JPET Fast Forward. Published on June 6, 2012 as DOI: 10.1124/jpet.112.194118

JPET# 194118

Phenanthrene Metabolism in Smokers: Use of a Two-step Diagnostic Plot Approach to Identify Subjects with Extensive Metabolic Activation

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Running Title: The Metabolic Activation of Phenanthrene in Humans

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Text pages:

Number of Tables: 5

Number of Figures: 6

Number of References: 47

Words in Abstract: 243

Words in Introduction: 884

Words in Discussion: 1615

JPET Fast Forward. Published on June 6, 2012 as DOI: 10.1124/jpet.112.194118
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Non-standard abbreviations:

polycyclic aromatic hydrocarbons (PAHs)

benzo[α]pyrene (BaP)

phenanthrene (Phe)

r-1,*t*-2,3,*c*-4-tetrahydroxy-1,2,3,4-tetrahydrophenanthrene (PheT)

deuterated phenanthrene ([D_{10}]Phe)

deuterated *r*-1,*t*-2,3,*c*-4-tetrahydroxy-1,2,3,4-tetrahydrophenanthrene ([D_{10}]PheT)

deuterated *anti*-1,2-dihydroxy-3,4-epoxy-1,2,3,4-tetrahydrophenanthrene ([D_{10}]PheDE)

r-7,*t*-8,9,*c*-10-tetrahydroxy-7,8,9,10-tetrahydrobenzo(α)pyrene (BaPT)

benzo[α]pyrene 7,8-diol-9,10-epoxide (BPDE)

Section: Metabolism, Transport, and Pharmacogenomics

JPET Fast Forward. Published on June 6, 2012 as DOI: 10.1124/jpet.112.194118
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ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) in cigarette smoke are among the most likely causes of lung cancer. PAHs require metabolic activation to initiate the carcinogenic process. Phenanthrene (Phe), a non-carcinogenic PAH, was used as a surrogate of benzo[α]pyrene (BaP) and related PAHs to study the metabolic activation of PAHs in smokers. A dose of 10 µg deuterated Phe ([D_{10}]Phe) was administered to 25 healthy smokers in a crossover design, either as an oral solution or by smoking cigarettes containing [D_{10}]Phe. Phe was deuterated to avoid interference from environmental Phe. Intensive blood and urine sampling was performed to quantitate the formation of deuterated *r*-1,*t*-2,3,*c*-4-tetrahydroxy-1,2,3,4-tetrahydrophenanthrene ([D_{10}]PheT), a biomarker of the diol epoxide metabolic activation pathway. In both the oral and smoking arms approximately 6% of the dose was metabolically converted to diol epoxides, with a large inter-subject variability in the formation of [D_{10}]PheT observed. Two diagnostic plots were developed to identify subjects with large systemic exposure and significant lung contribution to metabolic activation, respectively. The combination of the two plots led to the identification of subjects with substantial local exposure. These subjects produced, in one single pass of [D_{10}]Phe through the lung, a [D_{10}]PheT exposure equivalent to the systemic exposure of a typical subject, and may be an indicator of lung cancer susceptibility. Polymorphisms in PAH metabolizing genes of the 25 subjects were also investigated. The integration of phenotyping and genotyping results indicated that *GSTM1* null subjects produced approximately 2-fold more [D_{10}]PheT than did *GSTM1* positive subjects.

INTRODUCTION

According to the International Agency for Research on Cancer (IARC), tobacco smoking was associated with 90% of the lung cancer deaths in the United States (IARC, 2004). Polycyclic aromatic hydrocarbons (PAHs) are among the strongest carcinogens in cigarette smoke and are considered to be a major etiological factor in lung cancer (Hecht, 2003a; Pelkonen and Nebert, 1982). PAHs are very lipophilic and could theoretically accumulate in tissues and reach toxic concentrations (Lang and Pelkonen, 1999). Thanks to evolution, humans have developed the capacity to detoxify foreign chemicals, including carcinogens, by converting them to more water soluble metabolites, which may enhance their elimination from the body. Enzymes involved in the detoxification of PAHs include cytochrome P450 1A1 (CYP1A1), 1B1 (CYP1B1), glutathione *S*-transferases (GST), UDP-glucuronosyltransferases (UGT) and sulfotransferases (SULT) (Shimada, 2006; Hecht, 2010a). However, at the cost of solving the acute problem (lethal accumulation of xenobiotics), a small portion of PAHs is transformed to electrophilic intermediates. These intermediates do not cause lethal hazards in the short term, but may initiate carcinogenesis in the long term by attacking nucleophilic macromolecules of the cell, causing gene mutations which may eventually lead to the development of tumors (Hecht, 1999). The conversion of PAHs to active intermediates is called metabolic activation or bioactivation. Enzymes involved in metabolic activation include CYP1A1, cytochrome P450 1A2 (CYP1A2), and CYP1B1, as well as epoxide hydrolase (EH) (Shimada, 2006).

Fewer than 20% of life-long smokers develop lung cancer, indicating that some individuals are more susceptible than others (IARC, 1986; Peto et al., 2000). How to identify susceptible

subjects is still not clear. Numerous epidemiological studies have been conducted to investigate the relationship of cancer susceptibility to genetic polymorphisms in PAH metabolizing enzymes (Bartsch et al., 2000; Ishibe et al., 1997; Kawajiri et al., 1990; Kiyohara et al., 2002; Nakachi et al., 1991; Okada et al., 1994; Persson et al., 1999; Shi et al., 2008; Shields et al., 1993; Tefre et al., 1991; Williams, 2001; Xu et al., 1996). However, the results of these studies have been mixed and sometimes even contradictory, perhaps due to the complexity of PAH metabolism and the multiple factors involved. For example, these studies have not considered gene-environment interactions and tissue-specific metabolic enzyme expression. Subjects with a higher expression of activation enzymes and lower expression of detoxification enzymes would theoretically have larger systemic exposure to active intermediates than other subjects. In addition, if the pulmonary enzymes of these subjects played a major role in activation, then lung exposure would be substantial, which could lead to an increased susceptibility to lung cancer. Due to the important role of metabolic activation of PAHs in lung carcinogenesis, the present study focused on identification of subjects with large systemic and local (lung) exposure, and presumably high lung cancer risk.

Benzo[α]pyrene (BaP) is found in cigarette smoke and is a prototypic and widely studied compound for the investigation of carcinogenesis by PAHs (Jiang et al., 2007; Straif et al., 2005; Uno et al., 2006). A major bioactivation pathway of BaP (Figure 1) is its conversion to *r*-7,*t*-8,9,*c*-10-tetrahydroxy-7,8,9,10-tetrahydrobenzo(α)pyrene (BaPT) via the formation of the “bay region diol epoxide” benzo[α]pyrene 7,8-diol-9,10-epoxide (BPDE), which reacts

readily with DNA and is mutagenic and carcinogenic (IARC, 2010). BPDE is thought to be one electrophilic reactive intermediate responsible for BaP mutagenesis and carcinogenesis (Conney, 1982; Cooper et al., 1983; Geacintov et al., 1997; Hecht, 1999; Szeliga and Dipple, 1998), and related bay region diol epoxides are considered to be major ultimate carcinogens of a number of other PAHs (IARC, 2010). Since most PAHs like BaP are carcinogenic and cannot be administered to humans, there have not been any detailed pharmacokinetic (PK) studies of PAHs in humans. To address this issue, a novel biomarker approach has been proposed: the use of phenanthrene (Phe) as a surrogate of BaP (Hecht et al., 2003b). Phe is a non-carcinogenic PAH ubiquitous in the environment and can be safely administered to human subjects because all humans are exposed to Phe. The conversion of Phe to *r*-1,*t*-2,3,*c*-4-tetrahydroxy-1,2,3,4-tetrahydrophenanthrene (PheT) mimics the formation of the diol epoxide metabolic activation of BaP including the intermediates formed and enzymes involved (Figure 1) (IARC 1983; Carmella et al., 2004), although there are some differences (Hecht et al., 2010b).

In the present study, deuterated phenanthrene ([D_{10}]Phe) was administered to 25 subjects and the metabolism of [D_{10}]Phe to deuterated *r*-1,*t*-2,3,*c*-4-tetrahydroxy-1,2,3,4-tetrahydrophenanthrene ([D_{10}]PheT) was characterized in order to evaluate each individual's capacity to carry out the diol epoxide metabolism pathway. We used [D_{10}]Phe to avoid interference from ubiquitous exposure to environmental Phe. An intermediate formed during the activation of [D_{10}]Phe was deuterated *anti*-1,2-dihydroxy-3,4-epoxy-1,2,3,4-tetrahydrophenanthrene ([D_{10}]PheDE), a biomarker of

the diol epoxide pathway leading to BPDE from BaP (Figure 1). The primary objective of our study was to quantify the amount of [D_{10}]PheDE formed in the metabolism of [D_{10}]Phe. [D_{10}]PheDE cannot be quantified directly because it reacts rapidly with H_2O producing [D_{10}]PheT, which was measured in this study (Carmella et al., 2004).

Preliminary reports from a subset of subjects demonstrated the rapid formation of diol epoxides and potential immediate negative health consequences of smoking (Zhong et al., 2011a), and examined the metabolism of [D_{10}]Phe administered to 16 smokers either in a cigarette or orally as a biomarker of the activation pathway (Zhong et al., 2011b). With the completion of the clinical trial, a comprehensive analysis of the bioactivation of Phe is now reported.

METHODS

Clinical Study Design. The study was approved by the U.S. Food and Drug Administration and the University of Minnesota Institutional Review Board. Details on the recruitment of subjects and dosing protocols were previously reported (Zhong et al., 2011a; Zhong et al., 2011b). Subjects were recruited using advertisements on the radio, television or metropolitan and campus newspapers. Volunteers interested in the study called the University of Minnesota Transdisciplinary Tobacco Use Research Center (TTURC) and were informed about the study. The preliminary screening was performed over the phone to select subjects meeting the following specific inclusion criteria: the smoking of at least 10 cigarettes daily for the past year and good physical and mental health. Female subjects who were pregnant or nursing were excluded. Eligible subjects were further invited to the clinic site for an orientation visit to fill out a detailed questionnaire regarding their smoking and medical history. Pregnancy tests were also conducted. Subject recruitment incentives were used and an average of $500 was paid to each subject for the completion of the study.

