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Quantitation of Phenanthrene Dihydrodiols in the Urine of Smokers and Non-smokers by Gas Chromatography-Negative Ion Chemical Ionization-Tandem Mass Spectrometry

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Abstract

Polycyclic aromatic hydrocarbons (PAH) are well-established environmental carcinogens likely to be causative agents for some human cancers. Bay-region diol epoxides are ultimate carcinogenic metabolites of multiple PAH. Dihydrodiols are the important intermediate products of this pathway and can be further oxidized to form diol epoxides. We quantified two dihydrodiol metabolites of phenanthrene (Phe), the simplest PAH with a bay-region, in the 6 h urine of smokers (N=25) and non-smokers (N=25) using a newly developed and validated analytical method. After hydrolysis by β -glucuronidase and sulfatase, and solid phase extraction, the sample was silylated and analyzed by gas chromatography-negative ion chemical ionization-tandem mass spectrometry (GC-NICI-MS/MS). Levels (nmol/6 h urine) of Phe-1,2-dihydrodiol (Phe-1,2-D) and Phe-3,4-dihydrodiol (Phe-3,4-D) were 2.04 ± 1.52 and 0.51 ± 0.35 , respectively, in smokers, significantly higher than those in non-smokers (1.35 ± 1.11 of Phe-1,2-D, $p < 0.05$; 0.27 ± 0.25 of Phe-3,4-D, $p < 0.005$). Cigarette smoking also influenced the regioselective metabolism of Phe, presenting as a significant difference in the urinary distribution pattern of Phe-1,2-D and Phe-3,4-D between smokers and non-smokers: the ratio Phe-3,4-D: Phe-1,2-D increased from 0.20 in non-smokers to 0.28 in smokers ($p < 0.01$), which can be explained by the induction of the phenanthrene metabolizing enzymes CYP1A2 and CYP1B1 by cigarette smoke. The method described here is the first example of facile quantitation of an intact human dihydrodiol metabolite of any PAH with three or more aromatic rings and will be applicable in clinical and molecular epidemiology studies of PAH metabolism and cancer susceptibility.

Keywords

Phenanthrene; Dihydrodiol; Bioactivation; Cigarette Smoke

1. Introduction

Polycyclic aromatic hydrocarbons (PAH) are important environmental carcinogens believed to be among the causes of cancer induced by environmental pollutants and cigarette smoke. One member of this class of compounds, benzo[a]pyrene (BaP), is considered “carcinogenic

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to humans” by the International Agency for Research on Cancer [1]. Phenanthrene (Phe) is a main component of environmental PAH mixtures and occurs in various sources such as air and particulate matter [2–4], cigarette smoke [5, 6], soil and house dust [7–9], water and sediment [10, 11], diesel emissions [12, 13], as well as foodstuffs [14–16]. Phe metabolites have notable strengths as PAH exposure biomarkers. Although Phe is a non-carcinogenic PAH, it has structural features such as a bay region and an enzymology profile similar to that of carcinogenic PAH including BaP and may thus mirror the bioactivation and detoxification pathways of BaP. Phe metabolites are excreted in relatively large quantities in human urine and are easier to quantify than those of BaP. For example, the concentrations of Phe-tetraols are about 10,000 times higher than those of BaP-tetraol in human urine [17]. Thus Phe is of potentially high diagnostic value in the biomonitoring of occupational and epidemiological PAH exposures by providing information on both exposure and metabolic activation.

