Excretion of the Mercapturic Acid S-[2-(N\(^7\)-Guanyl)ethyl]-N-acetylcysteine in Urine following Administration of Ethylene Dibromide to Rats

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ABSTRACT

Administration of the carcinogen ethylene dibromide (EDB) to rats resulted in the urinary excretion of S-[2-(N\(^7\)-guanyl)ethyl]-N-acetylcysteine, which is derived from the nucleic acid adduct S-[2-(N\(^7\)-guanyl)ethyl]glutathione. This mercapturic acid was isolated from urine by reversed-phase and propyramino high-performance liquid chromatography and was quantified by measurement of fluorescence intensity. The urinary mercapturic acid was identified as S-[2-(N\(^7\)-guanyl)ethyl]-N-acetylcysteine on the basis of chromatography and UV, fluorescence, \(^1\)H nuclear magnetic resonance, and fast atom bombardment mass spectra, all of which were identical with the authentic synthesized material. The excretion of mercapturic acid into urine of rats given injections of various doses of EDB occurred in a dose-dependent, linear manner over the range of 0.5–37 mg EDB/kg body weight. A good correlation was found between the excretion of mercapturic acid and the (in vivo) formation of DNA adducts in liver and kidney DNA. The higher level of urinary mercapturic acid compared to the level of hepatic DNA adduct indicates that extra-hepatic DNA adducts and RNA adducts may contribute to the mercapturic acid production. The measurement of the mercapturic acid may provide a means of noninvasive estimation of DNA adducts derived from EDB exposure.

INTRODUCTION

EDB\(^3\) is mutagenic in a variety of test systems (1–3) and is a carcinogen that is capable of producing liver, lung, stomach, mammary, adrenal, spleen, skin, and kidney tumors in experimental animals (4–7). While epidemiology studies on humans exposed to high levels of EDB are inconclusive (8, 9), EDB is acutely toxic and has caused two human deaths (10).

EDB appears to require metabolic activation to exert its biological effects. While EDB is primarily metabolized via cytochrome P-450-mediated oxidation (11), several lines of experiments demonstrate that GSH S-transferase-mediated conjugation with GSH is involved in the genotoxic effects of this compound (1, 3, 12–16). The major DNA adduct formed via conjugation is S-[2-(N\(^7\)-guanyl)ethyl]GSH (13, 15–17). This DNA adduct is lost from DNA in vivo with a half-life of 70–100 h (15).

In this paper, we report the urinary excretion of S-[2-(N\(^7\)-guanyl)ethyl]-N-acetylcysteine from EDB-treated rats. The amount of this mercapturic acid excreted is dose-related and shows good correlation with the level of liver and kidney DNA adducts formed in vivo.

MATERIALS AND METHODS

Chemicals. Unlabeled EDB was purchased from Aldrich Co. (Milwaukee, WI) and [1,2-\(^14\)C]EDB (60 mCi/mmol) was obtained from New England Nuclear (Boston, MA). Hydroxylapistate was purchased from Calbiochem-Behring (San Diego, CA). All other chemicals used were of the highest quality commercially available.

Animals. Male Sprague-Dawley rats (200–220 g) were purchased from Harlan Industries (Indianapolis, IN) and housed in a controlled environment. The animals were maintained on Purina Lab Chow and tap water ad libitum.

Spectroscopy. FAB-MS analysis was done at the Vanderbilt Mass Spectrometry Laboratory by Dr. B. Sweetman. The spectra were obtained on a VG 70-250 system having extended geometry, a standard Iontech saddle field FAB gun producing xenon atoms of 8 kV energy, and a VG 11/250 data system. Glycerol was used as the matrix and the temperature was ambient. NMR spectra were obtained with a Bruker AM 400-NB Spectrometer (Billerica, MA) operating at 400.13 MHz for \(^1\)H. Chemical shifts are reported in ppm; DSS was used as an internal standard. UV spectra were determined in 1-cm pathlength quartz microcells using a Cary 210 spectrophotometer in the automatic baseline correction mode, and fluorescence spectra were recorded using a Varian SF-330 spectrofluorometer (Varian, Walnut Creek, CA).

