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Investigation of the Presence in Human Urine of Mercapturic Acids Derived from Phenanthrene, a Representative Polycyclic Aromatic Hydrocarbon

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Abstract

Polycyclic aromatic hydrocarbons (PAH) are environmental carcinogens implicated as causes of cancer in certain industrial settings and in cigarette smokers. PAH require metabolic activation to exert their carcinogenic effects. One widely accepted pathway of metabolic activation proceeds through formation of “bay region” diol epoxides which are highly reactive with DNA and can cause mutations. Phenanthrene (Phe) is the simplest PAH with a bay region and an excellent model for the study of PAH metabolism. In previous studies in which [D₁₀]Phe was administered to smokers, we observed higher levels of [D₁₀]Phe-tetraols derived from [D₁₀]Phe-diol epoxides in subjects who were null for the *glutathione-S-transferase M1* (*GSTM1*) gene. We hypothesized that Phe-epoxides, the primary metabolites of Phe, were detoxified by glutathione conjugate formation, which would result ultimately in the excretion of the corresponding mercapturic acids in urine. We synthesized the four stereoisomeric mercapturic acids that would result from attack of glutathione on Phe-epoxides followed by normal processing of the conjugates. We also synthesized the corresponding dehydrated metabolites and sulfoxides. These 12 standards were used in liquid chromatography-nanoelectrospray ionization-high resolution tandem mass spectrometry analysis of urine samples from smokers and creosote workers, the latter exposed to unusually high levels of PAH. Only the sulfoxide derivatives were consistently detected in the urine of creosote workers; none of the compounds was detected in the urine of smokers. These results demonstrate a new pathway of PAH-mercapturic acid formation, but do not provide an explanation for the role of *GSTM1* null status on Phe-tetraol formation.

Keywords

phenanthrene metabolism; mercapturic acids; human urine

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1. Introduction

Polycyclic aromatic hydrocarbons (PAH) are ubiquitous environmental pollutants formed during the incomplete combustion of organic matter. Multiple epidemiologic studies have investigated the relationship between exposure to PAH in occupational settings and the incidence of cancer [1]. The International Agency for Research on Cancer (IARC) concluded that there is sufficient evidence for the carcinogenicity to humans of occupational exposures which occur during coal gasification, coke production, coal-tar distillation, chimney sweeping, paving and roofing with coal-tar pitch, aluminum production, and carbon electrode manufacturing, all of which can entail considerable exposure to PAH [1]. Benzo[*a*]pyrene (BaP), the prototypic powerful PAH carcinogen, is also considered carcinogenic to humans by IARC [1]. Cigarette smoke is another significant source of PAH exposure, and these compounds are considered to be among the principal causes in smokers of lung cancer, a disease which kills 1.42 million people per year in the world [2–4].

PAH require metabolism to exert their carcinogenic effects [5–8]. Three general mechanisms of PAH metabolic activation have been studied in great detail: activation through the formation of bay region diol epoxides [5–7], through radical cation intermediates [9], or through redox cycling [10]. The study described here focuses on the bay region diol epoxide mechanism. Phenanthrene (Phe, **1**, Scheme 1) is the simplest PAH with a bay region. Although not carcinogenic, its structural features are ideal for investigating the role of bay region diol epoxides which are readily formed during its metabolism, as shown in Scheme 1 [11–14]. Thus, convincing evidence indicates that P450s stereoselectively catalyze the oxidation of the angular ring of Phe to Phe-1,2-epoxide (**2**) and Phe-3,4-epoxide (**12**), which are stereoselectively hydrated with catalysis by epoxide hydrolase (EH) to give Phe-(1*R*, 2*R*)-diol (**3**) and Phe-(3*R*,4*R*)-diol (**19**), established metabolites of Phe [15]. Further stereoselective oxidation of **3** and **19** by P450s produces the bay region diol epoxide Phe-(1*R*,2*S*)-diol-(3*S*,4*R*)-epoxide (**4**) and the “reverse diol epoxide” Phe-(3*S*,4*R*)-diol-(1*R*,2*S*)-epoxide (**20**), which upon hydrolysis give tetraols **5** and **21**, respectively. Alternatively, the simple epoxides **2** and **12** could be detoxified by reaction with glutathione ultimately producing sets of mercapturic acids such as **6**, **9**, **13**, **16** and **7**, **10**, **14**, **17** as well as their further oxidation products **8**, **11**, **15**, **18**. For the reasons described below, the possible metabolic formation of these three sets of mercapturic acid products is the focus of this study.

Only 11% of female and 24% of male lifetime cigarette smokers will get lung cancer by age 85 or greater, and this relatively small percentage is not due to competing causes of death from smoking [17]. A major goal of our research is to identify these susceptible smokers who could then be targeted for surveillance and early detection of lung cancer. We hypothesize that smokers who extensively metabolically activate PAH by the diol epoxide pathway will be at higher risk for lung cancer, all other factors considered equal. As a test of this hypothesis, we are administering [D₁₀]phenanthrene ([D₁₀]Phe, a single dose of 1 – 10 µg) to smokers and analyzing the amounts of [D₁₀]Phe-tetraols **5** and **21** excreted in 6h urine, a protocol which has been previously validated [18–20]. The use of [D₁₀]Phe allows us to focus only on the metabolic activation process, setting aside exposure variables such as

diet and polluted air which contain Phe and would complicate the interpretation of levels of unlabeled Phe-tetraols **5** and **21** excreted by smokers.

