurs some 10 ppm downfield from that of GSH (26.2 ppm) (16), demonstrating that the residue is bound to the aromatic ring by the sulfur atom. (The observed resonances of the carbon atoms were assigned to the amino acid residues following earlier work; in each case, the α carbon atoms of the glutamic acid residue and the cysteine residue could be interchanged.)

The UV spectra and molar extinction coefficients of the hydroquinone-GSH conjugates are shown in Fig. 3. The spectra are consistent with those reported for other GSH-substituted diphenols (10, 14). Each spectrum exhibited characteristic maxima at 231 nm and between 305 and 354 nm, with the position of the second peak shifted to longer wavelengths as the degree of GSH substitution increased.

Multiple glutathione addition to 1.4-benzoquinone. The majority of the reactions of quinones can be characterized as 1,4-reductive Michael additions (9). The initial hydroquinone product is, of course, susceptible to subsequent oxidation. In the present study, the initial addition of GSH to 1,4-benzoquinone results in the formation of hydroquinone and a monosubstituted GSH conjugate maintained in the oxidized form by the presence of excess quinone (Fig. 4A). As the concentration of GSH is increased and 1,4-benzoquinone concentrations decrease, the monosubstituted GSH conjugate begins to accumulate in the reduced form (Fig. 4, B and C) and di- and trisubstituted GSH adducts are formed at the expense of the oxidized form of the mono-GSH conjugate (Fig. 4C). The final pattern of products represents the reduced forms of each of the possible GSH addition products (Fig. 4D).

Electrochemistry of hydroquinone-glutathione conjugates. Hydrodynamic voltammograms of the various GSH conjugates are illustrated in Fig. 5. The voltammograms show that complete oxidation of all of the conjugates was achieved at +0.8–1.1 V. However, at lower potentials, substantial differences exist between the conjugates in the proportion residing in the oxidized or reduced form. For example, at an applied potential of +0.6 V, the per cent maximum response varies between 14 and 73%. The half-wave oxidation potentials ($E_{1/2}$) of the hydroquinone-GSH conjugates are shown in Table 1. There is an apparent correlation between the extent and position of GSH substitution and the $E_{1/2}$ such that there is a trend for the greater degree of substitution to result in higher oxidation potentials. Although the initial conjugation of GSH with 1,4-benzoquinone has little effect on the oxidation potential, subsequent oxidation and GSH addition results in adducts that become increasingly more difficult to oxidize. Thus, the $E_{1/2}$ of 2-(glutathion-*S*-yl)hydroquinone and of 2,5 and 2,6-(diglutathion-*S*-yl)hydroquinone are similar to that of unsubstituted hydroquinone (mean $E_{1/2}$ of these four compounds = 0.53 ± 0.02 V). The $E_{1/2}$ of 2,3-(diglutathion-*S*-yl)hydroquinone and 2,3,5-(triglutathion-*S*-yl)hydroquinone increases by approximately 0.15 V and the $E_{1/2}$ of 2,3,5,6-(tetraglutathion-*S*-yl)hydroquinone increases an additional 0.15 V. It appears that the addition of two GSH residues to one side of the quinone nucleus increases the $E_{1/2}$ by approximately 0.15 V and then addition to other side, to form the completely substituted isomer, increases the $E_{1/2}$ another 0.15 V. These data suggest that conjugation of quinones with GSH results in the formation of more redox-stable compounds only after multiple GSH addition.

Nephrotoxicity of hydroquinone-glutathione conjugates. The relative *in vivo* nephrotoxicity of the various (glutathion-*S*-yl)hydroquinone conjugates is shown in Fig. 6. Increases in the degree of GSH substitution, up to but excluding the completely substituted adduct, caused corresponding increases in nephrotoxicity as evidenced by elevations in BUN (Fig. 6) and histological alterations to the kidney (data not shown). The pathological changes observed were dose related and specifically localized and consisted of extensive coagulative necrosis of the proximal tubular cells in the cortico-medullary junction. Severe necrosis was localized to the S_3 segments of the proximal tubules, which contained eosinophilic cells with pyknotic nuclei. 2,3,5-(Triglutathion-*S*-yl)hydroquinone was the most potent nephrotoxicant and exhibited a steep dose-response curve. A dose of 20 μmol/kg was sufficient to cause a significant elevation of BUN. Each of the three disubstituted conjugates exhibited similar degrees of nephrotoxicity at doses of approximately 50 μmol/kg. Differences in the relative toxicity of the disubstituted conjugates were apparent at lower doses (30–50 μmol/kg), although the significance of these ob-