Preparation of S-[2-(N\(^7\)-Guanyl)ethyl]-N-acetylcysteine. S-[2-(N\(^7\)-Guanyl)ethyl]-N-acetylcysteine was chemically synthesized using methods similar to those described by Peterson et al. (17). N-Acetylcysteine (9.8 mg, 0.06 mmol) was stirred with sodium (7.5 mg, 0.33 mmol) in 3 ml of dry CH\(_2\)OH. When the material was dissolved, N\(^2\)-(bromoethyl)guanosine [12.9 mg, 0.03 mmol; prepared by the method of Peterson et al. (17)] was added. After 3 h, the reaction was complete as judged by HPLC. The reaction was quenched by the addition of 0.1 M ammonium acetate and CH\(_2\)OH was removed under reduced pressure. The resting solution was heated at 100°C for 1 h to remove the ribose moiety. The mercapturic acid was purified by reversed-phase HPLC as described elsewhere (17). The corresponding fractions were collected and lyophilized to give 4.8 mg of product (47% yield): \(^1\)H-NMR (D\(_2\)O): \(\delta 1.91\) (s, 3H, -COCH\(_3\)), 2.67 (dd, 1H, \(\beta\)-CH\(_3\), \(J = 14, 8.0\)), 2.77 (dd, 1H, \(\beta\)-CH\(_3\), \(J = 14, 4.5\)), 2.99 (m, 2H, -SO\(_2\)-), 4.26 (dd, 1H, \(\alpha\)-CH, \(J = 4.5, 8.0\)), 4.44 (m, 2H, -NHCH\(_2\)-), 7.97 (s, 1H, CH=H). Other spectra are presented under "Results."

Isolation and Quantitation of Mercapturic Acid in Urine Sample. EDB was administered i.p. (in 0.2 ml of (CH\(_2\))\(_2\)SO) and urine samples were collected from rats housed individually in metabolic cages. After collection, urine samples were adjusted to pH 5 by the addition of 1 N HCl and then two volumes of isopropanol were added. Precipitated materials were removed by centrifugation at \(10^5 \times g\) for 10 min. The resulting supernatants were concentrated under reduced pressure and then slowly percolated through C18 Sep-Pak cartridges (Waters Associates, Milford, MA). The cartridges were then washed successively with H\(_2\)O (5 ml), 10% aqueous CH\(_3\)OH (5 ml), and 50% aqueous CH\(_3\)OH (5 ml). The last fraction (of each sample) was collected and concentrated in vacuo to 0.5 ml. Samples were loaded onto an octadeclisyl (C18) column (Whatman Partisil 5-ODS, 5 \(\mu\)m, 4.6 × 250 mm) with a mobile phase of 80 mM ammonium acetate buffer (pH 5.0) containing 5% CH\(_3\)OH. Elution was achieved by running a gradient of 5–40% CH\(_3\)OH over 40 min at a flow rate of 1 ml/min. A fraction was collected which eluted 3 min on either side of the retention time of the authentic mercapturic acid (\(t_r 19.2\) min). This fraction was dried by lyophilization, dissolved in 8 mM ammonium acetate buffer, and then applied to an aminopropyl HPLC column (Altex Ultrasil-NH\(_2\), 10 \(\mu\)m, 4.6 × 250 mm). A linear gradient of 8–760 mM ammonium acetate buffer (pH 7.0, in 80% CH\(_3\)OH) was used to elute the column (flow rate, 1.0 ml/min). The pertinent fractions, which eluted 3 min on either side of the authentic mercapturic acid peak (\(t_r 18.3\) min) were collected, and the final purification was done by passing the sample over a C18 column (Partisil ODS, vide supra) with a 10 mM KH\(_2\)PO\(_4\) buffer (pH 2.1).

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2 Burroughs Wellcome Scholar in Toxicology (1983-1988). To whom requests for reprints should be addressed.

3 The abbreviations used are: EDB, ethylene dibromide; HPLC, high-performance liquid chromatography; FAB-MS, fast atom bombardment-mass spectrometry; NMR, nuclear magnetic resonance; GSH, glutathione; DSS, sodium 2,2-dimethyl-2-silapentane-5-sulfonate.
containing 5% CH₃CN. Elution was done with a linear gradient of 5–15% CH₃CN over 20 min and then 15% CH₃CN for 10 min (flow rate, 1 ml/min).

The mercapturic acid was quantitated by measuring fluorescence intensity of HPLC eluates with a Kratos FS900 Spectrofluoro Monitor connected in line with the column (290-nm excitation wavelength, 370-nm emission filter).

Estimation of DNA Adduct. EDB was administered i.p. [in 0.2 ml of (CH₂)₂SO] to rats at various doses. After 24 h, the livers and kidneys were removed and DNA was isolated from those organs by CHCl₃-phenol extraction and hydroxylapatite chromatography as described elsewhere (16). Adducts were released by neutral thermal hydrolysis (14). The samples were then chromatographed on an octadecylsilane (C18) HPLC column (Whatman Partisil ODS, vide supra) with 50 mM NH₄H₂PO₄ buffer (pH 2.5) containing 5% CH₃OH. After 5 min, a linear gradient of 5–25% CH₃OH was used to elute the column over 20 min (flow rate, 1 ml/min). Quantitation of the DNA adduct was made by measuring fluorescence intensity (290-nm excitation wavelength, 370-nm emission filter) and making comparison with external standards (17).