A total of twenty-five eligible subjects, 8 male, were recruited between April 2008 and September 2010. The age of these subjects was between 23 and 54 years with a mean age (± standard deviation) of 36.2 ± 10.4 years. Their weights ranged from 61 to 113 kg with a mean weight (± standard deviation) of 87.4 ± 15.4 kg. Twelve subjects were Caucasian, eight were African-American and 5 subjects reported being multi-racial.

A dose of 10 μg (53.2 nmol) [D_{10}]Phe was administered to 25 subjects, either as an oral solution or by smoking cigarettes to which [D_{10}]Phe had been added. [D_{10}]Phe (98%, containing 2% nondeuterated Phe) was purchased from Cambridge Isotope Laboratories, and then repurified in the University of Minnesota Molecular and Cellular Therapeutics GMP facility. The study had a randomized, open-label, single-dose, crossover design. The order of administration was randomized and each dose was separated by a washout period of at least one week. For the oral dose, each subject was given 10 μg [D_{10}]Phe (5 mL of 20% ethanol-80% water solution). The dosing bottle was rinsed twice with water to ensure accurate dosing. For the inhalation dose, subjects followed a standard smoking protocol designed to deliver 10 μg [D_{10}]Phe, as monitored by a smoking topography device (Zhong et al., 2011a). The administration of [D_{10}]Phe by smoking was performed in a specially ventilated room at TTURC. Subjects underwent an adaptation trial prior to smoking the cigarettes containing [D_{10}]Phe. Specific instructions about the puff volume (55 ml), puff duration (2 s) and puff interval (30 s) were given to the subjects. Subjects smoked the cigarettes through the smoking topography device which recorded the puff volume, puff duration and puff number. A Marlboro cigarette was used in the practice session, after which subjects were then allowed to smoke the cigarette containing [D_{10}]Phe. The smoking process was also observed by the clinician to ensure good compliance. The smoking session usually lasted about 4-5 min.

Blood samples of 10 mL each were taken prior to dosing and 15, 30, 45, 60, 90, 120, 150, 240, 360, 540 or 720, and 1440 min after the completion of administration. Blood samples were centrifuged to obtain plasma which was stored at -20 °C until analysis. Urine samples

were obtained pre-dosing and at the following post-dosing intervals: 0-30, 30-60, 60-120, 120-360, 360-720, 720-1440, 1440-2880 min. The volume of each urine collection was measured and an aliquot of 50 mL was stored at -20 °C until analysis. GC-MS was employed to analyze the plasma and urine samples as described previously (Zhong et al., 2011a). It should be noted that since approximately 90% of the PheT in human urine exists as sulfate and glucuronide conjugates (Hecht et al., 2003b), plasma and urine samples were incubated with β- glucuronidase and arylsulfatase before quantitation. Hence the reported level of [D_{10}]PheT in both plasma and urine is the sum of the free [D_{10}]PheT and its conjugates.

Pharmacokinetic Analysis.

Noncompartmental analysis was performed with the use of Phoenix WinNonlin™ (v6.1, PharSight, Cary, NC) to calculate the area under the [D_{10}]PheT plasma concentration-time curve ($AUC_{(PheT)}$) and the elimination half-life ($t_{1/2}$) of [D_{10}]PheT.

The clearance of [D_{10}]PheT (CL_7, Figure 2) was estimated from the slope of the urinary excretion rate *vs.* mid-point plasma concentration curve:

$$\Delta X/\Delta t = CL_7 \times Cp_{mid} \qquad \text{Equation 1}$$

where Cp_{mid} was the plasma concentration of [D_{10}]PheT at the mid-point of the urine collection interval. The urinary excretion rate ($\Delta X/\Delta t$) was determined from the amount of [D_{10}]PheT excreted in urine at each interval (ΔX) divided by the length of the collection interval (Δt). Urine collections at 720-1440 and 1440-2880 min were not included in the estimate of CL_7 because very low concentrations were present in urine in those intervals, and

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each of those intervals was > 1 half-life of [D_{10}]PheT (Gibaldi and Perrier, 1982).

The total amount of [D_{10}]PheDE formed during activation (A_{act}) was estimated by two methods (equations 2 and 3; see Appendix A for detailed derivation of the equations). The first method was using the product of $AUC_{(PheT)}$ and CL_7:

$$A_{act,plasma} = AUC_{(PheT)} \times CL_7 \qquad \text{Equation 2}$$

The second method was to calculate the amount of [D_{10}]PheT collected in urine up to 48 hr ($Xu_{(PheT),t=48}$):

$$A_{act,urine} = Xu_{(PheT),t=48} \qquad \text{Equation 3}$$

The percentage of dose activated (f_{act}) was calculated as:

$$f_{act} = \frac{A_{act}}{D} \times 100\% \qquad \text{Equation 4}$$

where D is the administered dose of [D_{10}]Phe (10 µg or 53.2 nmol).

$A_{act,plasma}$ and $A_{act,urine}$ are two indicators of *systemic* exposure to [D_{10}]PheT and thus formed the basis of diagnostic plot I (Figure 3, top).

Differences in A_{act} between smoking and oral dosing were calculated as:

$$A_{act\ (lung)} = A_{act,smk} - A_{act,oral} \qquad \text{Equation 5}$$

where $A_{act,smk}$ and $A_{act,oral}$ are A_{act} after smoking and oral dosing, respectively. $A_{act\ (lung)}$ is a measure of lung contribution to the formation of [D_{10}]PheT. A positive value of $A_{act\ (lung)}$ indicates that the lung contributed more to the formation of [D_{10}]PheT than liver, and hence played a major role in metabolic activation. In addition, the larger the value

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of $A_{act\ (lung)}$, the larger local (lung) exposure to [D_{10}]PheT.

The relative bioavailability of [D_{10}]PheT after oral dosing compared to the smoking administration was calculated from the ratio of oral/smoking $AUC_{(PheT)}$ values (F_{AUC}) and the ratio of oral/smoking $Xu_{(PheT),t=48}$ values (F_{Xu}). F_{AUC} and F_{Xu} were used as two indicators of *lung contribution to activation*, and formed the basis for diagnostic plot II (Figure 3, bottom). The combination of plots I and II allowed the identification of subjects with substantial *local* exposure.

According to equation A23 in Appendix A, the amount of [D_{10}]PheDE formed after oral dosing and smoking could be calculated as

$A_{act} = [(f_a \times f_m) + f_{act}] \times \text{Dose}$ Equation 6

where f_a, f_{act} and f_m are the fraction of the dose absorbed, the fraction of the dose converted to [D_{10}]PheDE during first-pass activation and the fraction of [D_{10}]Phe converted to [D_{10}]PheDE in the systemic circulation, respectively. Since changes in the route of administration would only affect f_a and f_{act}, but f_m would remain the same, equation 6 could be rewritten as 6.1 and 6.2 for the oral and smoking arm, respectively.

$A_{act\ (oral)} = [(f_{a\ (oral)} \times f_m) + f_{act\ (oral)}] \times \text{Dose}$ Equation 6.1

$A_{act\ (smk)} = [(f_{a\ (smk)} \times f_m) + f_{act\ (smk)}] \times \text{Dose}$ Equation 6.2

$f_{a\ (oral)}$ and $f_{a\ (smk)}$ are the fraction of [D_{10}]Phe dose absorbed after oral dosing and smoking, respectively. $f_{act\ (oral)}$ and $f_{act\ (smk)}$ are the fraction of [D_{10}]Phe converted to

active intermediates during first-pass metabolism after oral dosing and smoking, respectively.

Genotyping. Twelve polymorphisms of metabolizing enzymes of Phe were determined by the BioMedical Genomics Center at the University of Minnesota. Genotyping was performed using the iPLEX® Gold method (Sequenom, Inc, San Diego, CA). Similar methods have been reported (Hecht et al., 2006). Briefly, the method is based on the primer-extension reaction that generates allele-specific products with distinct masses detected by Matrix-Assisted Laser Desorption / Ionization Time-Of-Flight (MALDI-TOF) mass spectrometry. Briefly, the method started with polymerase chain reaction (PCR) amplification followed by shrimp alkaline phosphatase (SAP) treatment to remove unincorporated dNTPs. Single-base extension (SBE) was carried out by the addition of SBE primers, iPLEX® enzymes and buffers. SBE products were measured with the MassARRAY® system (Sequenom, Inc, San Diego, CA), and mass spectra were analyzed with TYPER® software (Sequenom, Inc, San Diego, CA). iPLEX reagents and protocols for multiplex PCR, SBE and generation of mass spectra were based on the manufacturer's instructions.

The polymorphisms of *CYP1A1* and *CYP1B1* investigated in the study were *CYP1A1MspI, CYP1A1I462V, CYP1B1R48G, CYP1B119S, CYP1B1L432V* and *CYP1B1N453S*. In addition, *EPHX1Y113H* and *EPHX1H139R,* two polymorphisms of microsomal epoxide hydrolase 1 (*EPHX1*), were also measured. The polymorphisms of the detoxification enzymes glutathione *S*-transferase P1 (*GSTP1*), T1 (*GSTT1*), and M1 (*GSTM1*) measured in the study were *GSTP1A114V, GSTP1I105V, GSTT1* null and *GSTM1* null.

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Statistical Analysis. A paired t-test was used to compare $t_{1/2}$, CL_7 and $AUC_{(PheT)}$ between the oral and smoking arm. The measurement of relative bioavailability by two methods (F_{AUC} *vs.* F_{Xu}) was compared by using a paired t-test after the logarithmic transformation of the original data. Two-way ANOVA was employed to investigate the influence of data source (plasma *vs.* urinary data) and route of administration (oral *vs.* smoking) on the estimates of systemic exposure to [D_{10}]PheT. One-way ANOVA was used to identify polymorphisms that might have effects on an individual's capacity to activate PAHs. A *p*-value of < 0.05 was considered to be significant.

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RESULTS

Pharmacokinetics Table 1 reports the half-life, clearance (CL_7) and $AUC_{(PheT)}$ of [D_{10}]PheT after oral dosing and smoking administration of [D_{10}]Phe. No significant difference was observed in $t_{1/2}$, CL_7, or $AUC_{(PheT)}$ between the oral and smoking arms of the study, consistent with the results reported previously for 16 subjects (Zhong et al., 2011b).

