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Quantitation of *N*-Acetyl-*S*-(9,10-dihydro-9-hydroxy-10-phenanthryl)-L-cysteine in Human Urine: Comparison with Glutathione-*S*-transferase Genotypes in Smokers

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Abstract

There are major inter-individual differences in carcinogenic polycyclic aromatic hydrocarbon (PAH) metabolism in humans, and it has been hypothesized that these differences may be related to cancer risk in smokers and other exposed people. One important pathway of PAH metabolism involves detoxification of epoxide and diol epoxide metabolites by reaction with glutathione, catalyzed by glutathione-*S*-transferases (GSTs). Interindividual differences in these pathways have been examined by genotyping methods, investigating polymorphisms in *GSTM1* and *GSTP1*. We are developing a phenotyping approach to assessing individual differences in PAH metabolism by quantifying human urinary metabolites of the ubiquitous PAH phenanthrene (**1**). In this study, we developed a method for quantitation of a mercapturic acid, *N*-acetyl-*S*-(9,10-dihydro-9-hydroxy-10-phenanthryl)-L-cysteine (PheO-NAC, **12**), the end product of reaction of phenanthrene-9,10-epoxide (**11**) with glutathione. [D_{10}]PheO-NAC was added to the urine as internal standard, and the PheO-NAC fraction was enriched by solid phase extraction. PheO-NAC was quantified by liquid chromatography-electrospray ionization-tandem mass spectrometry with selected reaction monitoring. The detection limit was approximately 4 fmol/mL urine. PheO-NAC was detected in the urine of 46 of 104 smokers, mean ($\pm$ S.D.) 57.9 ± 144 fmol/mL. PheO-NAC was detected significantly more frequently ($P < 0.0001$) in subjects who were *GSTM1* positive than in those who were *GSTM1* null, and the levels of PheO-NAC were significantly higher in the *GSTM1* positive subjects, consistent with a role for *GSTM1* in the detoxification of phenanthrene-9,10-epoxide. There were no significant relationships between PheO-NAC levels and the occurrence of two *GSTP1* polymorphisms. The results of this study provide the first evidence for a PAH-derived mercapturic acid in human urine and should be useful in the development of a phenotyping approach to assess individual differences in PAH metabolism.

Introduction

Polycyclic aromatic hydrocarbons (PAH) are considered to be among the most important carcinogens in cigarette smoke (1–3). Multiple PAH occur in smoke, and some of these are potent locally acting carcinogens, readily inducing tumors when applied to mouse skin or directly in the rodent respiratory tract (1). Fractions of cigarette smoke condensate enriched in PAH have tumor initiating activity (4). There is a similarity between the types of mutations observed in the *p53* gene in lung tumors from smokers and those seen in reactions of PAH diol epoxides with the *p53* gene in vitro, or in cells treated with benzo[*a*]pyrene (BaP), a prototypic

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PAH carcinogen (5,6). BaP has recently been classified as carcinogenic to humans by the International Agency for Research on Cancer (7).

PAH require metabolic activation to exert their carcinogenic effects, and there are competing detoxification pathways (8). Many studies have shown that there are large interindividual differences in human PAH metabolism, leading to the hypothesis that exposed individuals such as smokers who metabolically activate PAH effectively are at higher risk for cancer than those who detoxify these compounds effectively (9–16). This hypothesis has been tested in multiple investigations in the literature in which polymorphisms in PAH metabolizing genes, particularly *CYP* and *GST* genes, have been investigated with respect to cancer incidence in smokers (17–24). The results of these studies provide some support for the hypothesis that *CYP1A1* polymorphisms, particularly in combination with *GSTM1* null, may increase the risk for lung cancer in smokers.