In one study of subjects to whom [D₁₀]Phe was administered, we found that individuals who were null for the *GSTM1* gene, among 12 genetic polymorphisms tested, had a significantly higher approximately doubled level of [D₁₀]Phe-tetraols **5** and **21** than did those who were *GSTM1* competent [18]. The simplest explanation for this phenomenon would be that *GSTM1* catalyzes the detoxification of Phe-diol epoxides **4** and **20**, competing with tetraol formation; therefore the null individuals would have higher levels of [D₁₀]Phe-tetraols **5** and **21**. However, we have previously analyzed smokers' urine for the mercapturic acids which would be formed by GST-catalyzed detoxification of **4** and **20**, and found that formation of these mercapturic acids was only a minor pathway, with levels less than 14% of the amounts of Phe-tetraols **5** and **21** [19;21]. These results indicated that interception of Phe-diol epoxides **4** and **20** by GSTs was not the reason for the higher Phe-tetraol levels in the *GSTM1*-null individuals. Therefore, in the study reported here, we have investigated the role of GSTs in the detoxification of Phe-1,2-epoxide (**2**) and Phe-3,4-epoxide (**12**), as interception of these simple Phe-epoxides at an earlier point in the metabolic process leading to Phe-tetraols **5** and **21** could also explain the observation that *GSTM1*-null individuals had higher [D₁₀]Phe-tetraol levels in their urine. We synthesized the anticipated mercapturic acid products **6**, **9**, **13**, and **16** which would arise from GST-catalyzed detoxification of Phe-epoxides **2** and **12**, as well as the corresponding dehydrated mercapturic acids **7**, **10**, **14**, and **17**, and sulfoxides **8**, **11**, **15**, and **18**. We then analyzed urine samples for these metabolites using liquid chromatography-nanoelectrospray-high resolution tandem mass spectrometry (LC-NSI-HRMS/MS).

1. Materials and Methods

1.1. Chemicals

All reagents were obtained from Sigma-Aldrich (St. Louis, MO).

1.2. General Synthetic Methods

Racemic phenanthrene-1,2-epoxide (**2**) and phenanthrene-3,4-epoxide (**12**) were prepared as previously described [22]. Compounds **6**, **9**, **13**, and **16** (each as a mixture of unseparable diastereomers) were synthesized by reaction of the epoxides **2** and **12** with *N*-acetyl-L-cysteine (NAC). Briefly, 150 mg of the epoxide was dissolved in 200 μ L THF and added to a solution of 32 mg of KOH and 59 mg of NAC in 200 μ L of MeOH kept at 0 °C. The mixture was stirred for 1 h at 0 °C and then was brought to room temperature and stirred overnight. The solvent was removed under reduced pressure, the residue was dissolved in 1 mL H₂O and extracted with 1 mL ethyl acetate 3 times. The products were purified by HPLC, and the concentrations of **9**, **13**, and **16** were quantified by NMR essentially as described previously [23]. Compound **6** was quantified by MS/MS, by comparison to compound **9**.

Compounds **7**, **10**, **14**, and **17** were prepared by dehydrating compounds **6**, **9**, **13**, and **16**, respectively, by treatment with formic acid. Briefly, 500 μ g of the starting material was dissolved in 1 mL H₂O and 100 μ L HCOOH was added at 50 °C. The mixture was stirred

for 1 h, and then the pH was adjusted to 7 with NH_4OH . The mixture was purified by HPLC to obtain the desired products. The identities of the resulting products were confirmed by LC-NSI-HRMS/MS.

Compounds **8**, **11**, **15** and **18** were synthesized by dissolving the mercapturic acids **7**, **10**, **14**, and **17** (0.158 mg) in glacial acetic acid (1 mL) and adding a 30% solution of H_2O_2 (1 μL) at 4 °C. The mixture was stirred for 1 h. The progress of the reaction was monitored by HPLC. After stirring overnight, all the starting material was converted to the corresponding sulfoxide. The solvent was removed under reduced pressure, and the residue was redissolved in 15 mM NH_4OAc . The crude mixture was purified by HPLC to give the corresponding products. The identity of the compounds was confirmed by NMR and/or LC-NSI-HRMS/MS.

Purification of the mercapturic acids—Purification of **6**, **7**, **9**, **10**, **13**, **14**, **16**, and **17** via HPLC was carried out using an Agilent 1100 capillary flow instrument with a diode array UV detector operated at 254 nm (Agilent Technologies, Palo Alto, CA). A 250 × 10 mm, 5 μm , C18 Luna column (Phenomenex, Torrance, CA) was eluted with 75% 15 mM NH_4OAc and 25% CH_3CN at a flow rate of 1 mL/min.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

characterization of the mercapturic acids—The synthesized compounds were analyzed using a Finnigan TSQ Quantum Discovery instrument (Thermo Electron, San Jose, CA) coupled to an Agilent 1100 Capillary HPLC system. The MS was operated in the negative ion ESI mode. A 250 × 10 mm, 5 μm , C18 Luna column and a Krudkatcher disposable precolumn filter (Phenomenex, Torrance, CA) were used. The column was eluted at 10 $\mu\text{L}/\text{min}$ with a linear gradient of CH_3CN in 15 mM NH_4OAc . CH_3CN was brought from 15% to 75% in 30 min, then the organic solvent was held at 75% for 5 min followed by a 14 min reequilibration at 15% CH_3CN . The ESI source was set in the negative ion mode as follows: voltage 5 kV; current 50 μA ; heated ion transfer tube 330 °C. The metabolites were measured by MS/MS using selected reaction monitoring (SRM) mode. The collision energy for all transitions was 15 eV, and the Ar collision gas pressure was 1.0 mTorr. The MS settings of the ion source were optimized using standard solutions of compounds **6** and **9**, **7** and **10**, and **8** and **11** to produce maximum MS/MS signals at m/z 356 → m/z 209, m/z 338 → 209 and m/z 354 → 225, respectively.