A_{act} is an estimate of the systemic exposure to [D_{10}]PheT and was calculated by two methods: use of plasma data ($A_{act,plasma}$) and use of urinary data ($A_{act,urine}$). As shown in Table 2, $A_{act,plasma}$ and $A_{act,urine}$ were 4.23 ± 3.94 and 3.06 ± 1.91 nmol in the smoking arm, respectively. The percentage of the [D_{10}]Phe dose activated (f_{act}) was calculated as 7.96 ± 7.41 and 5.75 ± 3.60 based on plasma and urinary data, respectively. Similar results were obtained from the oral arm. Neither route of administration (smoking *vs.* oral) nor source of data (plasma *vs.* urine) had a significant impact on the estimate of A_{act}.

Although at the group level no difference was observed in the means of A_{act}, a large inter-subject variability (> 20 fold) in A_{act} was observed in both the smoking and oral arms (Table 2). One purpose of this study was to identify people with a potentially increased susceptibility to carcinogenesis by PAH because of their ability to carry out the bay region diol epoxide pathway. Since the route of administration of [D_{10}]Phe in the smoking arm mimicked the uptake of carcinogens by cigarette smokers, plasma and urinary data of the smoking arm were used to identify subjects with a large systemic exposure to [D_{10}]PheT, as shown in diagnostic plot I (Figure 3, top). Six subjects whose estimates of systemic exposure

(A_{act}) were in the top 30% of the population as measured by plasma data ($\geq$ 4.68 nmol by equation 2) and urine data ($\geq$ 4.29 nmol by equation 3) fell into zone A (large systemic exposure zone): subjects 1, 6, 14, 22, 23 and 24. Similarly, 5 subjects whose estimates of systemic exposure were in the lowest 30% of the population as measured by plasma data ($\leq$ 1.96 nmol by equation 2) and urine data ($\leq$ 1.58 nmol by equation 3), fell into zone B (low systemic exposure zone): subjects 2, 7, 12, 18 and 21.

The relative bioavailability of [D_{10}]PheT after oral dosing compared to the smoking administration was calculated from the ratio of oral/smoking $AUC_{(PheT)}$ values (F_{AUC}) and the ratio of oral/smoking $Xu_{(PheT),t=48}$ values (F_{Xu}). F_{AUC} and F_{Xu} were used as two indicators of lung contribution to metabolic activation. As shown in Figure 4, after the administration of [D_{10}]Phe by smoking, the parent molecule [D_{10}]Phe passes through the lung before it reaches the systemic circulation. After oral dosing the parent molecule passes through the liver before it reaches the systemic circulation. Therefore, if F_{AUC} and F_{Xu} are less than 1, the lung has a greater contribution to the formation of [D_{10}]PheT. As shown in Table 3, the relative bioavailability measured by F_{AUC} and F_{Xu} was 1.35 ± 1.33 and 1.35 ± 0.96, respectively. No significant difference was observed in relative bioavailability measured by the two methods. At the group level no significant difference was observed in the disposition of parent molecule by lung and liver. However, a large inter-subject variability was observed (Table 3), illustrating a greater than a 9-fold range in both F_{AUC} and F_{Xu}. Diagnostic plot II was then developed to identify subjects with significant lung contribution to metabolic activation. Nine subjects with both F_{AUC} and F_{Xu} values of less than 1 were identified in zone C (Figure 3,

bottom): subjects 1, 3, 4, 5, 6, 8, 13, 22 and 25.

The combination of zones A (diagnostic plot I) and C (diagnostic plot II) led to the identification of three subjects with both a large systemic exposure and a significant lung contribution to activation: subjects 1, 6 and 22. These three subjects formed much more [D_{10}]PheT after smoking than oral dosing (Table 4). In addition, $A_{act\ (lung)}$ of these 3 subjects was between 0.66 and 10.56 nmol. This amount of [D_{10}]PheT indicated substantial local exposure, especially considering that the total exposure to [D_{10}]PheT in a typical subject after smoking ranged from 3.06 to 4.23 nmol.

Table 5 shows the effects of metabolic enzyme polymorphisms on an individual's capacity to activate PAHs as measured by estimates of A_{act} (equations 2 and 3) of both the oral and smoking arms. Among 12 polymorphisms tested only the *GSTM1* polymorphism was associated with a difference in systemic exposure. Figure 5a (plasma data) and 5b (urine data) show the maximum, minimum and median of A_{act} in *GSTM1* negative and positive subjects after smoking. A more than two-fold difference was observed in A_{act} between *GSTM1* negative subjects ($A_{act,plasma}$: 5.87 ± 4.85 nmol; $A_{act,plasma}$: 4.16 ± 2.00 nmol; n = 12) and *GSTM1* positive subjects ($A_{act,plasma}$: 2.60 ± 1.76 nmol; $A_{act,plasma}$: 2.05 ± 1.16 nmol; n=13) after smoking ($p < 0.05$). To further confirm the effects of *GSTM1* on the metabolic activation, estimates of A_{act} from the oral arm were investigated (Figure 5 c and d) and similar results were observed.

Figure 6 integrates diagnostic plot I and *GSTM1* genotype information, and reveals an

interesting correlation between genotyping and phenotyping results. The occurrence of the *GSTM1* negative genotype was 100% (n = 6) in zone A compared with 48% in the group as a whole (n = 25). In contrast, the occurrence of the *GSTM1* negative genotype was only 20% (n = 5) in zone B. High occurrence of the *GSTM1* negative genotype in subjects with a large systemic exposure to [D_{10}]PheT, and low occurrence of the *GSTM1* negative genotype in subjects with a low systemic exposure to [D_{10}]PheT clearly suggests a correlation between *GSTM1* genotype and an individual's capacity to activate PAHs. It is of interest to note that the three subjects (1, 6 and 22) with both high systemic exposure and significant lung contribution to activation were all *GSTM1* negative and African American.

DISCUSSION

Since lung cancer treatment is not particularly effective (five-year survival rate < 16%), prevention is an important alternative, especially considering that tobacco smoking accounts for 90% of cases of the disease in the United States (IARC 2004; Spiro et al., 2005). The outcome of smoking cessation, a major prevention strategy, depends on the intensity of intervention. The rate of successful smoking cessation at 1 year is 3-5% when smokers simply try to stop, 7-16% when behavioral intervention is provided, and up to 24% when smokers receive both pharmacological treatment and behavioral support (Laniado-Laborín, 2010). Therefore, the successful identification of susceptible individuals could lead to increasing the intensity of intervention for these individuals. This would potentially improve the outcome of smoking cessation interventions. The current study aimed to quantitate an individual's capacity to metabolically activate PAHs (one group of carcinogens involved in lung cancer), and identify subjects with extensive activation and presumably higher lung cancer risk.

The large inter-subject variability (> 20 fold) in the capacity to activate [D_{10}]Phe was consistent with the large inter-subject variability in the activation of PAHs reported in the literature (Cohen et al., 1979), and further justified the use of a PK approach to identify highly susceptible individuals. In our PK study of 25 subjects, the fraction of [D_{10}]Phe converted to [D_{10}]PheT ranged from 0.49% to 15.04% ($A_{act,urine}$) in the smoking arm. Even though only a relatively small fraction of a PAH, as represented by [D_{10}]Phe, is metabolically activated in humans, it is believed to be critical in the initiation of carcinogenesis (Conney,

1982; Cooper et al. 1983; Dipple et al. 1984; Geacintov et al., 1997; Gelboin, 1980; Szeliga and Dipple, 1998). Metabolites not quantified in this study include phenols and dihydrodiols as well as unidentified material.

The quantitation of an individual's capacity to activate PAHs was the primary objective of the current study. The unique PK approach used here quantitated both systemic and local exposure to [D_{10}]PheT. There are no other published studies on PAH PK in humans. While the measurement of systemic exposure is important in the identification of susceptible subjects, local (lung) exposure is a physiologically more relevant measurement for two reasons: 1) it captures the magnitude of exposure to carcinogenic intermediates at the specific site where carcinogenesis occurs; 2) [D_{10}]Phe was employed as a surrogate of BaP and other related PAHs which generally have their strongest carcinogenic effects at the site of application, although there are exceptions (Hecht, 2003a; IARC, 1983; IARC, 2010). For locally acting carcinogens, lung exposure is much more relevant to tobacco smoke carcinogenesis of lung cancer than systemic exposure. Despite its crucial role in carcinogenesis by PAHs, local exposure is usually very difficult to measure in clinical trials. In the present study the integration of systemic exposure and relative bioavailability revealed important information regarding local exposure. The concept is that subjects with both large systemic exposure (zone A of plot I, Figure 3) and significant lung contribution to activation (zone C of plot II, Figure 3) had substantial local (lung) exposure. As shown in Figure 4, for a given individual, the difference in systemic exposure after oral dosing and smoking is due to the different pathways that [D_{10}]Phe takes before reaching the systemic circulation, i.e., one

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single pass of parent molecule through the liver or lung. In other words, if more [D_{10}]Phe were activated after smoking than oral dosing ($F_{AUC} < 1$ or $F_{Xu} < 1$), then this subject's lung contributed more to the activation than did liver. Therefore, subjects with relative bioavailability less than 1 as well as subjects with large systemic exposure were of interest.

If relative bioavailability is less than 1, then by definition $A_{act(oral)} < A_{act(smk)}$. According to equations 6.1 and 6.2, there are three scenarios when $A_{act(oral)} < A_{act(smk)}$:

1) $f_{a\ (oral)} < f_{a\ (smk)}$ and $f_{act\ (oral)} < f_{act\ (smk)}$

2) $f_{a\ (oral)} > f_{a\ (smk)}$ and $f_{act\ (oral)} < f_{act\ (smk)}$

3) $f_{a\ (oral)} < f_{a\ (smk)}$ and $f_{act\ (oral)} > f_{act\ (smk)}$

In the first case, since $f_{a\ (oral)} < f_{a\ (smk)}$, the lung allowed a larger fraction of [D_{10}]Phe to enter systemic circulation than did the liver, indicating a larger systemic exposure after smoking than oral dosing. In addition, since $f_{act\ (oral)} < f_{act\ (smk)}$, more active intermediates would be produced in lung than liver, indicating substantial local exposure. Therefore, case 1 was "large systemic exposure, large local exposure". Similarly, case 2 and 3 would be considered "low systemic exposure, large local exposure", and "large systemic exposure, low local exposure", respectively. Although in case 3 lung may receive lower exposure to the active intermediates than does the liver, given the substantial amount of active intermediates in the circulation, the attack of electrophilic intermediates against lung DNA would still probably be substantial. Therefore, for all the cases related to a relative bioavailability of less than 1, potentially negative health consequences due to substantial

local exposure, may be likely. As such, diagnostic plot II was developed to identify subjects with a significant lung contribution to metabolic activation.