PAH metabolism is extremely complex, with many different enzymes being involved (8). We believe that inter-individual differences in PAH metabolism are too complicated to be estimated by genotyping, and have therefore been developing a carcinogen metabolite phenotyping approach, in which PAH metabolites would be quantified in human urine. The advantage of this approach is that it integrates all genetic and environmental effects on PAH metabolism. We have focused on phenanthrene (Phe) metabolites in this research. Phe is the simplest PAH with a bay region, a feature closely associated with carcinogenicity, although Phe is not considered to be carcinogenic (25,26). We chose Phe because it is prevalent in the human environment and its metabolites are readily detected in urine, as opposed for example to those of BaP (27). In previous studies, we have described methods to quantify *r*-1,*t*-2,3,*c*-4-tetrahydroxy- 1,2,3,4-tetrahydrophenanthrene (PheT, **5**), the end product of the diol epoxide metabolic activation pathway common to many PAH, and phenanthrols (HOPhe, **6–10**) in human urine (Scheme 1) (28–31). In this study, we have focused on the detoxification of Phe by the glutathione-*S*-transferase (GST) pathway. Earlier reports demonstrated that a major mercapturic acid found in the urine of rats, rabbits, and guinea pigs dosed with Phe was *N*-acetyl-*S*-(9,10- dihydro-9-hydroxy-10-phenanthryl)-L-cysteine (PheO-NAC, **12**, Scheme 1) (32,33). Therefore, we have developed a method for quantitation of this metabolite in human urine. We are aware of no published studies on analysis of mercapturic acids of PAH in human urine.

Materials and Methods

Apparatus and chromatography

HPLC was carried out with a Waters system (Millipore, Waters Division, Milford, MA), equipped with a model 440 UV detector or a model 991 photodiode array detector. A 250 × 10 mm Phenomenex Luna 5 μm, C18 column (Phenomenex, Torrance, CA) was eluted with 65% 32 mM NH₄HCO₃ and 35% CH₃OH at a flow rate of 3 mL/min. This HPLC system was used for purification and identification of standard PheO-NAC and [D₁₀]PheO-NAC.

Oasis MAX and MCX LP extraction cartridges (6 cc, 500 mg) were purchased from Waters. Strata-X polymeric sorbent (33 μm, 30 mg/1 mL) was obtained from Phenomenex.

LC-ESI-MS/MS for characterization of PheO-NAC was carried out with a Thermo Finnigan LCQ Deca (Thermo Electron LC/MS Division, San Jose, CA) ion trap mass spectrometer interfaced with a Waters Alliance 2690 HPLC multi-solvent delivery system and equipped with an SPD-10 A UV detector (Shimadzu Scientific Instruments). A Prosphere 300 C18 guard column (7.5 × 4.6 mm, 5 μm, Alltech Associates Inc., Deerfield, IL) was connected to a Prosphere 300 PAH column (250 × 4.6 mm, 5 μm). The column was eluted with 32 mM NH₄HCO₃ and CH₃OH. The gradient program started with 100% 32 mM NH₄HCO₃ and the

proportion of CH₃OH was increased by 10% every 10 min for 60 min at a flow rate of 0.7 mL/min. The ESI source was set in negative ion mode as follows: voltage, 5 kV; current, 80 μ A; and capillary temperature, 275°C. MS/MS data were acquired with the following parameters: isolation width, 1.5; activation amplitude, 30.0%; activation Q, 0.25; and activation time, 30 min. The data were acquired and smoothed 7 times using the Gaussian algorithm provided with the ICIS data acquisition and processing software.