LC-NSI-HRMS/MS—The identity of the compounds was confirmed using a Nano LC-Ultra 2D HPLC (Eksigent, Dublin, CA) system equipped with a 5 μL injection loop, coupled with a LTQ Orbitrap Velos (Thermo Scientific, Waltham, MA). Separation was performed with a capillary column (75 μm ID, 10 cm length, 15 μm orifice) created by hand-packing a commercially available fused-silica emitter (New Objective, Woburn MA) with 5 μm Luna C18 bonded separation media (Phenomenex, Torrance, CA). The column was eluted at 1 $\mu\text{L}/\text{min}$ with 15% CH_3CN in 5 mM NH_4OAc for the first 5 min, then the flow was changed to 300 nL/min with a 15 min linear gradient from 15% to 45% CH_3CN followed by a 2 min hold at 90% CH_3CN to wash the column before returning to initial conditions. An injection volume of 2 μL per sample was used for the analysis.

The NSI source voltage was set at 2.3 kV, the capillary temperature was 350 °C, and the metabolites were quantified by HRMS/MS-selected reaction monitoring. Compounds **6**, **9**, **13** and **16** were analyzed in negative mode monitoring for the transition $m/z \rightarrow 209.0430$. The dehydrated compounds **7**, **10**, **14**, and **17** and the sulfoxide derivatives **8**, **11**, **15**, and **18**, were analyzed in positive mode monitoring for the transitions $m/z 340 \rightarrow m/z 209.0419$ and $m/z 356 \rightarrow m/z 209.0419$ respectively. The analysis was performed with accurate mass monitoring of fragment ions at 5 ppm tolerance utilizing the Orbitrap detector. These two parallel reaction monitoring events were performed using the HCD collision cell with 1 amu isolation width, collision energy of 50 and resolution set at 30,000 (at 400 amu) with an actual resolution of 55,000 (at 340 amu and 356 amu). A full scan event was also performed over a 100 – 500 amu range at a resolution setting of 30,000 to monitor for the accurate mass at 5 ppm of the protonated precursor ion of the analyte $m/z 340.1002$ and $m/z 356.0951$ to confirm analyte identity.

1.3. Spectral Data

1-(*N*-acetylcysteinyl)-2-hydroxy-1,2-dihydrophenanthrene (**6**). HRMS (NSI⁻) m/z calc'd 356.0962 [M – H]⁻, 338.0856 [M – H – H₂O]⁻, 227.0536 [M – CH₂CH(COOH)NHCOCH₃]⁻, 209.0430 [M – CH₂CH(COOH)NHCOCH₃ – H₂O]⁻, and 162.0230 [M – C₁₄H₁₁O]⁻; found m/z (relative intensity, %) 356.0963 (precursor ion), 338.0858 (5%), 227.0538 (20%), 209.0434 (100%), and 162.0233 (10%).

2-(*N*-acetylcysteinyl)-1-hydroxy-1,2-dihydrophenanthrene (**9**). ¹H NMR (500 MHz, DMSO-*d*₆) δ ppm 8.27 (d, J = 8.6 Hz, 1 H, C8-H), 7.90 (d, J = 7.3 Hz, 1 H, C5-H), 7.83 (d, J = 8.2 Hz, 1 H, C9-H), 7.48–7.57 (m, 3 H, C10-H, C6-H, and C7-H), 7.37 (d, J = 10.1 Hz, 1 H, C4-H), 6.66 (br. s., 1 H, N-H), 6.20 (dd, J = 10.2, 5.0 Hz, 1 H, C3-H), 5.84 (d, J = 4.9 Hz, 1 H, C1-OH), 4.77 (m, 1 H, C1-H), 3.94 (m, 1 H, -CH-COO), 3.72–3.76 (m, 1 H, C2-H), 3.04 (dd, J = 12.7, 5.0 Hz, 1 H, -S-CH₂), 2.81 (dd, J = 12.7, 6.0 Hz, 1 H, -S-CH₂'), 1.83 (s, 3 H, -CO-CH₃). COSY and HSQC spectra were consistent with these assignments. HRMS (NSI⁻) m/z calc'd 356.0962 [M – H]⁻, 338.0856 [M – H – H₂O]⁻, 227.0536 [M – CH₂CH(COOH)NHCOCH₃]⁻, 209.0430 [M – CH₂CH(COOH)NHCOCH₃ – H₂O]⁻, and 162.0230 [M – C₁₄H₁₁O]⁻; found m/z (relative intensity, %) 356.0961 (precursor ion), 338.0859 (4%), 227.0538 (21%), 209.0431 (100%), and 162.0234 (11%).

3-(*N*-acetylcysteinyl)-4-hydroxy-3,4-dihydrophenanthrene (**13**). ¹H NMR (500 MHz, DMSO-*d*₆) δ ppm 8.21 (d, J = 8.6 Hz, 1 H, C8-H), 7.88 (d, J = 8.3 Hz, 1 H, C5-H), 7.83 (d, J = 8.3 Hz, 1 H, C9-H), 7.56 (m, 1 H, C7-H), 7.47 (m, 1 H, C6-H), 7.35 (m, 1 H, C10-H), 6.62 (d, J = 9.2 Hz, 1 H, C1-H), 6.30 (br. s., 1 H, N-H), 6.13 (dd, J = 9.2, 5.8 Hz, 1 H, C2-H), 5.42 (s, 1 H, C4-H), 3.91 (m, 1 H, -CH-COO), 3.85 (d, J = 5.8 Hz, 1 H, C3-H), 2.99 (dd, J = 12.4, 4.4 Hz, 1 H, -S-CH₂), 2.90 (dd, J = 12.5, 5.8 Hz, 1 H, -S-CH₂'), 1.80 (s, 3 H, -CO-CH₃). COSY and HSQC spectra were consistent with these assignments. HRMS (NSI⁻) m/z calc'd 356.0962 [M – H]⁻, 338.0856 [M – H – H₂O]⁻, 227.0536 [M – CH₂CH(COOH)NHCOCH₃]⁻, 209.0430 [M – CH₂CH(COOH)NHCOCH₃ – H₂O]⁻, and 162.0230 [M – C₁₄H₁₁O]⁻; found m/z (relative intensity, %) 356.0961 (precursor ion), 338.0858 (4%), 227.0538 (23%), 209.0433 (100%), and 162.0235 (11%).