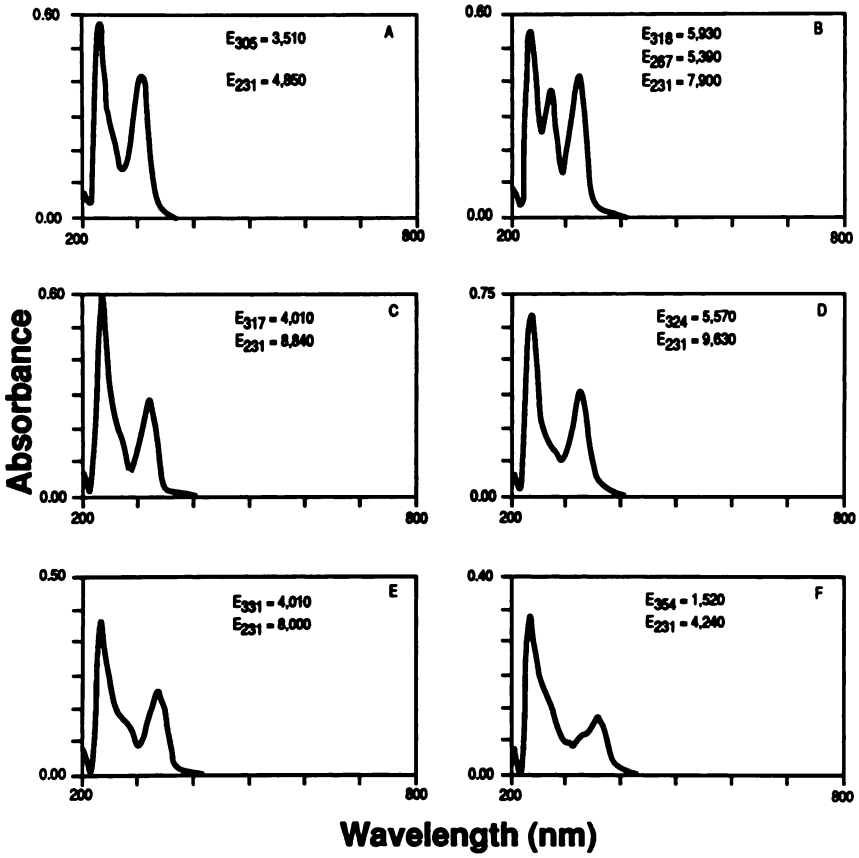

Fig. 3. UV absorbance spectra of hydroquinone-glutathione conjugates. Compounds were dissolved in 0.05 M phosphate/methanol/acetic acid (69:30:1) at a concentration of 1 mg/ml (49–120 μM). A, 2-(glutathion-*S*-yl)hydroquinone; B, 2,6-(diglutathion-*S*-yl)-hydroquinone; C, 2,5-(diglutathion-*S*-yl)hydroquinone; D, 2,3-(diglutathion-*S*-yl)hydroquinone; E, 2,3,5-(triglutathion-*S*-yl)hydroquinone; and F, 2,3,5,6-(tetraglutathion-*S*-yl)hydroquinone.