Quantitative LC-ESI-MS/MS for analysis of urine was performed using a Finnigan TSQ Quantum Discovery MAX or Ultra AM instrument (Thermo Electron, San Jose, CA) coupled to an Agilent 1100 Capillary HPLC system. The MS was operated in the negative ion ESI mode. An Agilent 1100 capillary flow HPLC (Agilent Technologies, Palo Alto, CA) equipped with a 250 $\times$ 0.5 mm 5 μ m C18 column (Agilent Zorbax SB-C18) and a Krudkatcher disposable pre-column filter (Phenomenex) was used. It was eluted with 0.1% aqueous HCOOH and CH₃CN, at 10 μ L/min. The gradient was as follows [% CH₃CN, time (min)]: 10%, 0–34 min; 28%, 34–45 min; 100%, 45–65 min. For confirmation of co-elution of PheO-NAC and [D₁₀] PheO-NAC in a second system, solvent A was 15 mM NH₄OAc and solvent B was CH₃OH. The gradient was as follows [% A, time(min)]: 90%–35%, 40 min; 0%, 15 min. The flow rate was 10 μ L/min. The ESI source was set in the negative ion mode as follows: voltage, 5 kV; current, 50 μ A; and heated ion transfer tube, 330 °C. The metabolites were measured by MS/MS using the selected reaction monitoring (SRM) mode. The collision energy for all of the transitions was 15 eV, and the Ar collision gas pressure was 1.0 mTorr. The MS settings of the ion source were optimized using a standard solution of PheO-NAC and [D₁₀]PheO-NAC to produce maximum signals at *m/z* 356 and *m/z* 366 respectively. For MS/MS, the settings were adjusted to obtain signal maxima at *m/z* 356 $\rightarrow$ 209 for PheO-NAC and *m/z* 366 $\rightarrow$ 218 for [D₁₀]Phe-NAC.

NMR spectra were obtained with a Varian Inova (Varian, Inc., Palo Alto, CA) instrument operating at 600 MHz and 25 °C.

Chemicals

N-Acetyl-*S*-(9,10-dihydro-9-hydroxy-10-phenanthryl)-L-cysteine (PheONAC, **12**) was prepared by the reaction of Phe-9,10-epoxide (**11**) (34) with *N*-acetyl-L-cysteine (Sigma-Aldrich, St. Louis, MO), essentially as described previously (35). The product was purified by HPLC to give 2 diastereomers of **12**. Spectral data for peak 1: UV, λ_{max} 209.8, 271.0; ¹H-NMR (DMSO-*d*₆) δ 7.86 (t, *J*=6.6 Hz, 2H, Phe-H4 and H5), 7.69 (br, 1H, NH), 7.39–7.25 (m, 6H, Phe-H1–3 and H6–8), 5.40 (br, 1H, OH), 4.68 (s, 1H, Phe-H9), 4.20 (s, 1H, Phe-H10), 4.15 (m, 1H, CHNH(COOH)), 2.92 (dd, *J*=4.2, 13.2 Hz, 1H, SCH₂a), 2.71 (dd, *J*= 7.2, 13.2 Hz, 1H, SCH₂b), 1.82 (s, 3H, CH₃); MS (negative ion) *m/z* (rel. intensity) 356 [M - H][−] (100); 338 [M - H₂O][−] (9); 227 [M - CH₂CH(COOH)NHC(O)CH₃][−] (29); 209 [M - CH₂CH(COOH)NHC(O)CH₃ - H₂O][−] (41). Spectral data for peak 2 were virtually identical to those of peak 1.

[D₁₀]PheO-NAC was prepared the same way from [D₁₀]Phe-9,10-epoxide, which in turn was synthesized from [D₁₀]Phe (Cambridge Isotope Laboratories, Andover, MA) (34). MS (negative ion) *m/z* (rel. intensity) 366 [M - H][−] (100); 237 [M - CH₂CH(COOH)NHC(O)CH₃][−] (30); 218 [M - CH₂CH(COOH)NHC(O)CH₃ - HDO][−] (31).

Subjects

The study was approved by the University of Minnesota Research Subjects' Protection Program Institutional Review Board Human Subjects Committee. Smokers were recruited through flyers posted on the University of Minnesota campus and near campus, and from advertisements in newspapers and on radio and television. Interested participants called the Tobacco Use Research Center and were told that the study would involve submission of urine

and blood samples. All subjects signed a consent form. One hundred and four subjects gave first morning urine samples and 30 mL of blood (collected in tubes containing EDTA). Nonsmokers were similarly recruited. Samples were stored at -20°C until analysis.