RESULTS

Isolation and Characterization of Urinary 5-[(N³-Guanyl)ethyl]-N-acetylcysteine. Preliminary experiments using rat urine to which had been added authentic mercapturic acid (500 ng/ml) indicated that more than 90% of the added compound was recovered after HPLC chromatography. The HPLC system described allowed detection of the mercapturic acid at levels as low as 80 pmol with the use of fluorescence monitoring. Thus, this system was used to isolate and to quantify urinary mercapturic acids.

Administration of [1,2-¹⁴C]EDB to a rat resulted in the urinary excretion of a radiolabeled compound chromatographically identical to synthetic 5-[(N³-Guanyl)ethyl]-N-acetylcysteine (Fig. 1). Cochromatography of the purified urinary compound with a measured amount of authentic mercapturic acid under the same HPLC chromatographic conditions (Fig. 2) indicated that the compounds from the two different preparations eluted with the same retention time. Therefore, this compound was subsequently isolated from the urine of five rats injected i.p. with EDB (37 mg/kg) and then subjected to various spectral analyses.

The UV spectra of the urinary compound were nearly identical to that of authentic mercapturic acid (Fig. 3). The mercapturic acids retained the prominent UV absorbance of guanine with the absorption maximum at 252 nm shifted to a longer wavelength (compared to 249 nm in guanine). Both the standard and the urinary product showed a shift of the UV peak to 281 nm in alkaline solution (data not shown). When excited at 290 nm (pH 2.1), this urinary compound showed fluorescence over a range of 310–400 nm with a maximum at 367 nm (data not shown). These results are consistent with the view that the urinary compound contains guanine, probably derivatized at the N³ position.

FAB-MS spectrometry of the urinary compounds yielded a molecular ion at m/z 339 in the negative ion mode and at 341 in the positive ion mode (Fig. 4). The results are consistent with the molecular weight of 340 for 5-[(N³-Guanyl)ethyl]-N-acetylcysteine derived from the DNA adduct 5-[(N³-Guanyl)ethyl]GSH. For comparison, FAB-MS spectra of the authentic mercapturic acid were obtained under identical conditions at the same time (Fig. 4). The molecular ion peaks of urinary compounds in both ion modes were somewhat weaker than those of authentic mercapturic acids, but the major fragments and the molecular ions clearly support the assigned structure of the urinary compound.

NMR spectroscopy (Fig. 5) provided further confirmation of the urinary mercapturic acid. Acetyl protons are easily assigned as the highest field signal, a singlet at δ 1.91. Three four-line patterns (dd) appear at δ 2.63, 2.77, and 4.16. They show mutual coupling and can be assigned to the two cys β-protons and to the cys α-proton, respectively. The two methylene groups of ethylene bridge appear at δ 2.97 and 4.36 and are assigned as
the assignment is believed to be correct in this sample.

Excretion of Mercapturic Acid by Rats Administered EDB. Determination of the total amounts of mercapturic acid excreted by rats at various times following i.p. injection of EDB (37 mg/kg body weight) indicated that the excretion occurred in a linear fashion for the first 24-h period and continued at a slower rate to 48 h (Fig. 6). Because animal-to-animal variation was small and the excretion showed a linear pattern for 24 h after dosing, the amounts of mercapturic acid recovered during this time were used to generate a dose-response curve. The excretion of mercapturic acid occurred in a dose-dependent, linear manner over a dose range of 0.5–37 mg/kg, as shown in Fig. 7 (at 0.5 mg/kg, $[^{14}C]$EDB was injected in order to detect the urinary mercapturic acid, as fluorescence measurements were not sensitive enough). In order to examine the relationship between the amount of mercapturic acid excreted into urine and the level of DNA adduct formed in vivo, DNA adducts were also determined 24 h after dosing. The DNA adduct levels in liver and kidney still remained high 24 h after dosing, as the in vivo half-life of the hepatic DNA adduct is 70–100 h (15). The urinary excretion of mercapturic acid was proportional to the level of DNA adducts both in liver and in kidney (Fig. 7).

**DISCUSSION**

The importance of DNA adducts in chemical carcinogenesis has been a significant focus of cancer research since the electrophilic theory of carcinogenesis was proposed (for review see Ref. 18 and references therein). In addition to studies on the nature of DNA adducts, the development of detection methods for adducts has been pursued in order to improve risk assessment related to carcinogen exposure. The measurement of
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Fig. 5. 'H-NMR spectrum of the urinary mercapturic acid. The spectrum was recorded in D2O at 400 MHz with approximately 40 µg of sample (1620 scans).

Fig. 6. Time course of the excretion of mercapturic acids into urine from rats after EDB administration. Rats were injected with a dose of 37 mg EDB/kg body weight.