4-(*N*-acetylcysteinyl)-3-hydroxy-3,4-dihydrophenanthrene (**16**). ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ ppm 8.40 (d, $J = 8.5$ Hz, 1 H, C8-H), 7.85 (d, $J = 8.5$ Hz, 1 H, C5-H), 7.77 (d, $J = 8.2$ Hz, 1 H, C9-H), 7.55–7.60 (m, 1 H, C7-H), 7.43–7.50 (m, 1 H, C6-H), 7.32 (d, $J = 8.3$ Hz, 1 H, C10-H), 6.85 (br. s., 1 H, N-H), 6.67 (d, $J = 9.5$ Hz, 1 H, C1-H), 6.15 (dd, $J = 9.5$, 5.2 Hz, 1 H, C2-H), 5.08 (d, $J = 5.2$ Hz, 1H, C3-OH), 4.87 (s, 1 H, C4-H), 4.40–4.44 (m, 1 H, C3-H), 3.88–3.95 (m, 1 H, -CH-COO), 3.19 (dd, $J = 12.8$, 4.9 Hz, 1 H, -S-CH₂), 3.02 (dd, $J = 13.1$, 4.6 Hz, 1 H, -S-CH₂'), 1.84 (s, 3 H, -CO-CH₃). COSY and HSQC spectra were consistent with these assignments. HRMS (NSI^-) m/z calc'd 356.0962 [$\text{M} - \text{H}$]⁻, 338.0856 [$\text{M} - \text{H} - \text{H}_2\text{O}$]⁻, 227.0536 [$\text{M} - \text{CH}_2\text{CH}(\text{COOH})\text{NHCOCH}_3$]⁻, 209.0430 [$\text{M} - \text{CH}_2\text{CH}(\text{COOH})\text{NHCOCH}_3 - \text{H}_2\text{O}$]⁻, and 162.0230 [$\text{M} - \text{C}_{14}\text{H}_{11}\text{O}$]⁻; found m/z (relative intensity, %) 356.0961 (precursor ion), 338.0857 (4%), 227.0537 (18%), 209.0432 (100%), and 162.0235 (10%).

1-(*N*-acetylcysteinyl)phenanthrene (**7**). HRMS (NSI^+) m/z calc'd 340.1002 [$\text{M} + \text{H}$]⁺, 235.0576 [$\text{M} - \text{COOH} - \text{NH}_2\text{COCH}_3$]⁺, 209.0419 [$\text{M} - \text{CH}_2\text{CH}(\text{COOH})\text{NHCOCH}_3$]⁺, 162.0219 [$\text{M} - \text{C}_{14}\text{H}_9$]⁺, and 130.0499 [$\text{M} - \text{C}_{14}\text{H}_9\text{S}$]⁺; found m/z (relative intensity, %) 340.1000 (precursor ion), 235.0576 (45%), 209.0420 (100%), 162.0220 (8%), and 130.0498 (6%).

2-(*N*-acetylcysteinyl)phenanthrene (**10**). HRMS (NSI^+) m/z calc'd 340.1002 [$\text{M} + \text{H}$]⁺, 235.0576 [$\text{M} - \text{COOH} - \text{NH}_2\text{COCH}_3$]⁺, 209.0419 [$\text{M} - \text{CH}_2\text{CH}(\text{COOH})\text{NHCOCH}_3$]⁺, 162.0219 [$\text{M} - \text{C}_{14}\text{H}_9$]⁺, and 130.0499 [$\text{M} - \text{C}_{14}\text{H}_9\text{S}$]⁺; found m/z (relative intensity, %) 340.1002 (precursor ion), 235.0576 (53%), 209.0417 (100%), 162.0220 (7%), and 130.0498 (6%).

3-(*N*-acetylcysteinyl)phenanthrene (**14**). HRMS (NSI^+) m/z calc'd 340.1002 [$\text{M} + \text{H}$]⁺, 235.0576 [$\text{M} - \text{COOH} - \text{NH}_2\text{COCH}_3$]⁺, 209.0419 [$\text{M} - \text{CH}_2\text{CH}(\text{COOH})\text{NHCOCH}_3$]⁺, 162.0219 [$\text{M} - \text{C}_{14}\text{H}_9$]⁺, and 130.0499 [$\text{M} - \text{C}_{14}\text{H}_9\text{S}$]⁺; found m/z (relative intensity, %) 340.1001 (precursor ion), 235.0571 (42%), 209.0415 (100%), 162.0216 (8%), and 130.0496 (5%).

4-(*N*-acetylcysteinyl)phenanthrene (**17**). HRMS (NSI^+) m/z calc'd 340.1002 [$\text{M} + \text{H}$]⁺, 235.0576 [$\text{M} - \text{COOH} - \text{NH}_2\text{COCH}_3$]⁺, 209.0419 [$\text{M} - \text{CH}_2\text{CH}(\text{COOH})\text{NHCOCH}_3$]⁺, 162.0219 [$\text{M} - \text{C}_{14}\text{H}_9$]⁺, and 130.0499 [$\text{M} - \text{C}_{14}\text{H}_9\text{S}$]⁺; found m/z (relative intensity, %) 340.1002 (precursor ion), 235.0570 (39%), 209.0414 (100%), 162.0215 (7%), and 130.0495 (5%).