Analysis of PheO-NAC in urine

A 5 mL urine sample was placed in an 8 mL silanized vial (Chrom Tech Inc., MN) and 1 pmol/mL $[\text{D}_{10}]\text{PheO-NAC}$ was added as internal standard. HCOOH (200 μL) was added to the urine. The sample was loaded onto an Oasis MCX cartridge that was previously equilibrated using 6 mL CH_3OH and 6 mL 2% aqueous HCOOH . The cartridge was then washed with the following solutions (% in H_2O): 5 mL of 2% HCOOH , 5 mL of 10% CH_3OH -2% HCOOH , 5 mL of 20% CH_3OH -2% HCOOH , 3 mL of 30% CH_3OH -2% HCOOH and 2 mL of 30% CH_3OH . The cartridge was then eluted with 5 mL of 80% aqueous CH_3OH to collect the analyte. One mL of 1% aqueous NH_4HCO_3 was added to the 80% CH_3OH eluate and this fraction was evaporated to dryness on a Speedvac using a silanized vial.

The sample was redissolved in 1 mL H_2O and loaded on an Oasis MAX cartridge that had been previously equilibrated with 6 mL CH_3OH and 6 mL of 0.2N KOH . The cartridge was washed twice with 6 mL of 0.2N KOH , twice with 6 mL 0.01N methanolic KOH , twice with 6 mL 1M NH_4OAc , once with 6 mL CH_3OH , and once with 6 mL acetone. The cartridge was then washed with 6 mL of 2% HCOOH in acetone to elute PheO-NAC. One and a half mL of 15% NH_4HCO_3 was added to this fraction and it was evaporated to dryness on a Speedvac using a silanized vial.

For desalting, the sample was dissolved in 3 mL H_2O and loaded onto a Strata-X polymeric sorbent that was previously activated using 1 mL CH_3OH and 1 mL H_2O . The cartridge was washed with 1 mL H_2O and 1 mL of 10% CH_3OH . The analyte was eluted using 1 mL of 90% CH_3OH . This fraction was collected in a 2 mL silanized vial and concentrated to approximately 300 μL on the Speedvac. The solution was then transferred to an insert vial and concentrated to dryness. The residue was dissolved in 40 μL H_2O , and 5 μL was analyzed by LC-ESI-MS/MS-SRM. A calibration curve was constructed using 50–5000 fmol PheO-NAC and 1000 fmol $[\text{D}_{10}]\text{PheO-NAC}$.

Genotyping

This was performed using Masscode by Bioserve Biotechnologies, Laurel, MD., as described previously (28).

Statistical analysis

All statistical analyses were implemented using SAS statistical software, version 9.1. Socio-demographic characteristics were summarized by standard descriptive statistical methods. Statistical comparisons of overall arithmetic means among groups were made using the Kruskal-Wallis test with 5% significance level. Pearson's chisquare test was applied to test associations between categorical variables.

Results

The method for analysis of PheO-NAC is summarized in Scheme 2. $[\text{D}_{10}]\text{PheO-NAC}$ was added to the urine as internal standard. PheO-NAC was enriched from the urine by three consecutive solid phase extraction steps. The first used a mixed reverse-phase cation exchange (MCX) cartridge. The reverse-phase properties of this cartridge retained PheO-NAC. The second used a mixed reverse-phase anion exchange (MAX) cartridge. The anion exchange properties of this cartridge effected further purification of PheO-NAC. After elution of PheONAC from the MAX cartridge with HCOOH , the mixture was neutralized with NH_4OAc