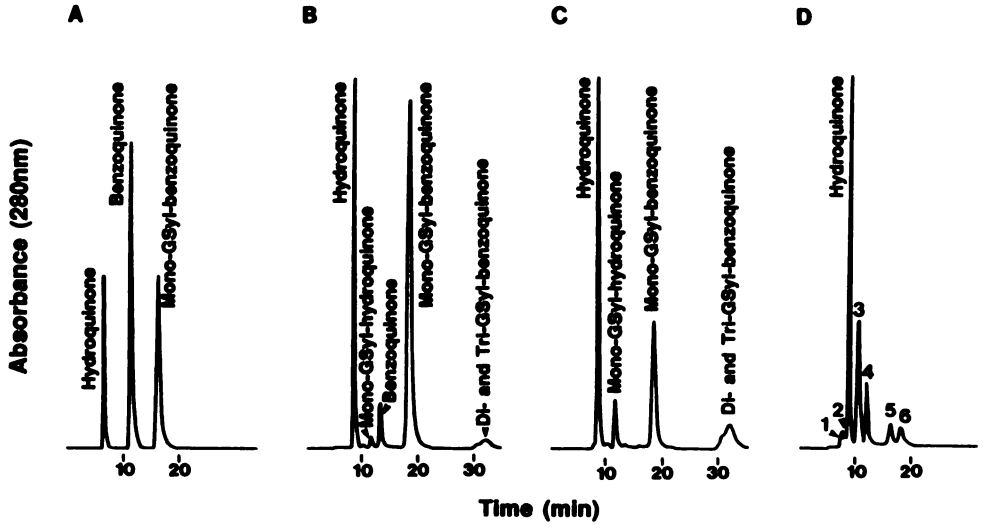

Fig. 4. Analysis of the products formed after the addition of increasing concentrations of GSH to 1,4-benzoquinone. GSH (334 μmol in 2 ml of water) was added dropwise to 1,4-benzoquinone (334 μmol in 2 ml of water) and 5–10-μl aliquots were analyzed by HPLC (condition as described in Fig. 1) after the addition of increasing amounts of GSH (A, 17 μmol of GSH; B, 117 μmol of GSH; C, 217 μmol of GSH; and D, 334 μmol of GSH). Peaks 1–6 in D are the same as those described in Fig. 1.

servations is unclear. The monosubstituted GSH conjugate required a dose of 250 μmol/kg before any increases in BUN appeared and it is clearly less toxic than either the tri- or disubstituted conjugates. The tetrasubstituted conjugate did not produce any signs of toxicity over the dose range studied (10–50 μmol/kg). Although the highest dose tested was only 50 μmol/kg, this dose is sufficient to cause significant elevations in BUN after administration of the tri- and disubstituted conjugates. The most potent nephrotoxicant, 2,3,5-(triglutathion-*S*-yl)hydroquinone, had no effect on SGPT values (Table 2) and none of the conjugates had effects on the liver, as determined by both macro- and microscopic examination or by monitoring SGPT concentrations (data not shown).

The role of both renal transport and renal metabolism in the nephrotoxicity of the most potent conjugate, 2,3,5-(triglutathion-*S*-yl)hydroquinone, was probed by using various inhibitors of these processes. The only agent capable of inhibiting toxicity, as evidenced by decreased BUN concentrations and by histological examination, was AT-125 (Table 2). AT-125 is an inhibitor of several glutamine-utilizing enzymes (18), including γ-glutamyl transpeptidase (19–21), and has previously been shown to inhibit the nephrotoxicity of various GSH conjugates (22). In contrast, aminooxyacetic acid, a general inhibitor of pyridoxal phosphate-utilizing enzymes including cysteine conjugate β-lyase, had no effect on 2,3,5-(triglutathion-*S*-yl)hydroquinone-mediated nephrotoxicity (Table 2). Moreover,

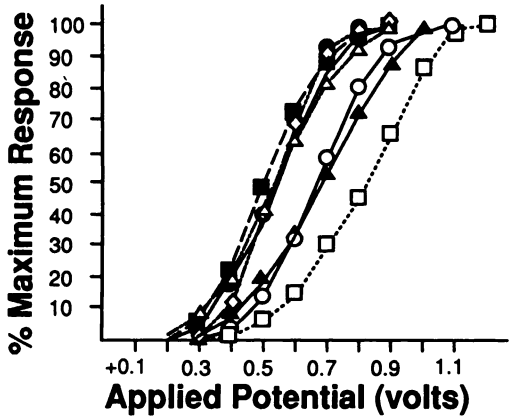

Fig. 5. Hydrodynamic voltammograms of hydroquinone-GSH conjugates. Compounds were dissolved in 0.05 M phosphate/methanol/acetic acid (69:30:1; pH 4.0) at a concentration of 10 μg/ml and 0.1 μg was analyzed by HPLC with electrochemical detection. The applied potential was varied from +0.2 to +1.2 V. ■, 2,5-(diglutathion-*S*-yl)hydroquinone; ●, 2-(glutathion-*S*-yl)hydroquinone; △, 2,6-(diglutathion-*S*-yl)hydroquinone; ◇, hydroquinone; ○, 2,3-(diglutathion-*S*-yl)hydroquinone; ▲, 2,3,5-(triglutathion-*S*-yl)hydroquinone; and □, 2,3,5,6-(tetraglutathion-*S*-yl) hydroquinone.