The metabolism of Phe by the PAH diol epoxide metabolic activation pathway (Scheme 1) yields dihydrodiols, diol epoxides, and tetraols, whereas phenanthrols signify detoxification [18–21]. Phe can be metabolized at three different molecular regions (1,2-; 3,4- and 9,10-positions) depending on various cytochrome P450 isoforms involved in the oxidation process [22]. Of particular interest is (1*R*,2*R*)-dihydroxy-1,2-dihydrophenanthrene (Phe-1,2-D), which is formed along with two other isomeric dihydrodiols (3*R*,4*R*)-dihydroxy-3,4-dihydrophenanthrene (Phe-3,4-D) and (9*R*,10*R*)-dihydroxy-9,10-dihydrophenanthrene (Phe-9,10-D) by metabolic steps competing with phenol formation. Phe-9,10-D is not representative of a metabolic activation pathway. Phe-1,2-D can be further oxidized to the bay-region (1*R*,2*S*)-dihydroxy-(3*S*,4*R*)-epoxy-1,2,3,4-tetrahydrophenanthrene (Phe-1,2-D-3,4-E), structurally analogous to the highly carcinogenic bay region (7*R*,8*S*)-dihydroxy-(9*S*,10*R*)-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (BPDE) of BaP, while Phe-3,4-D can only form the reverse diol epoxide (3*S*,4*R*)-dihydroxy-(1*R*,2*S*)-epoxy-1,2,3,4-tetrahydrophenanthrene (Phe-3,4-D-1,2-E) (Scheme 1), analogous to less carcinogenic diol epoxides [23]. Phe-1,2-D-3,4-E undergoes hydrolysis to produce (1*R*,2*S*,3*R*,4*S*)-tetrahydroxy-1,2,3,4-tetrahydrophenanthrene (Phe-(1*R*,2*S*,3*R*,4*S*)-tetraol), which was significantly correlated with Phe-(1*S*,2*R*,3*S*,4*R*)-tetraol and BaP-tetraol (hydrolysis product of BPDE) in human urine ($r = 0.72$, $p < 0.0001$) [17]. Thus we propose that Phe-1,2-D, the precursor of the “ultimate reactive metabolite” Phe-1,2-D-3,4-E, can be used as a proxy for the carcinogenic metabolism pathway of BaP and other PAH metabolically activated to bay region diol epoxides.

Most previous studies quantified Phe-1,2-D in human urine using an indirect method involving conversion of the dihydrodiol to phenols after 2–16 h acid treatment, then calculating the dihydrodiol concentration as the difference between the total phenols analyzed before and after acid treatment [18, 21, 24]. One direct method using methyl iodide as a derivatization reagent was reported, but this required a large urine volume (10 mL), tedious analysis time (16 h for derivatization) and relatively high consumption of organic solvents (110 mL per sample) during sample preparation, and consequently has not been widely applied [25].

The aim of this study was to develop a rapid and sensitive analytical method for the quantitation of Phe-1,2-D and Phe-3,4-D in human urine to investigate: 1) the concentration

of these compounds in the urine of smokers and non-smokers; 2) their potential use as biomarkers of exposure to PAH; and 3) the effect of cigarette smoking on regioselectivity in the metabolism of Phe.

2. Materials and Methods

2.1 Chemicals, Enzymes and Sample Preparation Supplies

Phe (purity 99.5%) and [1,2,3,4,4a,9a- $^{13}\text{C}_6$]Phe (purity ~95%) were obtained from Millipore Sigma (St. Louis, MO, USA) and Toronto Research Chemicals (North York, ON, Canada) respectively. Standards of Phe-1,2-D and [$^{13}\text{C}_6$]Phe-1,2-D were prepared by incubation of Phe and [$^{13}\text{C}_6$]Phe with cytochrome P4501A1 and cofactors, followed by HPLC purification, as described [26]. Purity (>95%) and concentration of Phe-1,2-D were determined by quantitative nuclear magnetic resonance (QNMR) (^1H NMR of Phe-1,2-D (500 MHz, $\text{DMSO}-d_6$) δ 8.17 (d, J = 8.6 Hz, 1H), 7.84 (d, J = 7.9 Hz, 1H), 7.79 (d, J = 8.3 Hz, 1H), 7.70 (d, J = 8.3 Hz, 1H), 7.48 (d, J = 7.6 Hz, 1H), 7.45 (d, J = 7.5 Hz, 1H), 7.20 (d, J = 10.2 Hz, 1H), 6.09 (d, J = 10.2 Hz, 1H), 5.53 (s, 1H), 5.19 (s, 1H), 4.65 (s, 1H), 4.28 (s, 1H).) and HPLC-UV. Standard Phe-3,4-D was provided by H. Yagi[27]. RC-2 [*bis*-Trimethylsilyltrifluoroacetamide (BSTFA) + 1% trimethylchlorosilane (TMCS)] was purchased from Regis Technologies (Morton Grove, IL, USA). β -Glucuronidase and arylsulfatase (from *Helix pomatia*) were obtained from Roche Diagnostics Corp. (Indianapolis, IN, USA). Strata SDBL styrene-divinylbenzene SPE cartridges (50 mg/1 mL, #8B-S014-DAK) were obtained from Phenomenex (Torrance, CA, USA). Oasis MAX (Mixed-mode Anion eXchange sorbent) SPE cartridges (60 mg/1 mL/# 186000378) were from Waters (Milford, MA, USA). All solvents used in this study including methanol and acetonitrile were HPLC grade and from Fisher Scientific (Fair Lawn, NJ, USA). Ultrapure H_2O from Millipore Milli-Q Advantage A10 Water Purification System was used throughout the study.