to prevent acid catalyzed dehydration during removal of solvents. A Strata-X cartridge was then used to remove the salts formed in this step. The appropriate fraction from the Strata-X cartridge was analyzed by LC-ESI-MS/MS-SRM for m/z 356 $\rightarrow$ 209 (PheO-NAC) and m/z 366 $\rightarrow$ 218 ([D₁₀]PheO-NAC). A typical chromatogram from a smoker's urine positive for PheO-NAC is illustrated in Figure 1. A clean peak matching the retention time of standard PheO-NAC was observed. This peak eluted 0.4–0.5 min later than the internal standard, [D₁₀]PheO-NAC. Further evidence for the identity of the analyte was obtained in a second HPLC system, which separated the two diastereomers of *trans*-PheO-NAC (36). The analyte diastereomers again matched the retention time of standard PheO-NAC, and eluted 0.4–0.5 min later than those of the internal standard (Figure 2). Similar chromatograms were obtained for all positive samples, including those from non-smokers. A chromatogram from a urine sample in which PheO-NAC was not detected is illustrated in Figure 3.

A calibration curve showing the relationship between ratios of PheO-NAC:[D₁₀]PheONAC and LC-ESI-MS/MS response is illustrated in Figure 4. The precision of the analysis was determined by replicate analyses of 8 aliquots of a non-smoker's urine sample, giving a relative standard deviation of 5.1%. The detection limit of PheO-NAC was approximately 4 fmol/mL urine. Recovery averaged 65%.

Our main focus in this study was analysis of PheO-NAC in the urine of smokers and the relationship of these data to *GST* genotypes. Urine samples from 104 smokers were analyzed. The smokers had a mean age of 38.3 ± 12.4 (S.D.) years (range 18 – 69). They were 36% male, 65% Caucasian, 32% African-American, and 3% other, and they smoked an average of 20.3 ± 9.2 cigarettes per day (range 5 – 60).

Forty-six of the 104 smokers' urine samples were positive for PheO-NAC, having analyte levels above 4 fmol/mL (Table 1). Levels of PheO-NAC in the positive samples ranged from 7 – 880 fmol/mL. The mean ($\pm$ S.D.) of all samples from smokers was 58 ± 140 fmol/mL (using a value of zero for the samples with no detectable analyte). There was no relationship between urinary PheO-NAC levels and number of cigarettes smoked per day, examined in categories of 1–15, 16–25, and 26–60 cigarettes per day. Levels of PheO-NAC were compared to those of PheT (5) and total HOPhe (6–10) in the same samples, which were 5.79 ± 8.00 pmol/mL and 4.14 ± 3.00 pmol/mL, respectively, approximately 100 times greater than PheO-NAC. Levels of PheO-NAC correlated weakly with those of PheT ($R=0.21$, $P=0.03$) and total HOPhe ($R=0.22$, $P=0.03$). Urine samples from 6 non-smokers were also analyzed; 5 were positive for PheO-NAC, with values ranging from 160 – 680 fmol/mL.

The relationship between PheO-NAC in urine and *GST* genotypes was investigated in the smokers. *GSTM1* genotype data were available for 102 subjects, of whom 44 were *GSTM1* positive and 58 had the null genotype. PheO-NAC was detected significantly more frequently ($P<0.0001$) in subjects who were *GSTM1* positive (30 of 44 subjects, 68%) than in those who were *GSTM1* null (15 of 58 subjects, 26%). Levels of PheO-NAC were significantly higher in smokers who were *GSTM1* positive (110 ± 210) than in those who were *GSTM1* null (16 ± 32 , $P<0.0001$).

For the *GSTP1* I105V polymorphism, there were 29 subjects with the normal genotype, 60 heterozygotes, 12 homozygotes, and 3 uncharacterized. For the *GSTP1A*114V polymorphism, there were 85 normals, 17 heterozygotes, no homozygotes, and 2 uncharacterized. There were no significant relationships between PheO-NAC levels and the occurrence of either of these polymorphisms, when comparing heterozygotes, homozygotes, and normals.