1-(*N*-acetylcysteinyl)phenanthrene sulfoxide (**8**). HRMS (NSI^+) m/z calc'd 356.0951 [$\text{M} + \text{H}$]⁺, 235.0576 [$\text{M} - \text{COOH} - \text{NH}_2\text{COCH}_3 - \text{O}$]⁺, 225.0369 [$\text{M} - \text{CH}_2\text{CH}(\text{COOH})\text{NHCOCH}_3$]⁺, 209.0419 [$\text{M} - \text{CH}_2\text{CH}(\text{COOH})\text{NHCOCH}_3 - \text{O}$]⁺, and 130.0499 [$\text{M} - \text{C}_{14}\text{H}_9\text{SO}$]⁺; found m/z (relative intensity, %) 356.0954 (precursor ion), 235.0571 (13%), 225.0363 (14%), 209.0414 (70%), and 130.0494 (100%).

2-(*N*-acetylcysteinyl)phenanthrene sulfoxide (**11**). HRMS (NSI^+) m/z calc'd 356.0951 [$\text{M} + \text{H}$]⁺, 235.0576 [$\text{M} - \text{COOH} - \text{NH}_2\text{COCH}_3 - \text{O}$]⁺, 225.0369 [$\text{M} - \text{CH}_2\text{CH}(\text{COOH})\text{NHCOCH}_3$]⁺, 209.0419 [$\text{M} - \text{CH}_2\text{CH}(\text{COOH})\text{NHCOCH}_3 - \text{O}$]⁺, and

130.0499 $[M - C_{14}H_9SO]^+$; found m/z (relative intensity, %) 356.0951 (precursor ion), 235.0572 (14%), 225.0365 (13%), 209.0416 (62%), and 130.0496 (100%).

3-(*N*-acetylcysteinyl)phenanthrene sulfoxide (**15**). Two diastereomers of **15** with respect to the sulfoxide chiral center were formed in equal quantities. Signals assigned to the corresponding diastereomeric protons are presented as δ/δ or as a range of the overlapping signals: 1H NMR (500 MHz, DMSO- d_6) δ ppm 9.08/9.26 (s/s, 1 H, C4-H), 8.85/8.89 (d/d, $J = 8.6$ Hz/ $J = 8.5$ Hz, 1 H, C5-H), 8.20/8.22 (d/d, $J = 8.2$ Hz/ $J = 8.2$ Hz, 1 H, C1-H), 8.05–8.08 (m, 1 H, C8-H), 7.95–7.99 (m, 2 H, C9-H and C10-H), 7.92/8.02 (d/d, $J = 8.5$ Hz/ $J = 8.2$ Hz, 1 H, C2-H), 7.75–7.80 (m, 1 H, C6-H), 7.69–7.75 (m, 1 H, C7-H), 6.68/7.28 (br. s./br. s., 1 H, -NH), 4.00/4.19 (m/m, 1 H, -CH-COO), 3.05–3.40 (m, 2 H, -S-CH₂), 1.77/1.78 (s/s, 3 H, -CO-CH₃). COSY spectra were consistent with the above assignments. HRMS (ESI⁻) m/z calc'd 354.0806 $[M - H]^-$ and 225.0380 $[M - CH_2CH(COOH)NHCOCH_3]^-$; found 354.0809 (precursor ion) and 225.0382 (100%). HRMS (NSI⁺) m/z calc'd 356.0951 $[M + H]^+$, 235.0576 $[M - COOH - NH_2COCH_3 - O]^+$, 225.0369 $[M - CH_2CH(COOH)NHCOCH_3]^+$, 209.0419 $[M - CH_2CH(COOH)NHCOCH_3 - O]^+$, and 130.0499 $[M - C_{14}H_9SO]^+$; found m/z (relative intensity, %) 356.0951 (precursor ion), 235.0573 (17%), 225.0366 (9%), 209.0417 (37%), and 130.0496 (100%).

4-(*N*-acetylcysteinyl)phenanthrene sulfoxide (**18**). HRMS (NSI⁺) m/z calc'd 356.0951 $[M + H]^+$, 235.0576 $[M - COOH - NH_2COCH_3 - O]^+$, 225.0369 $[M - CH_2CH(COOH)NHCOCH_3]^+$, 209.0419 $[M - CH_2CH(COOH)NHCOCH_3 - O]^+$, and 130.0499 $[M - C_{14}H_9SO]^+$; found m/z (relative intensity, %) 356.0950 (precursor ion), 235.0573 (12%), 225.0365 (13%), 209.0416 (65%), and 130.0496 (100%).

Analyses of mercapturic acid conjugates and their sulfoxides in human urine

Urine samples: A pooled smokers' urine sample was prepared by combining urine from 8 subjects, each of whom smoked about 20 cigarettes per day, and contributed between 150 and 320 mL from a 24 h urine collection. The smokers participated in ongoing studies at the University of Minnesota Tobacco Research Programs, approved by the Institutional Review Board. Urine samples from creosote workers were kindly provided by Dr. Mary Wolff (Mount Sinai Medical Center, New York). All samples were stored at $-20^\circ C$ until analysis.