As shown in plot I (Figure 3, top), systemic exposure was calculated by two methods (equation 2 and 3). Similarly, in plot II (Figure 3, bottom) the relative bioavailability was also estimated by two methods (F_{AUC} and F_{Xu}) to confirm the identification of subjects with significant lung contribution to activation. The combination of plot I and plot II led to the identification of 3 subjects. In these 3 subjects one single pass through lung produced the same level of [D_{10}]PheT as total exposure in a typical subject. This suggests that these subjects may have higher susceptibility than the rest of the study group.

There was a statistically significant difference in systemic exposure to [D_{10}]PheT between *GSTM1* negative and positive subjects. Since GSTM1 is a detoxification enzyme that catalyzes the conjugation of electrophilic intermediates with glutathione, the deletion of the *GSTM1* gene reduces the efficiency of the detoxification pathways. As a result, more [D_{10}]Phe may go through the activation pathway and more end product of the activation pathway was observed in *GSTM1* negative subjects. Furthermore, about a two-fold difference in activation caused by GSTM1 might lead to clinically different outcomes considering the important role of PAHs in lung carcinogenesis and decades of uptake of PAHs from cigarette smoking. It has been reported that *GSTM1* negative subjects have higher PAH-DNA adducts in the lung than *GSTM1* positive subjects (Rojas et al., 1998; Rojas et al., 2000). It is worth noting that the three subjects identified by diagnostic plots I and II were all *GSTM1* negative.

In addition, all three subjects were African American. The development of a population PK model is in progress which will estimate the population mean and inter-subject variability of the efficiency of the activation pathway. Age, gender, weight, race, renal function, genotype, and smoking history will be incorporated in the population PK analysis to examine their influence on lung cancer susceptibility.

In other studies, we have found that conjugation of Phe diol epoxides by glutathione is a relatively minor pathway, at least based on the amount of the appropriate N-acetylcysteine conjugate found in urine (Hecht et al., 2008). The amounts appear to be too low to account for the decreased levels of [D_{10}]PheT in the *GSTM1* competent vs. *GSTM1* null individuals observed here. It is possible that glutathione conjugation occurs earlier in the pathway that produces [D_{10}]PheT, for example by reaction with Phe-1,2-epoxide (Figure 1).

Although no effects of other polymorphisms on systemic exposure were observed, it is likely that multiple genes are involved in the metabolic activation of PAHs (Shimada, 2006; Hecht, 2010a). The low frequency of minor alleles and relatively small number of subjects in the present study may explain the failure to detect an association of these polymorphisms with our measures of exposure.

One limitation of the present study was the lack of a PK profile of the parent compound [D_{10}]Phe due to its extremely low concentrations in plasma (< 25 pg/mL) and urine (< 5 pg/mL) following the 10 µg dose of [D_{10}]Phe (Zhong et al., 2011b). Animal PK studies have

reported using a dose of 4.5-10 mg/kg which was at least 30,000- fold higher than the dose used in this study (Chu et al., 1992; Schober et al., 2010). The half-life and oral clearance of phenanthrene in a mouse study with an oral dose of 4.5 mg/kg were reported to be 0.32 hr and 2.18 L/hr·kg, respectively. Another study in rat and guinea pig indicated that more than 90% of the parent compound was converted to metabolites after oral administration of 10 mg/kg ^{14}C-labelled Phe. The distribution of Phe to tissues was not well characterized, but Phe is probably extensively distributed to tissues because of its lipophilicity. Collectively, the low dose of [D_{10}]Phe in human study, extensive metabolism and rapid distribution are likely to contribute to the low concentration of [D_{10}]Phe in plasma and urine.

In summary, in the present study the metabolic activation of Phe in humans has been investigated. Plasma and urinary data of the smoking arm indicated a more than 20-fold difference in an individual's capacity to activate PAHs, and formed the basis of diagnostic plot I to identify subjects with large systemic exposure. The relative bioavailability between oral dosing and smoking also showed a large inter-subject variability (> 9 fold), and formed the basis of diagnostic plot II to identify subjects with significant lung contribution to activation. The combination of plots I and II led to the identification of subjects with substantial lung exposure. This approach may have significant potential in the prediction of lung cancer risk. The integration of phenotyping and genotyping results indicated that *GSTM1* played an important role in the detoxification of Phe.

ACKNOWLEDGMENTS

We thank Dr. Andrea Yoder, Miss Claire Brookmeyer and Dr. Peter Villalta for their consultancy on GC/MS.

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FOOTNOTES

This study was supported by [Grant CA-92025] from the National Cancer Institute

FIGURE LEGNEDS

Figure 1. Metabolic activation pathways of BaP and Phe (modified from Carmella SG et al., 2004)

Figure 2. The PK view of metabolic activation of [D_{10}]Phe after administration (detailed description in Appendix A)

Figure 3. Diagnostic plot I (top) and II (bottom)

Figure 4. Comparison of metabolic activation of [D_{10}]Phe in the oral and smoking arm

Figure 5. Comparison of *GSTM1* negative (-) and positive (+) subjects after smoking (a and b) and oral dosing (c and d) (dotted line: median, * $p < 0.05$, ** $p < 0.01$)

Figure 6. Correlation between genotyping and phenotyping (triangle: *GSTM1* positive; circle: *GSTM1* negative)

JPET Fast Forward. Published on June 6, 2012 as DOI: 10.1124/jpet.112.194118
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Table 1 Half-life, clearance and AUC of [D_{10}]PheT after oral dosing and smoking

ID	$t_{1/2}$ (hr)		CL_7 (L/hr)		$AUC_{(PheT)}$ (nmol*hr/L)	
	Oral	SMK	Oral	SMK	Oral	SMK
1	7.2	10.9	7.8	13.2	1.07	1.43
2	17.7	8.1	3.1	7.3	0.13	0.11
3	12.6	11.4	5.8	6.4	0.97	1.06
4	11.0	6.7	4.9	6.4	0.37	0.38
5	3.9	4.8	5.5	4.8	0.24	0.48
6	6.3	7.5	4.3	7.3	1.10	1.49
7	12.6	8.1	4.5	13.0	0.97	0.13
8	5.5	5.9	5.4	4.6	0.46	0.51
9	6.4	9.8	3.2	1.3	1.04	0.81
10	6.9	7.5	5.1	7.1	0.55	0.48
11	4.9	4.5	7.5	3.1	0.85	0.70
12	4.4	6.4	3.2	3.6	0.43	0.48
13	7.9	9.9	6.0	7.0	0.58	0.76
14	7.4	7.1	4.0	4.5	1.70	1.11
15	8.8	6.0	4.6	4.0	0.94	0.53
16	5.1	5.2	4.4	4.5	0.93	0.87
17	9.3	7.5	1.9	1.9	1.98	1.27
18	6.1	11.0	2.2	2.2	0.80	0.65
19	5.4	N/A [a]	1.8	N/A [a]	0.90	N/A [a]

20	5.2	6.2	3.7	4.1	1.04	1.02
21	7.4	9.8	3.2	2.9	1.45	0.69
22	5.8	8.9	2.8	4.7	0.82	1.53
23	7.0	6.7	3.0	5.1	2.08	1.45
24	4.8	7.3	5.3	3.0	1.29	1.57
25	6.3	6.9	2.4	2.9	0.65	0.79
Mean	7.4	7.7	4.2	5.2	0.93	0.85
SD	3.2	2.0	1.6	3.0	0.49	0.44
p value [b]	N.S.		N.S.		N.S.	

a: Not available because plasma concentration of [D_{10}]PheT of subject 19 after smoking was below the limit of quantitation

b: Paired t-test was performed to compare the effect of route of administration; N.S. not significant, $p > 0.05$

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Table 2 A_{act} of oral and smoking arm calculated by two methods

ID	Oral arm				Smoking arm			
	$A_{act,plasma}$		$A_{act,urine}$		$A_{act,plasma}$		$A_{act,urine}$	
	nmol	% of dose	nmol	% of dose	nmol	% of dose	nmol	% of dose
1	8.25	15.51	5.62	10.56	18.81	35.36	8.00	15.04
2	0.41	0.77	0.28	0.53	0.79	1.48	0.43	0.81
3	5.57	10.47	3.37	6.33	6.74	12.67	3.45	6.48
4	1.79	3.36	1.33	2.50	2.41	4.53	2.06	3.87
5	1.32	2.48	1.49	2.80	2.30	4.32	2.27	4.27
6	4.62	8.68	6.37	11.97	10.77	20.24	7.03	13.21
7	4.36	8.20	3.29	6.18	1.71	3.21	1.08	2.03
8	2.45	4.61	1.89	3.55	2.30	4.32	2.46	4.62
9	3.24	6.09	5.29	9.94	1.00	1.88	3.76	7.07
10	2.83	5.32	2.28	4.29	3.44	6.47	2.22	4.17
11	6.31	11.86	6.11	11.48	2.14	4.02	3.22	6.05
12	1.36	2.56	1.72	3.23	1.73	3.25	1.58	2.97
13	3.43	6.45	2.45	4.61	5.26	9.89	2.96	5.56
14	6.75	12.69	5.85	11.00	4.91	9.23	4.40	8.27
15	4.3	8.08	4.45	8.36	2.10	3.95	3.29	6.18
16	4.08	7.67	4.20	7.89	3.92	7.37	4.13	7.76
17	3.72	6.99	3.89	7.31	2.34	4.40	2.33	4.38
18	1.74	3.27	1.68	3.16	1.39	2.61	0.77	1.45

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19	1.59	2.99	1.31	2.46	N/A [a]	N/A [a]	0.26	0.49
20	3.83	7.20	3.43	6.45	4.11	7.73	2.86	5.38
21	4.6	8.65	2.66	5.00	1.96	3.68	1.42	2.67
22	2.29	4.30	2.33	4.38	7.14	13.42	4.29	8.06
23	6.24	11.73	5.95	11.18	7.37	13.85	5.26	9.89
24	6.77	12.73	5.63	10.58	4.68	8.80	5.13	9.64
25	1.57	2.95	1.07	2.01	2.31	4.34	1.87	3.52
Mean	3.74	7.02	3.36	6.31	4.23	7.96	3.06	5.75
SD	2.06	3.87	1.86	3.50	3.94	7.41	1.91	3.60
p value [b]	N.S.							