Discussion

The results of this study provide convincing evidence for the presence of a PAH mercapturic acid, PheO-NAC (**12**, Scheme 1), in human urine. The identity of **12** is supported by the following observations. First, the solid phase extraction properties of the analyte were consistent with those of standard **12**. Second, LC-ESI-MS/MS with SRM for m/z 356 $\rightarrow$ 209, corresponding to the fragmentation pattern shown below, produced a clean peak in positive urine samples, and this peak had the same relative retention time as standard **12**, determined by comparison to that of the internal standard, [D₁₀]PheO-NAC (Figure 1).

Third, chromatography in a second system, which separated the diastereomers of **12**, again demonstrated that the analyte eluted at the proper retention time for standard PheO-NAC, as determined by comparison to the internal standard (Figure 2). The different ratios of isomer 1 and isomer 2 in the analyte vs. the internal standard (Figure 2) probably result from differing stereochemistry of addition in the enzymatic reaction vs. in the synthesis of [D₁₀]PheO-NAC from Phe-9,10-epoxide and *N*-acetyl-L-cysteine. Quantitation of this metabolite provides the first method for phenotyping human PAH metabolism by the mercapturic acid pathway.

Bay region and fjord region diol epoxides of PAH, which are major ultimate carcinogens of compounds such as BaP and benzo[*c*]phenanthrene, are good substrates for human GSTA1, GSTP1, and GSTM1 enzymes, representing cytosolic GSTs of the alpha, pi, and mu classes, based on extensive in vitro studies (37–40). These studies demonstrate that, depending on stereochemistry and other factors, PAH diol epoxides should be detoxified by GSTs in humans. Similar studies do not appear to have been performed with *anti*-PheDE (**4**). Thus, GSTs can be expected to be protective against PAH carcinogenesis in smokers and other exposed individuals. While a phenotyping method for the mercapturic acids resulting from detoxification of *anti*-PheDE by GSTs would likely be a more direct way of assessing this detoxification pathway in humans, we have carried out our initial work on PheO-NAC because studies in laboratory animals indicated that this mercapturic acid might be more readily detected. We are presently investigating methods for analysis of mercapturic acids derived from *anti*-PheDE.

We found a highly significant relationship between detectability of PheO-NAC in the urine of smokers and *GSTM1* genotype. Furthermore, the levels of PheO-NAC were significantly higher in the urine of *GSTM1* positive individuals than in those with *GSTM1* null. These results are completely consistent with in vitro studies, beginning with those of Warholm, *et al.* (41), which demonstrate that GSTM1-1 is a good catalyst of PAH epoxide detoxification, as well as for related compounds such as *trans*-stilbene oxide (37–39,42). Nevertheless, it should be noted that PheO-NAC was detectable in 26% of the *GSTM1* null individuals and was not detected in 32% of the individuals with *GSTM1* present. The detection of PheO-NAC in *GSTM1* null individuals is undoubtedly due to the presence of other GST enzymes, as well as possible non-enzymatic formation of this metabolite. Its lack of detection in *GSTM1* competent individuals could be influenced by the flux of metabolites through the Phe-9, 10-epoxide pathway. We observed no relationship between polymorphisms in the *GSTP1* gene and levels of PheO-NAC in urine. The *GSTP1*I105V polymorphism codes for a protein with higher activity toward several PAH diol epoxides than the common form (43). Further processing of

the initially formed GSH conjugates to mercapturic acids might also affect the amount of PheONAC in urine (44).

The lack of correlation between cigarettes per day and PheO-NAC levels was not surprising. First, there are significant environmental sources of Phe exposure other than cigarette smoke. In studies on PheT and HOPhe in human urine, we have detected these metabolites in every sample, be they from smokers or non-smokers (29–31). While PheT levels were higher in smokers than non-smokers, HOPhe levels were not (29,30). Based on the limited number of non-smokers analyzed in this study, there do not appear to be major differences between levels of PheO-NAC in the urine of smokers and non-smokers. The effects of cigarette smoking on levels of Phe metabolites in urine are blunted by alternate sources of exposure to Phe as well as inter-individual differences in metabolism.