2.2 Urine Samples

Urine samples from 50 volunteers (25 smokers: 15 males / 10 females; 25 non-smokers: 13 males / 12 females) were obtained from ongoing studies at the Tobacco Research Programs, University of Minnesota, approved by the Institutional Review Board. The volume of their 6 h urine was measured and aliquots were stored at -20°C until analysis. Smoking status of all the subjects were determined by measurement of urinary cotinine [28]. Those who had cotinine 30 ng/mL were classified as smokers. Our previous results have shown that 6-h urinary excretion of racemic [D_{10}]Phe-1,2,3,4-tetraol, the major end product of the Phe diol epoxide pathway, was sufficient to reflect the extent of metabolism of [D_{10}]Phe to this metabolite in smokers who received an oral or inhalation dose of [D_{10}]Phe, because it was highly correlated with 48-h excretion [29].

2.3 Sample Preparation

The analytical method is summarized in Scheme 2. An aliquot of 1.0 mL urine was added to a 2.0 mL glass vial containing 1.0 mL of 0.5 M NaOAc buffer (pH 5), β -glucuronidase (1000 units) and arylsulfatase (8000 units), and 5 pmol of [$^{13}\text{C}_6$]Phe-1,2-D as internal standard. The mixture was incubated overnight with shaking at 37°C . After incubation, the

sample was purified by loading onto a Strata SDBL cartridge preconditioned with 1.0 mL of CH₃OH and 1.0 mL of H₂O. The cartridge was then washed in order with 1.0 mL of 0.28% NH₄OH (wt%) /10% CH₃OH (vol%) in H₂O, 1.0 mL of H₂O, 1.0 mL of freshly prepared 1% formic acid (vol%)/10% CH₃OH (vol%) in H₂O and 1.0 mL of H₂O, and the analyte was eluted with 0.8 mL×2 of CH₃OH. The Strata SDBL we used is from Phenomenex. The packing material is styrene-divinylbenzene copolymer, which is used in organic trace analysis for solid phase extraction preconcentration. It has been successfully used in previous studies for the assay of phenanthrene tetraol. It can give a good recovery of the dihydrodiols and remove most of the urine matrix with a basic and acid wash. The solvents of the analyte fraction from the Strata SDBL were removed using a Speedvac concentrator (Thermo Scientific, San Jose, CA). The residue was redissolved in 1.0 mL of 10% CH₃OH (vol%) in H₂O with sonication and loaded onto an Oasis MAX cartridge preconditioned with 1.0 mL of CH₃OH and 1.0 mL of 0.84% NH₄OH (wt%) in H₂O. The cartridge was washed with 1.0 mL of 0.84% NH₄OH (wt%) in H₂O and the dihydrodiols were eluted with 1mL CH₃OH. The solvent was removed on a Speedvac and the residue was dissolved in 100 µL × 2 times of CH₃OH with sonication, transferred to an insert vial (Chrom Tech, 300 µL insert/vial, CP-0952–03SIL), concentrated to dryness, and dissolved in 10 µL of BSTFA plus 1% TMCS with sonication and vortexing. The samples were heated at 60 °C for 60 min to complete the derivatization, and 3 µL was analyzed by GC-NICI-MS/MS.

2.4 GC-NICI-MS/MS Analysis

Gas chromatography-negative ion chemical ionization- tandem mass spectrometry (GC-NICI-MS/MS) was carried out with a TSQ Quantum instrument (Thermo Scientific, San Jose, CA). Phe-1,2-D and [¹³C₆]Phe-1,2-D were detected as their trimethylsilyl derivatives. The GC was equipped with a 0.25 mm i.d. × 0.15 µm film thickness × 30 m DB17-MS column (Agilent Technologies, Palo Alto, CA). The oven temperature program was 80 °C for 1 min, then 80–200 °C at 35 °C /min, then 200–215 °C at 3 °C /min, then 215–320 °C at 35 °C /min, and then held for 2 min. The carrier gas was He at a constant flow rate of 1.0 ml/min. The programmed temperature vaporizer (PTV) injection port (Topaz 2.0 mm ID Baffled Inlet Liner for Thermo TRACE GCs equipped with PTV inlets, 2 mm × 2.75 × 120, Cat.# 23438, operated in the splitless mode) was set up as follows: 60 °C for 0.5 min, then 60–90 °C at 0.5 °C /sec, hold for 1 min, then 90–250 °C at 3 °C /sec, and then held for 15.5 min. The use of a PTV injector starting at a low temperature was found to be critical in preventing the decomposition of the silylated Phe-1,2-D and Phe-3,4-D. The MS transfer line was kept at 320 °C. The NICI-MS/MS conditions were as follows: CI gas, argon at 2.0 ml/min; source temperature, 250 °C; and emission current 70 mA. Selected reaction monitoring (SRM) with a collision energy of 30 eV, electron energy of 50 eV, scan width of m/z 0.02, and dwell-time 0.03 sec was used to detect Phe-1,2-D and [¹³C₆]Phe-1,2-D at m/z 193 → m/z 165 and m/z 199 → m/z 171, respectively (Scheme 2).