TABLE 1

Half-wave oxidation potentials of hydroquinone-glutathione conjugates

Oxidation potentials were determined by HPLC with electrochemical detection. Each compound was dissolved in a mixture of 0.05 M potassium phosphate (pH 4.0), methanol, and acetic acid (69:30:1) at a concentration of 10 μg/ml. The applied potential was varied from +0.2 to +1.2 V.

Compounds	$E_{1/2}$
	V
Hydroquinone	0.55
2-(Glutathion-*S*-yl)hydroquinone	0.53
2,5-(Diglutathion-*S*-yl)hydroquinone	0.51
2,6-(Diglutathion-*S*-yl)hydroquinone	0.54
2,3-(Diglutathion-*S*-yl)hydroquinone	0.67
2,3,5-(Triglutathion-*S*-yl)hydroquinone	0.69
2,3,5,6-(Tetraglutathion-*S*-yl)hydroquinone	0.83

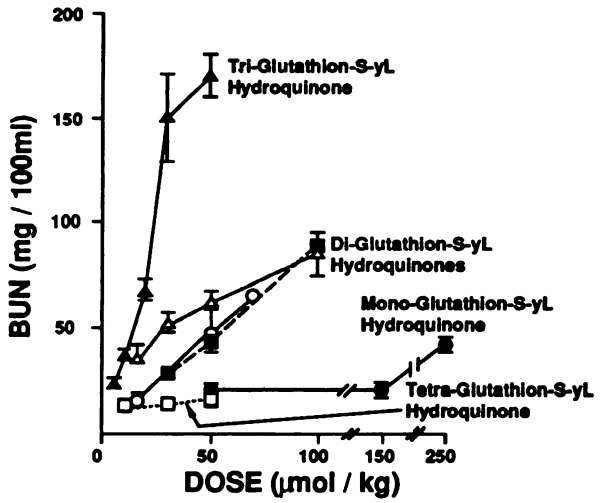

Fig. 6. Comparative nephrotoxicity of hydroquinone-GSH conjugates. ▲, 2,3,5-(Triglutathion-*S*-yl)hydroquinone; ■, 2,5-(diglutathion-*S*-yl)hydroquinone; △, 2,6-(diglutathion-*S*-yl)hydroquinone; ○, 2,3-(diglutathion-*S*-yl)hydroquinone; ●, 2-(glutathion-*S*-yl)hydroquinone; and □, 2,3,5,6-(tetraglutathion-*S*-yl)hydroquinone.

TABLE 2

The effect of γ-glutamyl transpeptidase, cysteine conjugate β-lyase, and organic anion transport inhibitors on 2,3,5-(triglutathion-S-yl)hydroquinone nephrotoxicity

$HQ-(GSH)_3$, 2,3,5-(triglutathion-*S*-yl)hydroquinone. AT-125 (10 mg/kg; intraperitoneally), probenecid (0.88 mmol/kg; intraperitoneally) or aminooxyacetic acid (0.62 mmol/kg; intraperitoneally) in 0.85% phosphate-buffered saline were given to rats either alone or 1 hr before $HQ-(GSH)_3$ (20 μmol/kg; intravenously). Control animals were given vehicle (0.85% phosphate-buffered saline) only. BUN and SGPT were determined after 24 hr according to Sigma Technical bulletins 535 and 505, respectively. Data represent the mean ± standard error (three experiments). Statistical analyses were performed by Student Newman Kuel's test.