a: Not available because plasma concentration of [D_{10}]PheT after smoking was below the limit of quantitation

b: Two-way ANOVA was performed to evaluate the influence of route of administration and data source (plasma, urine); N.S. not significant, $p > 0.05$

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Table 3 Relative bioavailability by two methods (F_{AUC} and F_{Xu})

ID	Relative Bioavailability	
	F_{AUC}	F_{Xu}
1	0.75	0.70
2	1.20	0.66
3	0.92	0.98
4	0.98	0.65
5	0.50	0.66
6	0.73	0.91
7	7.32	3.05
8	0.91	0.77
9	1.29	1.41
10	1.14	1.03
11	1.21	1.90
12	0.88	1.09
13	0.76	0.83
14	1.53	1.33
15	1.80	1.36
16	1.06	1.02
17	1.56	1.67
18	1.22	2.19
19	N/A [a]	5.01

20	1.02	1.20
21	2.11	1.87
22	0.53	0.54
23	1.43	1.13
24	0.82	1.10
25	0.83	0.57
Mean	1.35	1.35
SD	1.33	0.96
90% CI [b]	(0.93 -1.35)	(0.95 -1.38)
p value [c]	N.S.	

a: Not available because plasma concentration of [D_{10}]PheT after smoking was below the limit of quantitation

b: 90% CI of geometric means

c: Paired t test was performed to compare two methods of calculating relative bioavailability; N.S. not significant, $p > 0.05$

JPET Fast Forward. Published on June 6, 2012 as DOI: 10.1124/jpet.112.194118
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Table 4 Significant lung contribution to metabolic activation in subject 1, 6 and 22

ID	$A_{act,plasma}$ (nmol)		$A_{act(lung)}$ [b]	$A_{act,urine}$		$A_{act(lung)}$ [c]
	Smoking	Oral	(nmol)	Smoking	Oral	(nmol)
1	18.81	8.25	10.56	8.00	5.62	2.38
6	10.77	4.62	6.15	7.03	6.37	0.66
22	7.14	2.29	4.85	4.29	2.33	1.96
A typical subject[a]	4.23	3.74	-	3.06	3.36	-

a. Data of a typical subject was based on the group mean (n = 25)

b. The difference of $A_{act,plasma}$ between smoking and oral dosing

c. The difference of $A_{act,urine}$ between smoking and oral dosing

Table 5 Effects of polymorphisms on individual's capacity to activate PAHs

Gene Polymorphism	% of Occurrence			*p* values[a]			
				Oral		Smoking	
	Normal	Heterozytes	Homozygotes	$A_{act,plasma}$	$A_{act,urine}$	$A_{act,plasma}$	$A_{act,urine}$
CYP1A1 MspI (rs4646903)	64	36	0	N.S.	N.S.	N.S.	N.S.
CYP1A1 I462V (rs1048943)	88	12	0	N.S.	N.S.	N.S.	N.S.
CYP1B1 R48G (rs10012)	32	60	8	N.S.	N.S.	N.S.	N.S.
CYP1B1 A119S (rs1056827)	8	92	0	N.S.	N.S.	N.S.	N.S.
CYP1B1 L432V (rs1056836)	20	52	28	N.S.	N.S.	N.S.	N.S.
CYP1B1 N453S (rs1800440)	84	16	0	N.S.	N.S.	N.S.	N.S.
GSTP1 I105V (rs1695)	36	52	12	N.S.	N.S.	N.S.	N.S.
GSTP1 A114V (rs1138272)	84	16	0	N.S.	N.S.	N.S.	N.S.
EPHX1 Y113H (rs1051740)	32	68	0	N.S.	N.S.	N.S.	N.S.

EPHX1 H139R (rs2234922)	64	28	8	N.S.	N.S.	N.S.	N.S.
GSTT1	12 (presence)		88 (null)	N.S.	N.S.	N.S.	N.S.
GSTM1	52 (presence)		48 (null)	*	*	*	**

a: One way ANOVA was performed to determine the influence of genotype on A_{act}; N.S. not significant $p > 0.05$, * $p < 0.05$, ** $p < 0.01$