The major adduct formed from EDB in DNA in vitro and in vivo in rat liver or kidney is S-[2-(N7-guanyl)ethyl]GSH (13, 15–17). More than 95% of the binding of EDB to DNA has been shown to be in the form of S-[2-(N7-guanyl)ethyl]GSH (15). Although the role of this particular adduct in EDB-induced genotoxicity has not yet been proven to be critical, several lines of experiments (3, 22, 23), taken together with the dose-dependent formation of this adduct (Fig. 7), suggest that the extent to which it is formed may be correlated with the toxic and carcinogenic effect of EDB. Once this adduct is released from DNA in vivo, it is presumably excreted in urine after further processing to the mercapturic acid derivative (Fig. 8).

As indicated in this report, the urinary mercapturic acid isolated was clearly identified as S-[2-(N7-guanyl)ethyl]-N-acetylcysteine on the basis of its UV, fluorescence, 'H-NMR, and mass spectra and cochromatography with authentic mercapturic acid. Radioactivity was also eluted at the same HPLC retention time when [1,2-14C]EDB was administered to rats to form the urinary compound. The HPLC fluorescence detection method presented herein was shown to be both sensitive and reproducible, allowing quantitation of mercapturic acid excreted from rats given a dose as low as 2 mg EDB/kg. The dose-dependent excretion of mercapturic acid under experimental conditions used here and a good correlation with the extent of DNA adduct formed in vivo allows an estimate of the administered EDB dose (and adduct load) to be made on the basis of the amount of adduct excreted in urine.

The higher level of mercapturic acid compared to that of liver DNA adduct was somewhat unexpected, for the DNA adduct S-[2-(N7-guanyl)ethyl]GSH is relatively persistent in DNA. Our previous report indicated that the biological half-life of adduct is 70–100 h and is relatively constant among several different tissues examined (15). This half-life is somewhat shorter than the chemical half-life of 150 h measured in 50 mM KH2PO4 buffer (pH 7.0, 37°C) (15). It is not clear whether the release of adduct is due to enzymatic DNA repair. We have found that EDB can induce unscheduled DNA synthesis in primary human hepatocyte cultures4 and Nachtomi et al. (24) reported that DNA single-strand breaks are produced at high doses of EDB; these results suggest that the DNA adducts may be released partially as a result of DNA repair. About 20% of adduct is released during the first 24 h period after dosing, as judged by the experimentally measured half-life (15). Therefore, the excretion of mercapturic acid originates not only from liver DNA adducts, but apparently RNA adducts and extrahepatic DNA or RNA adducts may also contribute to the production of the mercapturic acid. We previously reported that S-[2-(N7-guany1)ethyl]GSH is found in extrahepatic tissues such as kidney, lung, and stomach (15). EDB also gives rise to RNA binding and RNA binding is greater than that of DNA (12, 24–26). We also recently found that binding of [1,2-14C]EDB to hepatic RNA in vivo was 1.5 times that of DNA binding (on the basis of adducts/mg nucleic acid, data not shown). RNA is present in rat liver in a quantity approximately five times greater than DNA (27). Therefore, the total amount of S-[2-(N7-guany1)ethyl]GSH in hepatic and kidney DNA and the excretion of mercapturic acids in the urine of rats administered with various doses of EDB. The data for DNA adduct represent the amount present in DNA 24 h after injection. Total amounts of DNA adducts were calculated on the basis of DNA content: 1.75 ± 0.18 mg/g liver, 2.03 ± 0.33 mg/g kidney (four animals, X ± SE). The levels of urinary mercapturic acid are those excreted within 24 h after injection.

Fig. 7. Comparison of dose responses for the formation of the DNA adduct S-[2-(N7-guanyl)ethyl]GSH in hepatic and kidney DNA and the excretion of mercapturic acids in the urine of rats administered with various doses of EDB.

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Fig. 8. Postulated scheme for bioactivation and degradation of EDB-derived DNA adducts.

References


13. Ozawa, N., and Guengerich, F. P.: Evidence for formation of an S-[2-(N7-guanyl)ethyl]GSH in RNA is approximately seven times higher than in DNA. The RNA adducts have not been fully characterized yet, though about 44% of RNA binding was demonstrated to be S-[2-(N7-guanyl)ethyl]GSH by HPLC analysis after acid hydrolysis. Although the stability of RNA adduct in vivo is not fully understood, it is clear that the amount of RNA adduct might also contribute to the mercapturic acid production and further studies will be required to evaluate the role of the RNA adducts.

The results presented in this paper were obtained under well-defined experimental conditions. It is not known how generally applicable this approach to risk assessment of EDB will be. More information about the relationship between excretion of mercapturic acid and the formation of DNA adduct in different systems is required. Furthermore, the sensitivity of this system to detect the mercapturic acid in urine needs to be enhanced in order to apply the approach to human risk assessment related to EDB exposure.

References


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