Compound	BUN	SGPT
	mg/100 ml	*units/liter*
$HQ-(GSH)_3$	67.4 ± 2.9[a]	63.4 ± 1.5
AT-125 + $HQ-(GSH)_3$	15.6 ± 2.0[b]	50.3 ± 2.5
Probenecid + $HQ-(GSH)_3$	59.8 ± 8.4[a]	67.6 ± 4.3
Aminooxyacetic Acid + $HQ-(GSH)_3$	66.7 ± 4.3[a]	43.6 ± 2.2
AT-125	18.6 ± 2.6	49.4 ± 2.4
Probenecid	22.3 ± 0.5	60.1 ± 3.6
Aminooxyacetic Acid	29.3 ± 1.1	60.1 ± 9.9
Control	17.6 ± 3.0	55.7 ± 1.6

[a] Values are statistically significant when compared with control animals at $p < 0.001$.

[b] Value is significantly different from rats that received $HQ-(GSH)_3$ alone at $p < 0.001$.

in contrast to nephrotoxic aliphatic (haloalkane) GSH/cysteine conjugates, probenecid-sensitive, Na^+ dependent transport mechanisms do not appear to contribute to the nephrotoxicity of hydroquinone-GSH conjugates (Table 2) inasmuch as probenecid, an inhibitor of renal organic anion transport, had no effect on 2,3,5-(triglutathion-*S*-yl)hydroquinone nephrotoxicity.

Discussion

In the present manuscript we have shown that the reaction of 1,4-benzoquinone with GSH results in the formation of 2-(glutathion-*S*-yl)hydroquinone, 2,3-(diglutathion-*S*-yl)hydroquinone, 2,5-(diglutathion-*S*-yl)hydroquinone, 2,6-(diglutathion-*S*-yl)hydroquinone; 2,3,5-(triglutathion-*S*-yl)hydroquinone; and 2,3,5,6-(tetraglutathion-*S*-yl)hydroquinone (Fig. 2). Successive oxidations and GSH addition (Fig. 4) results in multisubstituted GSH conjugates, in which the extent and position of GSH addition determines the increase in oxidation potential (Table 1). Paradoxically, however, with the exception of the completely substituted hydroquinone, the severity of the renal necrosis correlated with an increase in the extent of GSH substitution (Fig. 6). Thus, the conjugation of GSH with hydroquinones does not necessarily result in detoxification, even when the resulting conjugate is more stable to oxidation. Rather, multiple GSH conjugation results in the formation of extremely potent and selective nephrotoxicants.

The selective nephrotoxicity of hydroquinone-linked GSH conjugates is probably a consequence of their selective uptake into cells containing γ-glutamyl transpeptidase (Table 2). Thus, the site of the proximal tubular necrosis correlates with the localization of this enzyme on the luminal brush border membrane. Moreover, the activity of γ-glutamyl transpeptidase is essential for the nephrotoxicity of hydroquinone-linked GSH conjugates inasmuch as inhibition of this enzyme by pretreatment with AT-125 completely protected animals from the nephrotoxicity of 2,3,5-(triglutathion-*S*-yl)hydroquinone (Table 2).

The activity of γ-glutamyl transpeptidase is probably required for the transport of the latent quinone into renal proximal tubular cells as the corresponding cysteine conjugate. GSH might therefore act as a carrier of redox-active compounds through the body, in which the initial conjugation can be followed by release and uptake of the reactive quinone at another site, catalyzed by γ-glutamyl transpeptidase. The preferential uptake of these compounds into cells rich in γ-glutamyl transpeptidase suggests that, in addition to the kidney, tissues such as the pancreas, spleen, and seminal vesicles might also be found to be susceptible to the toxic effects of these conjugates. Moreover, some preneoplastic cells are known to express elevated levels of γ-glutamyl transpeptidase (23–28). It seems possible that the targeting of quinone-linked GSH conjugates to such cells may play a role in the mutagenicity and carcinogenicity of some quinones. Conversely, the high concentrations of γ-glutamyl transpeptidase in tumors, in addition to its specific localization within the brush border membrane of the proximal convoluted tubules of the kidneys, has stimulated interest in the potential that this enzyme might be exploited for the site-selective delivery of drugs (29, 30). γ-Glutamyl derivatives of dopamine (31) and sulfamethoxazole (32) have been designed as kidney-specific prodrugs and those of phenylene diamine mustard (33, 34) and 3- and 4-aminophenol show potential as antitumor agents (35, 36). The potential chemotherapeutic activity of hydroquinone-linked GSH compounds might therefore be an area worthy of investigation.