2.5 Statistical Analysis

The *t*-Test: Paired Two Sample for Means (Excel 16.27) was used to analyze the difference of analytes between smokers and non-smokers, and $p < 0.05$ was considered statistically significant.

3. Results

The method used in this study for the analysis of Phe-1,2-D and Phe-3,4-D was different from those reported previously, because the silylated analyte was quantified directly using a smaller amount of urine and a shorter analysis time. A typical chromatogram obtained upon analysis of a 6 h urine sample from a smoker is illustrated in Figure 1, showing clear quantifiable peaks for Phe-1,2-D, [$^{13}\text{C}_6$]Phe-1,2-D, and Phe-3,4-D as their TMS derivatives.

The average recovery of [$^{13}\text{C}_6$]Phe-1,2-D spiked into the samples was $56.3 \pm 6.81\%$ ($N=3$). The method limit of detection (LOD) calculated from the peak signal-to-noise ratio of real samples with a minimal value of 3 was 0.29 ± 0.036 nM for Phe-1,2-D and 0.084 ± 0.0067 nM for Phe-3,4-D. The analyte showed excellent linearity ($r^2 > 0.99$) in the range of 1–400 nM in the instrumental calibration curve.

The accuracy was determined by adding various amounts of Phe-1,2-D and Phe-3,4-D to the urine of a nonsmoker. As shown in Table 1, there was excellent agreement between the added and quantified amounts, the regression equation was $y=0.80x+0.85$ (y : determined level (nM), x : spiked level (nM) ($R^2=0.9992$) for Phe-1,2-D and $y=1.04x+0.10$ ($R^2=0.9998$) for Phe-3,4-D, and the y intercept, corresponding to 0.85 nM and 0.10 nM for Phe-1,2-D and Phe-3,4-D, respectively, agreed with the level determined in the urine of this nonsmoker, 0.77 ± 0.06 nM and 0.09 ± 0.01 nM. The intra-day precision was determined by analyzing six aliquots of a nonsmoker's urine. The results were 0.84 ± 0.10 nM for Phe-1,2-D ($CV=12.3\%$) and 0.10 ± 0.01 nM for Phe-3,4-D ($CV=8.7\%$). The inter-day precision was determined by analyzing 3 aliquots of a spiked (5.00 nM of Phe-1,2-D and 0.50 nM of Phe-3,4-D) nonsmoker's urine on 3 different days. The results were 4.76 ± 0.11 nM, 4.50 ± 0.41 nM and 4.63 ± 0.24 nM for Phe-1,2-D ($CV=2.8\%$), and 0.60 ± 0.02 nM, 0.57 ± 0.03 nM and 0.55 ± 0.03 nM for Phe-3,4-D ($CV=4.2\%$).

The concentrations of Phe-1,2-D and Phe-3,4-D were 1.87 ± 1.27 nM (0.43–5.69 nM) and 0.36 ± 0.26 nM (0.08–0.90 nM) in nonsmokers, and 4.05 ± 3.70 nM (0.54–17.65 nM) and 1.03 ± 0.92 nM (0.17–4.28 nM) in smokers. Levels of Phe-1,2-D and Phe-3,4-D in the 6 h urine of 50 subjects (25 smokers and 25 non-smokers) are summarized in Table 1. The overall mean and median amounts of Phe-1,2-D were 2.04 ± 1.52 and 1.69 nmol/ 6 h urine in smokers, significantly higher than those of non-smokers (1.35 ± 1.11 and 0.93 nmol/ 6 h urine, $p < 0.05$). The mean and median amounts of Phe-3,4-D were also significantly higher in smokers (0.51 ± 0.35 and 0.45 nmol/ 6 h urine) than in non-smokers (0.27 ± 0.25 and 0.21 nmol/ 6 h urine) to ($p < 0.005$). To characterize the regioselectivity of the metabolism of Phe by the diol epoxide pathway, the concentration ratio of [Phe-3,4-D/Phe-1,2-D] was calculated, and found to be significantly higher in smokers (0.28 ± 0.11) than in non-smokers (0.20 ± 0.08 , $p < 0.01$).