Figure 1

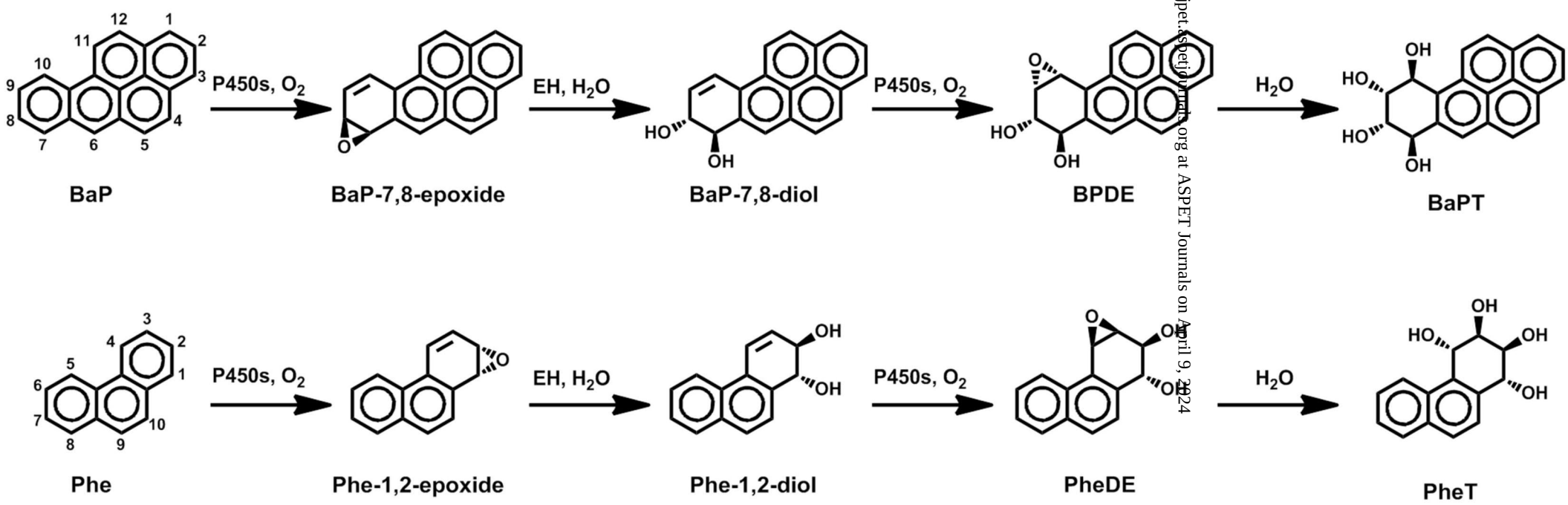

Figure 2

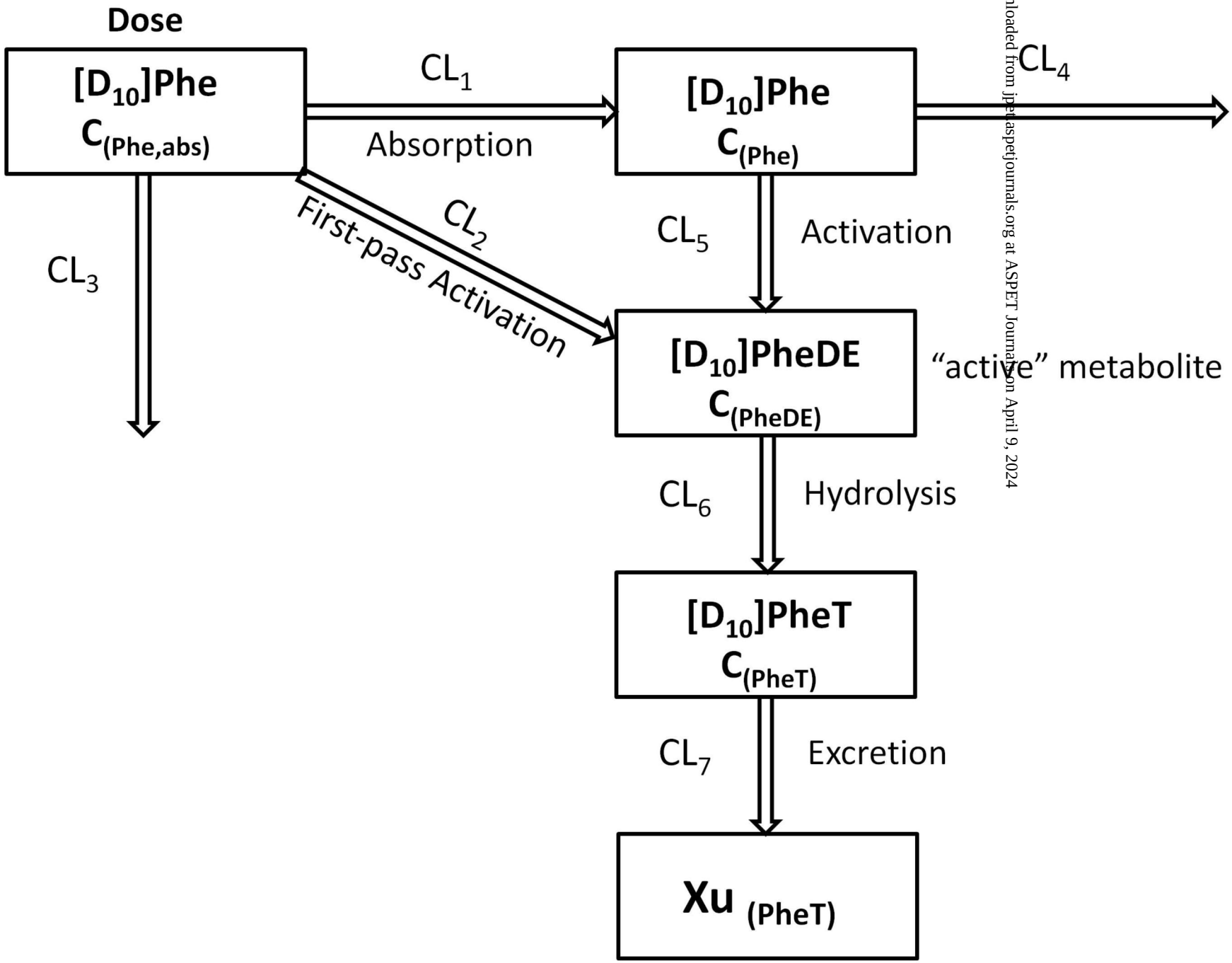

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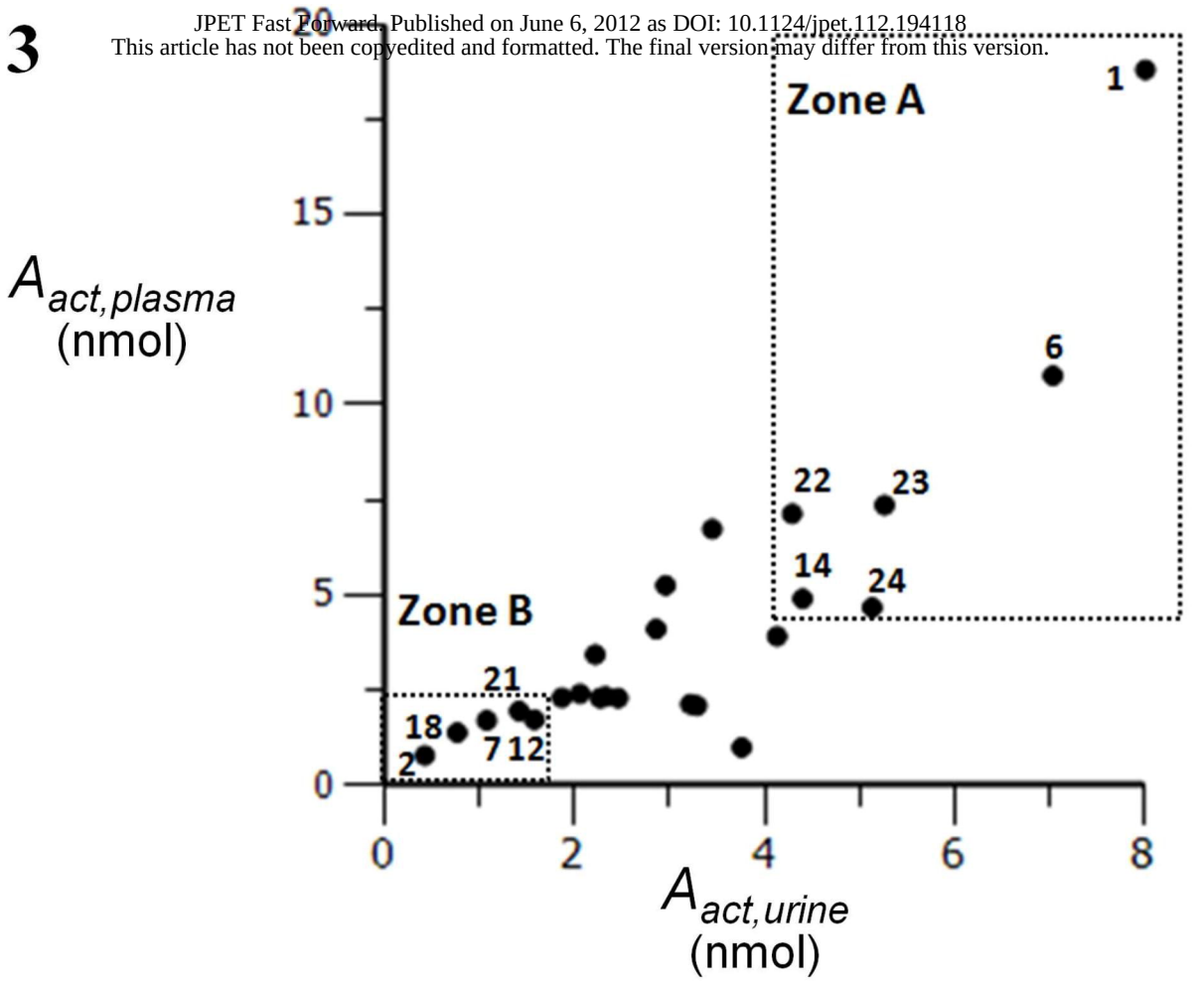
Figure 3



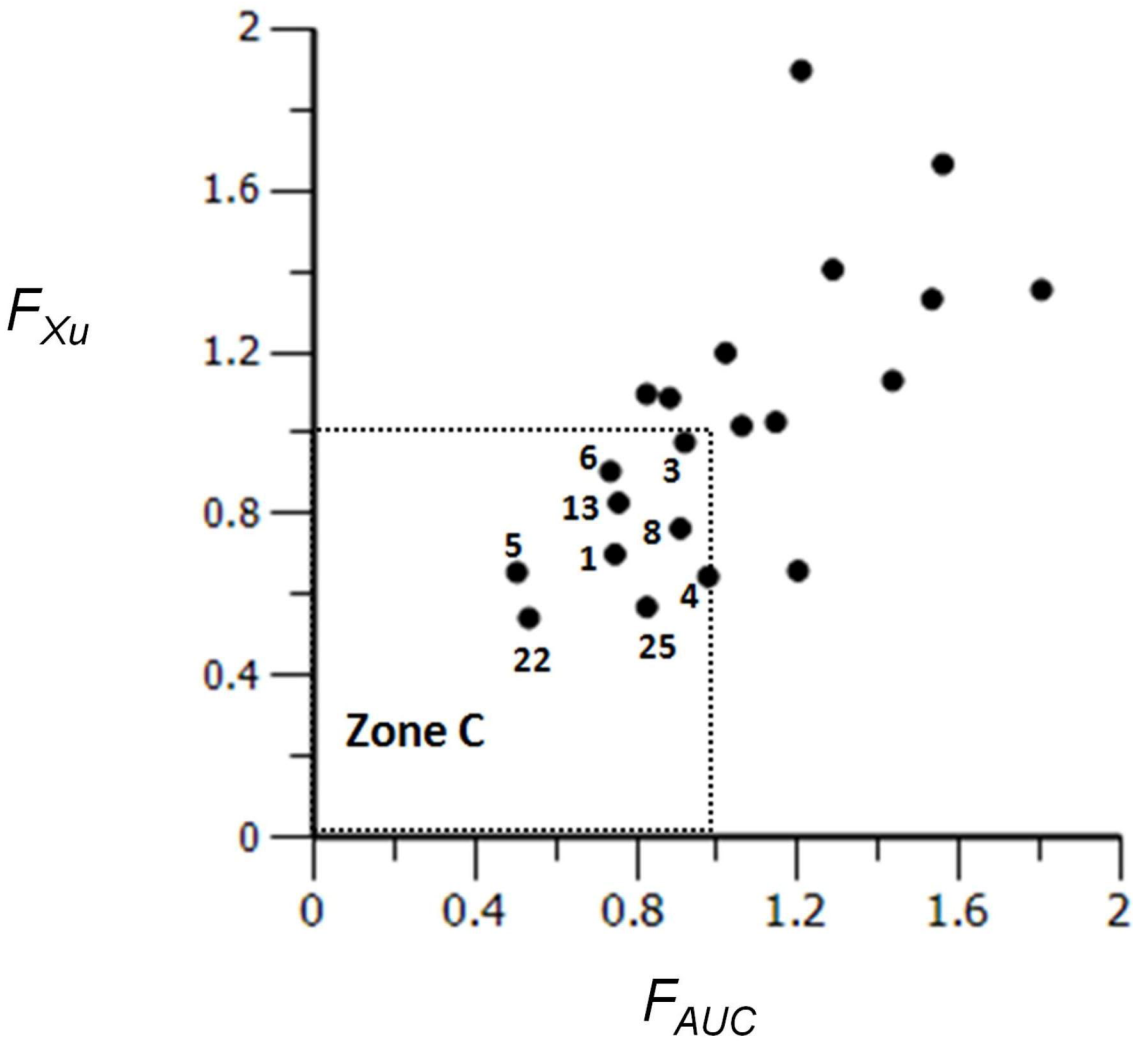
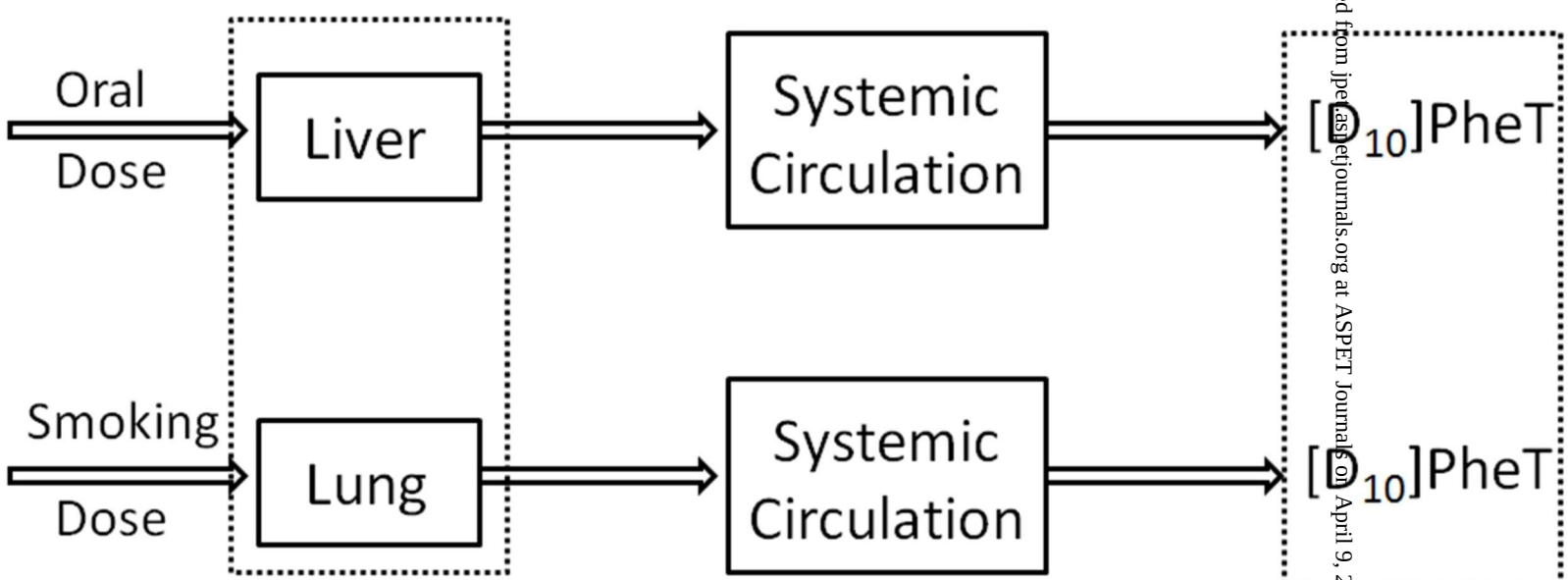