Analysis of urine: The pooled smokers' urine sample was used as a positive control for method optimization. The sample (1 mL) was neutralized to pH 7 with NH_4OH and loaded onto an Oasis MAX cartridge (1 mL, 30 mg, Waters) previously equilibrated using 1 mL MeOH, and 1 mL 2% aqueous NH_4OH . The cartridge was then washed with the following solutions (% in H_2O): 1 mL of 2% NH_4OH , 1 mL MeOH, 1 mL 2% HCOOH, 1 mL 30% MeOH and 2% HCOOH, 1 mL 50% MeOH and 2% HCOOH. The cartridge was then eluted with four 1 mL aliquots of 90% MeOH and 2% HCOOH to collect the analytes. The four 1 mL fractions were combined and evaporated to dryness in a silanized vial on a Speedvac. The residue was dissolved in 20 μL of 15 mM NH_4OAc , and 1–2 μL were analyzed by LC-NSI-HRMS/MS-negative mode for compounds **6**, **9**, **13**, and **16**, and positive mode for compounds **7**, **10**, **14** and **17**, and **8**, **11**, **15**, and **18**. When purifying the samples for the

analysis of compounds **7**, **10**, **14** and **17**, the urine samples were added to 100 μ L HCOOH and heated at 50 $^{\circ}$ C for 1 h, before following the protocol described above.

2. Results

We investigated the presence in human urine of twelve potential metabolites of Phe (**1**, Scheme 1) which could result from detoxification of Phe epoxides **2** and **12** by the mercapturic acid pathway: epoxide ring opening products **6**, **9**, **13**, and **16**; their dehydrated analogues **7**, **10**, **14**, and **17**; and the corresponding sulfoxides **8**, **11**, **15**, and **18** (Scheme 1). Racemic Phe epoxides **2** and **12** were prepared as described [22]. Reaction of each epoxide with *N*-acetylcysteine produced the ring opened products **6**, **9**, **13**, and **16** (each as a mixture of unseparable diastereomers). Treatment of these products with formic acid yielded the dehydrated compounds **7**, **10**, **14**, and **17**. Oxidation of these compounds with H₂O₂ in acetic acid provided the sulfoxides **8**, **11**, **15**, and **18**. Each standard was purified by HPLC and their structures were confirmed by ¹H-NMR and/or HRMS/MS.

The synthesized compounds were used to optimize the conditions for analysis of these mercapturic acids in urine. A mixed-mode reversed-phase anion exchange (MAX) cartridge was used to desalt and enrich the mercapturic acid-containing fraction.

Standard solutions of compounds **13** and **16** in H₂O (50 fmol/mL) were analyzed by LC-MS/MS in the negative ion mode. The following transitions were monitored: m/z 356 $\rightarrow$ m/z 209 and m/z 356 $\rightarrow$ m/z 227. These transitions correspond to loss of the mercapturate moiety, with or without dehydration of the resulting hydroxyphenanthrene fragment. The areas of the corresponding peaks, with retention times of 19.3 min, were measured and their ratio was 20:1, with the dehydration transition (m/z 356 $\rightarrow$ m/z 209) being strongly favored. This ratio of fragment ions was consistent across each of our standards **6**, **9**, **13**, and **16**. The presence of these compounds was investigated in the pooled smokers' urine sample (4 mL). Peaks with the same retention time and MS transitions were observed, and the peaks co-eluted with the standards when co-injected, however the areas of the peaks corresponding to the two transitions monitored had a ratio of 1:10, as shown in Figure 1, indicating that these peaks were not mainly comprised of compounds **6**, **9**, **13**, and **16**. Further analysis of the same samples using LC-NSI-HRMS/MS in the negative ion mode which did not show any peak corresponding to the accurate mass of compounds **6**, **9**, **13** or **16** in the pooled smokers' urine sample.

We next investigated the possible presence in the pooled smokers' urine sample of the dehydrated mercapturic acids **7**, **10**, **14**, and **17**. The urine was treated with HCOOH to cause dehydration of any **6**, **9**, **13**, or **16** that might have been present, and analyzed using LC-NSI-HRMS/MS in positive ion mode. We could not confirm the presence of any peak corresponding to the accurate mass of the analyte. The same analysis was repeated following a more thorough purification of the pooled smokers' urine sample using HPLC with the gradient which we used to purify the standards. Fractions were collected starting at 5 min, and 5 fractions of 5 min each were collected. The collected fractions were analyzed for the mercapturic acids **6**, **9**, **13**, and **16** as well as their dehydrated analogues **7**, **10**, **14**, and **17** using LC-NSI-HRMS/MS, but no corresponding trace was detected.

Creosote workers are exposed to far greater amounts of Phe than smokers. Therefore, to further investigate the existence of the mercapturic acid detoxification pathway of Phe epoxides in humans, we analyzed urine samples from six creosote workers for the dehydrated metabolites **7**, **10**, **14**, and **17**. Six samples (1 mL) were treated with acid and purified following the method optimized for analysis of **7**, **10**, **14** and **17** in smokers' urine, followed by LC-NSI-HRMS/MS analysis in the positive ion mode.

This analysis of the transition for the expected mercapturic acids (compounds **7**, **10**, **14**, and **17**) revealed the presence of **14** in some samples, but not any other peak. However the transition for the corresponding sulfoxides **8**, **11**, **15**, and **18**, m/z 356.0951 $\rightarrow$ 209.04194 (positive ion mode), showed a clear signal in all samples. Recovery and detection limits for the sulfoxides were investigated. One mL aliquots of the pooled smokers' urine sample were spiked with the sulfoxide standards **15** and **18** in increasing amounts: 50 fmol, 100 fmol, 250 fmol, 500 fmol, and 1000 fmol. The same amounts were also spiked into 1 mL H₂O. The spiked samples were purified and enriched following the protocol optimized for the urine analysis, and analyzed by LC-NSI-HRMS/MS in the positive ion mode. The detection limit was 100 fmol of **15** or **18** per mL in urine, the on-column detection limit was approximately 10 fmol of each, and the overall recovery was 50% (in H₂O) and 5% (in urine).