The mechanism of toxicity of hydroquinone-linked GSH conjugates is unclear. The nephrotoxicity of various haloalkane-GSH/cysteine conjugates appears to be dependent upon their metabolism to a reactive thiol, catalyzed by renal cysteine conjugate β-lyase (37). However, metabolism of hydroquinone-linked GSH conjugates to reactive thiols by β-lyase is unlikely to be the mechanism of toxicity. In support of this view, pretreatment of rats with aminooxyacetic acid, a β-lyase inhibitor, did not protect them from 2,3,5-(triglutathion-*S*-yl)hydroquinone-mediated nephrotoxicity (Table 2). Moreover, structure-activity studies have shown that the selective nephrotoxicity of 6-bromo-2,5-dihydroxythiophenol, a putative β-lyase-catalyzed metabolite of 2-bromo-(glutathion-*S*-yl)hydroquinone conjugates, is a function of the quinone moiety; bromothiophenols were not nephrotoxic (38). Thus, toxicity is probably a result of the oxidation of the hydroquinone-linked conjugate to the quinone form and the relatively high oxidation potentials exhibited by these conjugates suggests that this oxidation may be enzymatic in nature. Subsequent interaction of the GSH and/or cysteine-linked quinone with critical cellular macromolecules, either by direct alkylation and/or by the oxidation of protein thiols, is a possible explanation of toxicity.

The initial interaction of GSH with quinones apparently does little to affect their oxidation potential (Fig. 5; Table 1). A similar observation has also been made with menadione and its GSH conjugate, 2-methyl-3-(glutathion-*S*-yl)-1,4-naphthoquinone (39), and the addition of aryl thiols to 1,4-benzoquinones results in only small differences in redox values (9). However 2-bromo-(diglutathion-*S*-yl)hydroquinone is more stable to oxidation than either of three mono-GSH-substituted isomers (11). In the present study we have shown that 2-(glutathion-*S*-yl)hydroquinone and 2,5- and 2,6-(diglutathion-*S*-yl)hydroquinone exhibit oxidation potentials approximately equal to that of hydroquinone itself (Table 1). Therefore, the initial conjugation of quinones with GSH does not result in the formation of compounds more stable to oxidation. However, the presence of a third GSH residue on hydroquinone results in a compound that is both more stable to oxidation and, paradoxically, more toxic. However, the conjugates that are more stable to oxidation will also be more difficult to reduce. Indeed, the ability of ascorbate to inhibit the *in vitro* covalent binding of 2-bromo-(glutathion-*S*-yl)hydroquinone conjugates correlated with their $E_{1/2}$ values (11). Thus, the toxic quinone-linked GSH conjugates are probably less susceptible to reduction by cellular antioxidants such as ascorbate, GSH, and NAD(P)H. This property increases their ability to interact with critical cellular macromolecules. Moreover, the lack of toxicity of 2,3,5,6-(tetraglutathion-*S*-yl)hydroquinone is probably due to a combination of its high oxidation potential and, perhaps more importantly, the inability of the resulting quinone to alkylate tissue macromolecules. The reason(s) for the differential nephrotoxicity of quinol-linked GSH conjugates exhibiting differing degrees of GSH substitution are unclear but may be due to differences in their oxidation potentials as described above and in their differential uptake into renal cells (40). Further studies are warranted to investigate these possibilities.

We have demonstrated that the conjugation of GSH with quinones (and possibly other redox active compounds) does not necessarily result in detoxification, even when the resulting conjugate is more stable to oxidation. The selective nephrotoxicity of these GSH conjugates is probably a consequence of their selective uptake into renal cells as the corresponding cysteine conjugate, mediated by γ-glutamyl transpeptidase, followed by their oxidation to the quinone. Thus, such conjugates might produce toxicity in other cells that exhibit high levels of γ-glutamyl transpeptidase. Such a mechanism may contribute to the mutagenicity and carcinogenicity of certain quinones. Finally, physiological, biochemical, and electrochemical factors mediate the differential nephrotoxicity of multisubstituted GSH-linked hydroquinone conjugates.

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Send reprint requests to: Terrence J. Monks, The University of Texas System Cancer Center, Science Park-Research Division, P.O. Box 389, Smithville, TX 78957.