4. Discussion

The simple, accurate, and reproducible method developed in this study allowed sensitive and rapid analysis of Phe dihydrodiols using smaller volumes of urine compared with previous

studies, and has the potential to be applied in large-scale studies. This is critical for clinical and molecular epidemiology studies using Phe metabolite analysis to predict cancer risk.

Several metabolites including 1-hydroxypyrene (1-OHP), Phe phenols (OHPhe) and Phe tetraols have been used as biomarkers to assess human exposure to PAH via cigarette smoking. Levels of urinary 1-OHP were about twice (1.5–2.7) as great in cigarette smokers as in non-smokers [30–32]. Levels of 1-, 2-, 3- and 4-OHPhe in smokers were 1.7–2.9 times higher in smokers than nonsmokers [32]. And smokers had a significantly higher PheT level (~ 2.6 fold, $p=0.0073$) than nonsmokers [33]. The total urinary concentrations of Phe-1,2-D and Phe-3,4-D were 1.6-fold higher in smokers than non-smokers in this study ($p<0.05$), generally consistent with the other biomarkers mentioned above.

Although the number of subjects was relatively small, we observed that males had higher levels of Phe-1,2-D and Phe-3,4-D than females (Table 2), particularly among smokers. Male smokers had 2.6- and 1.6-times higher levels of Phe-1,2-D and Phe-3,4-D than females, while among non-smokers, the levels of Phe-1,2-D and Phe-3,4-D in males were only 1.1- and 1.04-times higher than those of females. Further research with larger sample sizes is needed to investigate potential gender differences in PAH metabolism, health risks, and related mechanisms. The U.S. CDC's National Health and Nutrition Examination Survey (NHANES) (2001–2014) investigated 1-, 2-, 3- and 4-OHPhe, the main metabolites of the Phe detoxification pathway, in males and females, and found that males had higher levels of all the analytes (1.1–1.5 times) than females [34].

Oxidation of Phe can occur at three different molecular regions (1,2-; 3,4- and 9,10-positions) mainly catalyzed by different cytochrome P450 isoforms. The ratios of Phe-3,4-oxidation to 1,2-oxidation were only 0.07 and 0.17 for human CYP1A1 in different studies, but 0.91 and 0.77 for human CYP1A2- and 0.56 for human CYP1B1, indicating that 3,4-oxidation is more favored in the case of CYP1A2 and CYP1B1 than with CYP1A1 [35, 36]. Activities and polymorphisms of human CYP1A2 and CYP1B1 have been confirmed in studies of caffeine and 17 β -estradiol (E2) at their different positions, therefore affecting their metabolite ratios and individual risk of toxicity and disease caused by these compounds [37–42]. Cigarette smoke is a potent inducer of CYP1A2 and CYP1B1 enzyme activity in both animals and humans [36, 43–46]. The ratio [3-OHPhe+4-OHPhe] / [1-OHPhe+2-OHPhe] increased from 0.39 to 0.57 with cigarette consumption in NHANES (2011–2012) with a large sample size [32]. In the present study, the ratio of Phe-3,4-D to Phe-1,2-D significantly increased from 0.20 in non-smokers to 0.28 in smokers ($p<0.05$), consistent with the observation that cigarette smoke can promote 3,4-oxidation of Phe in humans via a CYP-mediated pathway.

The results of this study showed that mean urinary Phe-1,2-D was 4.01 and 5.08 times higher than Phe-3,4-D in smokers and non-smokers, respectively. Previous studies found contrasting results for their downstream products: urinary levels of Phe-(1*S*,2*R*,3*S*,4*R*)-tetraol (the tetraol enantiomer resulting mainly from the reverse diol epoxide Phe-3,4-D-1,2-E that would be formed from Phe-3,4-D, Figure 1) were 17.7 and 6.77 times higher than those of Phe-(1*R*,2*S*,3*R*,4*S*)-tetraol (the tetraol enantiomer mainly resulting from the bay-region diol epoxide Phe-1,2-D-3,4-E that would be formed from Phe-1,2-D) in smokers

(N=30) and creosote workers (N=26) [17]. Incubations of human hepatocytes with Phe metabolites also demonstrated that conversion of Phe-3,4-D to tetraols was 2.45–29.5 times more than that of Phe-1,2-D [47] which can explain the predominance of Phe-(1*S*,2*R*,3*S*,4*R*)-tetraol in spite of the higher concentration of Phe-1,2-D than Phe-3,4-D.