The presence of these compounds in 1 mL urine samples from 6 creosote workers, 2 nonsmokers, and the pooled smokers' urine sample was investigated. Chromatograms from the analysis of a standard solution of sulfoxides **15** and **18** and from the analysis of a creosote workers' urine sample are illustrated in Figure 2A, B. Sulfoxides **8** and **11** also coeluted with **15** and **18**. A clear peak corresponding to the accurate mass of the analyte, matching the retention time of sulfoxide standards **8**, **11**, **15** and **18** was observed in all samples of creosote workers' urine. Our standard solution of compound **15** showed two peaks, which suggests that the sulfoxide diastereomers were separated by HPLC. HPLC analysis of compound **18** also showed two peaks, and these were very similar in retention time to the diastereomers of **15**. For this reason, it is unclear whether the two peaks in Figure 2B from the analysis of creosote workers' urine correspond to **8**, **11**, **15**, or **18**, or a combination of these compounds, and quantitative analysis was not performed. These sulfoxides were not detected in the pooled smokers' urine sample or in urine from non-smokers.

3. Discussion

The results of this study do not support our hypothesis that *GSTM1*-null smokers would be less able to detoxify Phe-epoxides **2** and **12** than *GSTM1*-competent subjects, therefore leading to the observed higher levels of Phe-tetraols **5** and **21** previously observed in *GSTM1*-null smokers. We found no evidence for the existence of a GST detoxification pathway of Phe-epoxides **2** and **12** in smokers, with a detection limit of approximately 0.1 pmol/mL, which would correspond to less than 3% of the amounts of Phe-tetraols **5** and **21** typically found in smokers' urine. We did however find convincing evidence for further oxidation of the anticipated mercapturic acids **7**, **10**, **14**, and **17** in analyses of urine from creosote workers exposed to high levels of PAH, by detection of sulfoxides typified by **8**, **11**, **15**, and **18**, although these were also non-detectable in the urine of smokers. Taking these

results together with our previous studies, neither GST detoxification of simple Phe-epoxides **2** and **12** nor of Phe-diol epoxides **4** and **20** can account for the observed higher levels of Phe-tetraols in *GSTM1*-null individuals.

It is possible of course that our previous observations regarding the effects of *GSTM1* null status on levels of [D₁₀]Phe-tetraols **5** and **21** after administration of [D₁₀]Phe were due to chance [18]. The study involved only 25 subjects who received 10 µg [D₁₀]Phe either orally or by smoking cigarettes to which [D₁₀]Phe had been added so that the cigarettes delivered approximately the same dose of [D₁₀]Phe as in the oral administration arm [19].

Nevertheless, we observed significantly higher levels of [D₁₀]Phe-tetraols **5** and **21** in both plasma and urine of the 12 *GSTM1* null subjects in that study, independent of the method of dosing, which adds considerable strength to the observation. In another study of Phe metabolism in 346 smokers who were genotyped for 11 polymorphisms in genes involved in PAH metabolism, including *GSTM1*, we found that the combination of *CYP1A1*462V and *GSTM1* null resulted in significantly higher levels of the ratio of Phe-tetraols to 3-hydroxy-Phe, an indicator of metabolic activation capacity [13]. We also observed that *GSTM1* null status in that study resulted in a nearly significant (*P* = 0.07) increase in the Phe-tetraols to 3-hydroxy-Phe ratio. Collectively, these data strongly suggest that there is a genuine effect of *GSTM1* null status on the metabolism of Phe to Phe-tetraols via the pathways illustrated in Scheme 1.

In the absence of convincing evidence for significant levels of detoxification of either the simple Phe-epoxides **2** and **12** or the diol epoxides **4** and **20** by the GST pathway, how can we account for the effect of *GSTM1* null status on levels of Phe-tetraols **5** and **21**? One possibility, not examined in this study, is further oxidation of the phenanthrene ring of the mercapturic acids **6**, **9**, **13**, and **16** or **7**, **10**, **14**, and **17**. There is precedent for the existence of such products. For example, *N*-acetyl-*S*-(phenacyl)cysteine has been identified as a minor metabolite of styrene in both human and rat urine [24]. It is also possible that *GSTM1* null status is in some way linked to other major pathways of Phe-epoxide or diol epoxide detoxification which were not thoroughly examined in this study.

Sulfoxides have been previously observed as human urinary metabolites of a number of compounds including acrylamide [25], furan [26], and the anti-cancer agent bendamustine [27]. These observations encouraged us to analyze for sulfoxides **8**, **11**, **15**, and **18** in this study. We obtained convincing evidence for the presence of these metabolites in the urine of creosote workers, but quantitation is uncertain. The average amount of Phe-tetraols **5** plus **21** in 26 urine samples from creosote workers analyzed in a previous study was 837 ± 692 pmol/mL urine, whereas levels of Phe-tetraols **5** plus **21** in smokers' urine averaged 4.60 pmol/mL [16]. Based on this ratio, we might expect only about 1/200th the amount of the sulfoxide metabolites in smokers' urine as in creosote workers' urine which may explain our inability to detect them in smokers' urine.

The workers exposed to creosote in this study were dock workers who employed creosote, a distillate of coal tar, as a wood preservative. Creosote is comprised mainly of PAH, particularly the lower molecular weight compounds such as naphthalene and phenanthrene [1]. The major exposure to these compounds in the dock workers appears to have been

through their skin [28–30]. The high exposure to PAH in creosote workers has been confirmed in multiple previous studies, mainly by analysis of 1-hydroxypyrene in urine [1]. Some studies have shown higher risks of skin cancer and lung cancer in creosote workers but the data are not consistent. The International Agency for Research on Cancer concluded that there is limited evidence in humans for the carcinogenicity of creosotes [1].