Carcinogen metabolite phenotyping may be an effective way to determine the relationship of PAH metabolism to cancer risk. With the development of the PheO-NAC assay described here, we now have three assays for urinary Phe metabolites - PheT, HOPhe, and PheONAC - that can be used in this approach. In ongoing studies, we have demonstrated that the PheT:HOPhe ratio is reasonably stable over time in most subjects, and that this ratio varies widely in smokers (28,29). The PheT:HOPhe ratio is an activation:detoxification ratio, and PheO-NAC could be envisioned as another component of the denominator of this ratio.

In summary, the results of this study demonstrate, for the first time, the presence in human urine of a mercapturic acid derived from a PAH, Phe. This metabolite, PheO-NAC (**12**) was detected and quantified in the urine of 46 of 104 smokers. There was a significant correlation between detectability and levels of this mercapturic acid and *GSTM1* genotype, demonstrating a solid phenotype-genotype relationship for this gene. There were no effects of *GSTP1* polymorphisms on analyte levels. However, factors other than *GST* genotype apparently influence the presence of PheO-NAC, suggesting that phenotyping may be a better approach than genotyping for determining PAH detoxification via the *GST* pathway. We hope to incorporate this assay as part of a carcinogen metabolite phenotyping ratio to assess the relationship between PAH metabolism and cancer in exposed individuals.

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Figure 1.

Chromatogram obtained upon LC-ESI-MS/MS-SRM analysis of PheO-NAC in human urine (HCOOH/CH₃CN elution system, see Materials and Methods). Upper panel, SRM for m/z 356 $\rightarrow$ 209 (PheO-NAC); lower panel, m/z 366 $\rightarrow$ 218 ($[D_{10}]$ PheO-NAC, internal standard)

Figure 2. Chromatogram obtained upon LC-ESI-MS/MS-SRM analysis of PheO-NAC in human urine (NH₄OAc/CH₃OH elution system, see Materials and Methods). Upper panel, SRM for m/z 356 → 209 (PheO-NAC); lower panel, m/z 366 → 218 ([D₁₀]PheO-NAC, internal standard)

Figure 3. Chromatogram obtained LC-ESI-MS/MS-SRM analysis of a urine sample negative for PheO-NAC ($\text{NH}_4\text{OAc}/\text{CH}_3\text{OH}$ elution system, see Materials and Methods). Upper panel, SRM for m/z 356 $\rightarrow$ 209 (PheO-NAC); lower panel, m/z 366 $\rightarrow$ 218 ($[\text{D}_{10}]$ PheO-NAC, internal standard)

Figure 4. Calibration curve relating ratio of PheO-NAC:[D₁₀]PheO-NAC to LC-ESI-MS/MS peak areas, $R^2 = 0.99$. The calibration curve was constructed using 50 – 5000 fmol PheO-NAC and 1000 fmol [D₁₀]PheO-NAC

Scheme 1.
Overview of Phenanthrene (Phe) Metabolism

Scheme 2.
Analytical Method for Quantitation of PheO-NAC in Human Urine

Table 1Levels of PheO-NAC (**12**) in smokers' urine (positive samples only)

Smoker	PheO-NAC (fmol/ml)	Smoker	PheO-NAC (fmol/ml)
1	560	24	30
2	99	25	100
3	20	26	10
4	42	27	310
5	71	28	23
6	100	29	21
7	42	30	25
8	48	31	92
9	30	32	51
10	190	33	31
11	41	34	55
12	55	35	26
13	70	36	140
14	570	37	95
15	880	38	40
16	530	39	68
17	680	40	82
18	150	41	52
19	7	42	35
20	70	43	33
21	47	44	15
22	19	45	98
23	53	46	210