Figure 4



Differences in the disposition of $[D_{10}]$Phe between liver and lung

Differences in systemic exposure between oral and smoking arm

Figure 5

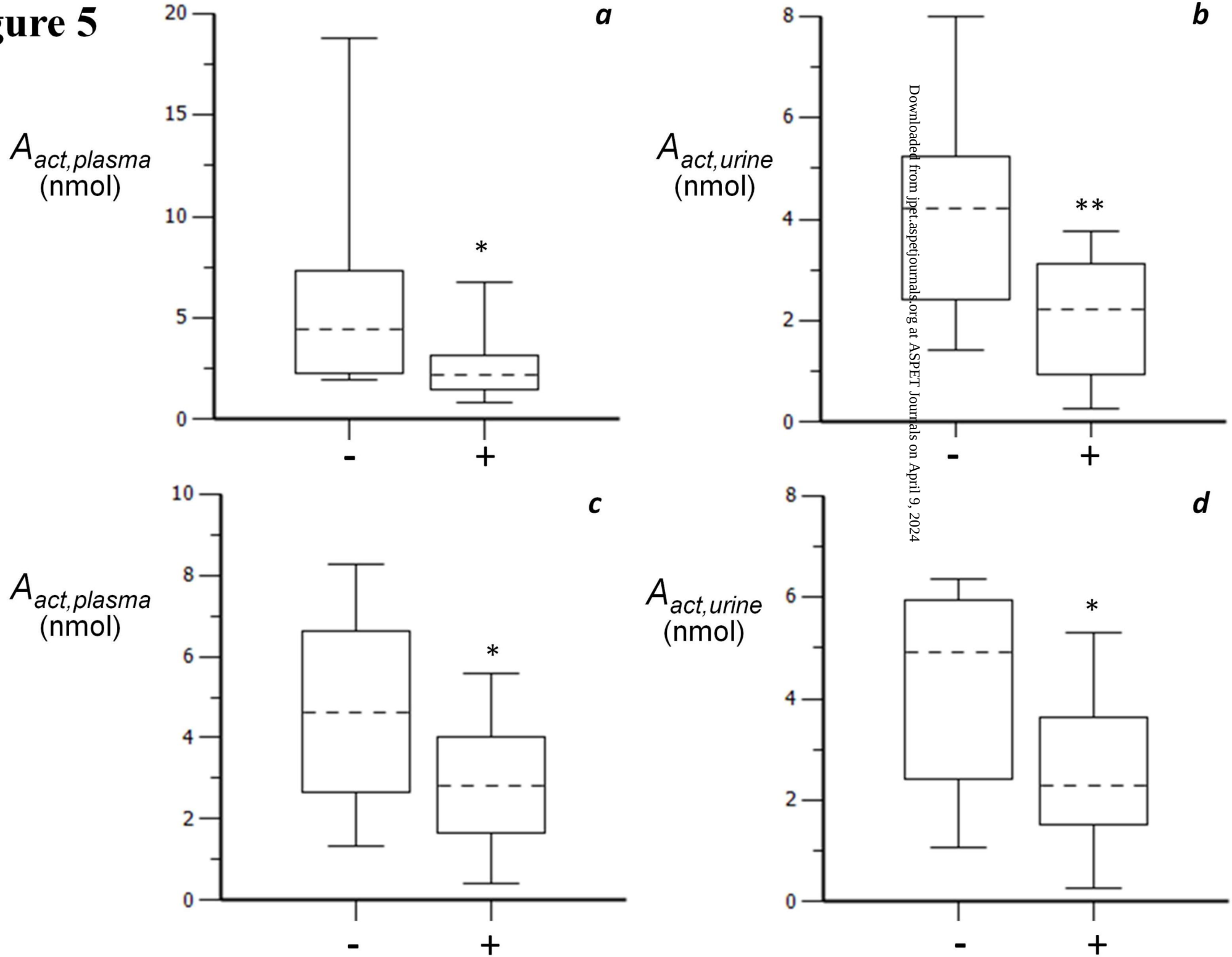
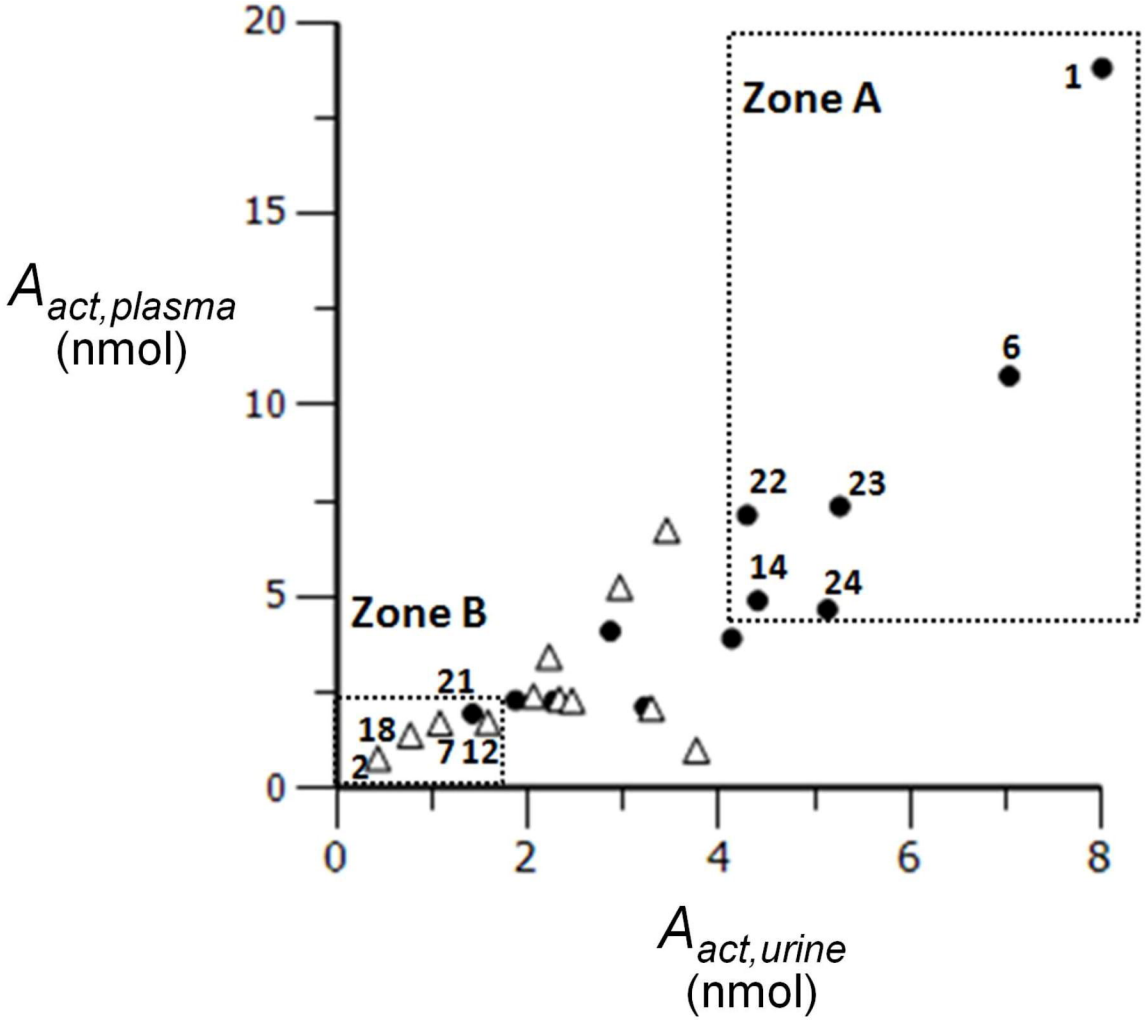


Figure 6



Appendix A

In the PK approach to identify susceptible subjects, one primary objective was to quantitate the [D_{10}]PheT formed and use it as a surrogate for the amount of "active" metabolite [D_{10}]PheDE formed during activation (A_{act}). This is an indicator of systemic exposure. As shown in Figure 1, [D_{10}]PheDE is the surrogate for the active intermediate BPDE, which binds to DNA and initiates carcinogenesis. However, direct measurement of [D_{10}]PheDE was very difficult because of the poor stability of this intermediate. Our investigation turned to [D_{10}]PheT, the end product of activation pathway. Based on our understanding of the metabolic activation of Phe as shown in Figure 2, A_{act} could be estimated by quantitating [D_{10}]PheT in plasma or urine.

The absorption and disposition of [D_{10}]Phe after extravascular administration (oral or smoking) could be summarized in 4 steps (Figure 2). Step 1: after delivery to the site of absorption, [D_{10}]Phe was transferred to systemic circulation intact (absorption), in the form of [D_{10}]PheT (first-pass activation), and in the form of other metabolites; the clearances of these three pathways of step 1 were CL_1, CL_2 and CL_3, respectively. Step 2: after entering the systemic circulation, a small fraction of [D_{10}]Phe was converted to "active" metabolite [D_{10}]PheDE (activation) and the majority of [D_{10}]Phe was transformed to other metabolites; the clearances of these two pathways of step 2 were CL_5 and CL_4, respectively. Step 3: [D_{10}]PheDE was converted to [D_{10}]PheT (hydrolysis) and the clearance of this process was CL_6; Step 4: [D_{10}]PheT was further converted to sulfate or glucuronide conjugates followed by renal excretion. The clearance of the process that eliminates [D_{10}]PheT from the body was

CL_7.