While extensive studies have investigated the detoxification of PAH diol epoxides by GSTs in various *in vitro* systems [31–35], there are to our knowledge only two reports, both from our laboratory, that have characterized human urinary mercapturic acids derived from PAH diol epoxides [19;21]. These reports quantified the mercapturic acid **22** derived from Phe-diol epoxide **20**. The corresponding mercapturic acid from Phe-diol epoxide **4** was not detected. As mentioned earlier, the levels of **22** in smokers' urine were quite low compared to the amounts of Phe-tetraols **5** and **21** [19;21]. We also characterized the mercapturic acid derived from Phe-9,10-epoxide in smokers' urine (mean 0.058 pmol/mL urine), and its formation was related to *GSTM1* status, being lower in the *GSTM1*-null individuals [36].

Collectively, our results indicate that mercapturic acid metabolites of phenanthrene, as well as the corresponding sulfoxides, are at most minor urinary metabolites in cigarette smokers. These results are surprising in view of the well-established activity of this pathway based on *in vitro* studies and the ease of detection of urinary mercapturic acids of other xenobiotics such as acrolein, acrylonitrile, and benzene [37]. One possible explanation for the low levels of these phenanthrene metabolites in human urine is that they are excreted mainly in feces, which were not collected or analyzed in this study.

The relationship between *GST* polymorphisms and PAH metabolism may be more complex than previously recognized. For example, in one recent study, we analyzed Phe tetraols **5** plus **21** in the urine of 2,097 cigarette smokers and found a highly significant relationship between number of *GSTT1* copies and levels of these metabolites ($P = 3.4 \times 10^{-16}$). However, the relationship was in a different direction than might have been expected – the levels of **5** plus **21** were *lowest* in the nulls and increased with increasing copies of *GSTT1*, which is opposite to our observations with *GSTM1* [38].

The synthesis of standards for this study was not trivial. Although the syntheses of the starting materials Phe-epoxides **2** and **12** have been previously reported [22], we note that these are multi-step methods resulting in relatively low overall yields. These syntheses start with tetrahydrophenanthrenones which must be prepared and separated to isolate the appropriate tetrahydrophenanthrene precursor from linear byproducts. This is followed by reduction of the tetrahydrophenanthrenones to the corresponding alcohols, dehydration to dihydrophenanthrenes, bromination, hydrolysis of the resulting dibromide to provide bromohydrin precursors, conversion of these to trifluoroacetates which are then brominated to provide dibromo esters, the immediate precursors to the labile Phe-epoxides **2** and **12**. Thus, considerable effort was invested in the preparation of the characterized standards **6**, **9**, **13**, **16**, **7**, **10**, **14**, **17**, **8**, **11**, **15**, and **18** for this project.

In summary, we have carried out a thorough investigation of potential mercapturic acid formation from Phe-epoxides **2** and **12**, using urine samples from cigarette smokers and creosote workers, the latter being exposed to unusually high levels of Phe and other PAH. Our results clearly demonstrate the existence of a new mercapturic acid pathway for PAH, illustrated by detection of sulfoxide metabolites. However, in most samples we were unable to detect the simple mercapturic acids hypothesized to be formed from Phe-epoxides **2** and **12**, which might have helped to explain the influence of *GSTM1* null status on levels of Phe-tetraols **5** and **21** in cigarette smokers.

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Nonstandard abbreviations

PAH	polycyclic aromatic hydrocarbons
<i>GSTM1</i>	<i>glutathione-S-transferase M1</i>
NAC	<i>N</i> -acetyl-L-cysteine
LC-NSI-HRMS/MS	liquid chromatography-nanoelectrospray-high resolution tandem mass spectrometry
LC-MS/MS	liquid chromatography-tandem mass spectrometry

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Highlights

- Human urine was analyzed for mercapturic acids from phenanthrene epoxides.
- The standards were synthesized from phenanthrene epoxides and *N*-acetylcysteine.
- The corresponding sulfoxide metabolites were also prepared.
- The sulfoxides were detected in the urine of highly exposed creosote workers.
- None of the metabolites were detected in the urine of cigarette smokers.

Figure 1.

Chromatograms obtained upon analysis of the pooled smokers' urine sample for compounds **13** and **16** via LC-MS/MS in negative mode with monitoring for the transitions m/z 356 $\rightarrow$ m/z 209 (top channel) and m/z 356 $\rightarrow$ m/z 227 (bottom channel). Panel A) standard **13** and **16**. The area ratio of the two peaks is of 20:1. Panel B) pooled smokers' urine sample. The area ratio of the two peaks is 1:10. Panel C) co-injection of the pooled smokers' urine sample and the standard.

Figure 2.

Extracted ion chromatograms from LC-NSI-HRMS/MS analyses of sulfoxides and MS/MS fragmentation spectra. A) Analysis for sulfoxides **8**, **11**, **15**, and **18** in creosote worker's urine. B) Synthetic standard of sulfoxide **15**. Proposed structures for the observed fragment ions are presented to the right.

Scheme 1.

Metabolism of phenanthrene (Phe) to intermediates and products discussed in the text.

Stereoselectivity in the formation of Phe epoxides and subsequent metabolites has been established based on previous studies (see for example [14], [15] and [16]). Compounds **6**, **9**, **13**, **16**; **7**, **10**, **14**, **17**; and **8**, **11**, **15**, **18** synthesized here were racemic mixtures.