In summary, we have developed a straightforward GC-NICI-MS/MS method for the quantitation of Phe-1,2-D and Phe-3,4-D, as their trimethylsilyl ethers, in human urine. This method has the potential to be applied in clinical and molecular epidemiology studies of PAH metabolism. While Phe is non-carcinogenic, these metabolites are important because they represent the first step in the diol epoxide and reverse diol epoxide metabolic activation pathways of carcinogenic PAH. The results demonstrate that urinary levels of Phe-1,2-D, the metabolite on the bay region diol epoxide pathway of Phe metabolism, are higher in smokers than non-smokers, indicating its potential use as a biomarker of both exposure to, and metabolic activation of PAH. This is the first example of facile quantitation of an intact human dihydrodiol metabolite of any PAH with three or more aromatic rings.

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Figure 1.

Typical GC-NICI-MS/MS chromatograms obtained upon analysis of a 6 h urine sample from a smoker. **A**, Phe-1,2-D (3.33 nM) and Phe-3,4-D (1.13 nM), as their di-TMS derivatives, observed in the urine sample; **B**, internal standard [¹³C₆]Phe-1,2-D-di-TMS.

Scheme 1.

Metabolic activation of benzo[a]pyrene and phenanthrene via the diol epoxide pathway.

Scheme 2.

Outline of the method for the analysis of Phe-1,2-D and Phe-3,4-D, and fragmentation in the daughter ion spectrum of di-trimethylsilyl-Phe-1,2-D; TMS = (CH₃)₃Si.

Table 1.

Precision and accuracy ^a, levels (nmol/6 h urine) and ratios of Phe-1,2-D and Phe-3,4-D in human urine.

	Spiked Concentration (nM)	Determined Concentration (nM)	Precision (CV, %)	Accuracy (%) ^b	Smokers (N=25)		Non-smokers (N=25)	
					mean±SD	median (interquartile)	mean±SD	median (interquartile)
Phe-1,2-D	0.00	0.766±0.063	8.16					
	0.200	0.920±0.071	7.72	77.1				
	1.00	1.75±0.239	13.6	98.8				
	5.00	4.76±0.105	2.21	79.9	2.04±1.52	1.69 (0.95~2.56)	1.35±1.11 ^c	0.93 (0.58~1.85)
	10.0	9.19±0.159	1.73	84.2				
	20.0	16.8±1.04	6.24	79.9				
Phe-3,4-D	0.00	0.090±0.008	9.26					
	0.0500	0.132±0.014	10.4	84.0				
	0.100	0.192±0.015	7.89	101				
	0.500	0.600±0.022	3.63	102	0.51±0.35	0.45 (0.28~0.64)	0.27±0.25 ^d	0.21 (0.11~0.29)
	1.00	1.19±0.054	4.56	110				
	5.00	5.28±0.050	0.95	104				
				Ratio = $\frac{\text{Phe} - 3,4}{\text{Phe} - 1,2} - \frac{D}{D}$	0.28±0.11	0.26 (0.20~0.34)	0.20±0.08 ^e	0.18 (0.14~0.24)

^a Phe-1,2-D and Phe-3,4-D were added to 1 mL aliquots of a nonsmoker's urine (N=3 for each concentration point).

^b The accuracy was reported as recovery (%) = (determined concentration – basal concentration) / spiked concentration × 100 (%).

^c $p < 0.05$, comparing non-smokers and smokers

^d $p < 0.005$, comparing non-smokers and smokers

^e $p < 0.01$ comparing non-smokers and smokers.

Table 2.

Levels of Phe-1,2-D and Phe-3,4-D in males and females (nmol/6 h urine).

	Smokers (N=25)		Non-smokers (N=25)	
	Males (N=15)	Females (N=10)	Males (N=13)	Females (N=12)
	mean±SD	mean±SD	mean±SD	mean±SD
Phe-1,2-D	2.71±1.61	1.05±0.53	1.42±1.00	1.28±1.25
Phe-3,4-D	0.60±0.38	0.37±0.26	0.27±0.19	0.26±0.31