Several assumptions were made in step 3 and 4, respectively. The first assumption is that in step 3 all [D_{10}]PheDE was converted to [D_{10}]PheT. The second assumption is that in step 4 all the [D_{10}]PheT formed was excreted in urine in 48 hr.

As such, the following differential equations were derived

$$\frac{dA_{(Phe,abs)}}{dt} = -(CL_1 + CL_2 + CL_3) \times C_{(Phe,abs)} \quad \text{(A1)}$$

$$\frac{dA_{(Phe)}}{dt} = CL_1 \times C_{(Phe,abs)} - (CL_4 + CL_5) \times C_{(Phe)} \quad \text{(A2)}$$

$$\frac{dA_{(PheDE)}}{dt} = CL_2 \times C_{(Phe,abs)} + CL_5 \times C_{(Phe)} - CL_6 \times C_{(PheDE)} \quad \text{(A3)}$$

$$\frac{dA_{(PheT)}}{dt} = CL_6 \times C_{(PheDE)} - CL_7 \times C_{(PheT)} \quad \text{(A4)}$$

$$\frac{dXu_{(PheT)}}{dt} = CL_7 \times C_{(PheT)} \quad \text{(A5)}$$

Equation A1 could also be written as

$$dA_{(Phe,\ abs)} = -(CL_1 + CL_2 + CL_3) \times C_{(Phe,abs)}\, dt \quad \text{(A6)}$$

The integration of equation A6 with regard to time from t = 0 to t = ∞ provides the following expression:

$$\int_{A(Phe,\ abs),\ t=0}^{A(Phe,\ abs),\ t=\infty} dA_{(Phe,\ abs)} = -(CL_1 + CL_2 + CL_3) \int_0^{\infty} C_{(Phe,abs)}\, dt$$

$A_{(Phe,abs)}$ integrated between t = 0 and t = ∞ is equal to the dose of [D_{10}]Phe (D).

Therefore, $D = (CL_1 + CL_2 + CL_3) \times AUC_{(Phe,\ abs)}$

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$$AUC_{(Phe,\,abs)} = \frac{D}{CL_1 + CL_2 + CL_3} \quad \text{(A7)}$$

Similarly, the integration of equations A2, A3, A4 and A5 from t = 0 to t = ∞ provides the following expressions:

$$CL_1 \times AUC_{(Phe,abs)} = (CL_4 + CL_5) \times AUC_{(Phe)} \quad \text{(A8)}$$

$$CL_2 \times AUC_{(Phe,abs)} + CL_5 \times AUC_{(Phe)} = CL_6 \times AUC_{(PheDE)} \quad \text{(A9)}$$

$$CL_6 \times AUC_{(PheDE)} = CL_7 \times AUC_{(PheT)} \quad \text{(A10)}$$

$$Xu_{(PheT),t=\infty} = CL_7 \times AUC_{(PheT)} \quad \text{(A11)}$$

It is assumed that $Xu_{(PheT),t=\infty} \approx Xu_{(PheT),t=48}$; that is, almost all of the [D_{10}]PheT formed is excreted into the urine in 48 hours.

The integration of equation A3 from t = 0 to t = T (0 < T < ∞) provides the following expressions:

$$A_{(PheDE),t=T} - A_{(PheDE),t=0} = CL_2 \times AUC_0^T{}_{(Phe,\,abs)} + CL_5 \times AUC_0^T{}_{(Phe)} - CL_6 \times AUC_0^T{}_{(PheDE)}$$

Since $A_{(PheDE),t=0}$ equals 0, then

$$A_{(PheDE),t=T} = CL_2 \times AUC_0^T{}_{(Phe,\,abs)} + CL_5 \times AUC_0^T{}_{(Phe)} - CL_6 \times AUC_0^T{}_{(PheDE)} \quad \text{(A12)}$$

Similarly, the integration of equations A4 and A5 from t = 0 to t = T could result in equations A13 and A14:

$$A_{(PheT),t=T} = CL_6 \times AUC_0^T{}_{(PheDE)} - CL_7 \times AUC_0^T{}_{(PheT)} \quad \text{(A13)}$$

$$Xu_{(PheT),t=T} = CL_7 \times AUC_0^T{}_{(PheT)} \quad \text{(A14)}$$

At time T, the [D_{10}]PheDE that has been produced from activation can exist in three different

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forms: [D_{10}]PheDE in the body, [D_{10}]PheT in the body and [D_{10}]PheT in urine. Therefore, the amount of [D_{10}]PheDE formed at time T is the sum of $A_{(PheDE),t=T}$, $A_{(PheT),t=T}$ and $Xu_{(PheT),t=T}$.

Combining equations A12, A13 and A14

$$A_{(PheDE),t=T} + A_{(PheT),t=T} + Xu_{(PheT),t=T} = CL_2 \times AUC_{0\ (Phe,\,abs)}^{T} + CL_5 \times AUC_{0\ (Phe)}^{T} \quad \text{(A15)}$$

When time T approaches infinity, equation A15 can be written as

$$A_{(PheDE),t=\infty} + A_{(PheT),t=\infty} + Xu_{(PheT),t=\infty} = CL_2 \times AUC_{(Phe,\,abs)} + CL_5 \times AUC_{(Phe)} \quad \text{(A16)}$$

By definition, the left side of equation A16 is equal to A_{act}:

$$A_{act} = A_{(PheDE),t=\infty} + A_{(PheT),t=\infty} + Xu_{(PheT),t=\infty} \quad \text{(A17)}$$

Combining equations A16 and A17 to obtain equation A18

$$A_{act} = CL_2 \times AUC_{(Phe,\,abs)} + CL_5 \times AUC_{(Phe)} \quad \text{(A18)}$$

Substituting equations A7 and A8 into A18 to obtain equation A19

$$A_{act} = CL_2 \times \frac{D}{CL_1 + CL_2 + CL_3} + CL_5 \times \frac{CL_1}{CL_4 + CL_5} \times \frac{D}{CL_1 + CL_2 + CL_3} \quad \text{(A19)}$$

If f_a , f_{act} and f_m are defined as the following

$$f_a = \frac{CL_1}{CL_1 + CL_2 + CL_3} \quad \text{(A20)}$$

$$f_{act} = \frac{CL_2}{CL_1 + CL_2 + CL_3} \quad \text{(A21)}$$

$$f_m = \frac{CL_5}{CL_4 + CL_5} \quad \text{(A22)}$$

then

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$$A_{act} = [(f_a \times f_m) + f_{act}] \times D \qquad \text{(A23)}$$

where f_a, f_{act} and f_m are the fraction of the dose absorbed, the fraction of the dose converted to [D_{10}]PheDE during first-pass activation and the fraction of [D_{10}]Phe converted to [D_{10}]PheDE in the systemic circulation, respectively

Since $A_{(PheDE),t=\infty}$ and $A_{(PheT),t=\infty}$ both equal 0, equation A17 could be reduced to

$$A_{act} = Xu_{(PheT),t=\infty} \qquad \text{(A24)}$$

In addition, it is assumed that all the [D_{10}]PheT formed was excreted in urine in 48 hr.

Therefore,

$$A_{act} = Xu_{(PheT),t=48} \qquad \text{(A25, equation 3 in text)}$$

The combination of equations A25 and A11 leads to equation A26

$$A_{act} = AUC_{(PheT)} \times CL_7 \qquad \text{(A26, equation 2 in text)}$$

Abbreviations (all mass and concentration units are on a molar basis)

A_{act} : total amount of [D_{10}]PheDE formed during metabolic activation

$A_{(Phe,abs)}$: amount of [D_{10}]Phe at the site of absorption

$A_{(Phe,abs),t=0}$: amount of [D_{10}]Phe at the site of absorption at time 0

$A_{(Phe,abs),t=T}$: amount of [D_{10}]Phe at the site of absorption at time T

$A_{(PheDE)}$: amount of [D_{10}]Phe in the body

$A_{(PheDE),t=0}$: amount of [D_{10}]PheDE in the body at time 0

$A_{(PheDE),t=T}$: amount of [D_{10}]PheDE in the body at time T

$A_{(PheDE),t=\infty}$: amount of [D_{10}]PheDE in the body at time infinity

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$A_{(PheT)}$: amount of [D_{10}]PheT in the body

$A_{(PheT),t=0}$: amount of [D_{10}]PheT in the body at time 0

$A_{(PheT),t=T}$: amount of [D_{10}]PheT in the body at time T

$A_{(PheT),t=\infty}$: amount of [D_{10}]PheT in the body at time infinity

$AUC_{(Phe,\,abs)}$: area under $C_{(Phe,abs)}$-time curve from time 0 to infinity

$AUC_0^T{}_{(Phe,\,abs)}$: area under $C_{(Phe,abs)}$-time curve from time 0 to T

$AUC_{(Phe)}$: area under $C_{(Phe)}$-time curve from time 0 to infinity

$AUC_0^T{}_{(Phe)}$: area under $C_{(Phe)}$-time curve from time 0 to T

$AUC_{(PheDE)}$: area under $C_{(PheDE)}$-time curve from time 0 to infinity

$AUC_0^T{}_{(PheDE)}$: area under $C_{(PheDE)}$-time curve from time 0 to T

$AUC_{(PheT)}$: area under $C_{(PheT)}$-time curve from time 0 to infinity

$AUC_0^T{}_{(PheT)}$: area under $C_{(PheT)}$-time curve from time 0 to T

$C_{(Phe,\,abs)}$: concentration of [D_{10}]Phe at the site of absorption

$C_{(Phe)}$: plasma concentration of [D_{10}]Phe

$C_{(PheDE)}$: plasma concentration of [D_{10}]PheDE

$C_{(PheT)}$: plasma concentration of [D_{10}]PheT

f_a: the fraction of dose absorbed

f_{act}: the fraction of dose converted to [D_{10}]PheDE during first-pass activation

f_m: the fraction of [D_{10}]Phe converted to [D_{10}]PheDE in the systemic circulation

$Xu_{(PheT)}$: cumulative amount of [D_{10}]PheT collected in the urine

$Xu_{(PheT),t=T}$: amount of [D_{10}]PheT collected in the urine at time T

$Xu_{(PheT),t=48}$: amount of [D_{10}]PheT collected in the urine at 48 hr

$Xu_{(PheT),t=\infty}$: amount of [D_{10}]PheT collected in the urine at time infinity