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Effect of creatinine and specific gravity normalization techniques on xenobiotic biomarkers in smokers' spot and 24-h urines

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Abstract

Renal excretion mechanisms are xenobiotic-specific; therefore, accurate exposure assessment requires an understanding of relationships of xenobiotic biomarker concentration and excretion rate to urine flow, specific gravity and creatinine concentration. Twenty-four-hour urine collection for xenobiotic exposure assessment is considered the "gold standard" procedure. Random spot-urine collection is convenient and minimizes subject compliance concerns but requires that normalization techniques be employed to account for diuresis and diurnal variation in xenobiotic biomarker excretion. This paper examines and makes recommendations concerning normalization techniques and conditions under which spot-urine results most accurately reflect 24-h urine results. Specific gravity, creatinine, and xenobiotic biomarkers were determined in smokers' spot and 24-h urines. Normalization techniques were applied, variance-component analyses were performed to estimate variability, spot urines were pooled mathematically to simulate 24-h urines and analyses of variance were performed to evaluate spot urines' ability to reflect 24-h urine concentrations. For each xenobiotic biomarker concentration, log-linear relationships were observed with urine flow, specific gravity, and creatinine. For most xenobiotic biomarker excretion rates, log-linear relationships were observed with urine flow; creatinine, however, was unaffected by urine flow. The conventional creatinine ratio-normalization technique demonstrated greater variability (within-day, between-day and between-subject) than other normalization techniques. Comparisons of simulated 24-h urines to spot urines suggest that spot-urine collection be performed only between 2 p.m. and 2 a.m. and that the modified specific-gravity-adjusted-creatinine ratio-normalization technique and the creatinine-regression normalization technique yield the best agreement between spot- and simulated 24-h urine results.

Keywords: Nicotine; Cotinine; Creatinine; Xenobiotic biomarkers; Specific gravity; Urinary flow rate; 24-h urine; Spot urine; LC-MS/MS; Normalization

1. Introduction

Although 24-h urine collection as a means of assessing exposure to xenobiotics is considered the "gold standard" sample collection technique, it has numerous disadvantages [1–6]. Urine sample integrity and completeness are essential to exposure-assessment research and absence of compliance with the collection protocol is a fundamental concern to the researcher, yet subject honesty and their questionnaire responses are the only tools available to ensure and assess the degree of compliance. In order to increase the likelihood that sample integrity and completeness have not been compromised and to reduce research costs, a more reliable, convenient, less expensive, and more manageable sample collection technique is required.

Random spot-urine sample collection, i.e., single urine-void collection, has the potential for ameliorating problems associated with 24-h urine collection as it is a more convenient, manageable and less expensive alternative. It also reduces sample-integrity and subject-compliance concerns [3,4,6,7]. The excretion rate of a xenobiotic biomarker may increase or decrease with changes in urinary flow (due to varying states of hydration), and relying solely on the xenobiotic biomarker concentration ([XenoBio]) may frustrate xenobiotic-uptake assessment [8]. The total renal elimination mechanism in the nephron (glomerular filtration, active secretion, passive diffusion and tubular reabsorption [8–11], antidiuretic hormone secretion [10,11], urine pH [12,13] and the pK_a of the biomarker in question [9]) complicates interpretation of simple concentra-

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tion data. Because [XenoBio] in spot-urine samples may be subject to dilution effects and variable excretion rates, several techniques for normalization of [XenoBio] using creatinine (CRE) concentration ([CRE]) and specific gravity (SG) have been employed. These include [CRE]-ratio-normalization of [XenoBio]-to-[CRE], regression-normalization of [XenoBio]-to-[CRE] and SG-ratio-normalization of [XenoBio]-to-SG.

Although [CRE]-ratio-normalization of [XenoBio]-to-[CRE] has been utilized for xenobiotic biomarkers in general [4,6,14–18] and for nicotine (NIC) xenobiotic biomarkers in particular [19–27], it is subject to a number of limitations [1,2,8,26,28]. For an individual, [CRE]-ratio-normalization has the potential to be a valid and effective technique to reduce variability provided that the renal elimination mechanism of the xenobiotic biomarker is similar to the renal elimination mechanism of CRE. CRE is removed from the plasma primarily by glomerular filtration and, for an individual, is considered generally to be excreted at a relatively constant rate throughout the day and from day to day [8]. Unfortunately, many xenobiotic biomarkers are removed by the other aforementioned mechanisms. Consequently, [CRE]-ratio-normalization may be ill-advised and complicate the interpretation of results [8]. CRE also exhibits inter-individual variability due to factors including gender, age, muscularity, physical activity, diet, disease-state, pregnancy, and creatine intake [1,8,17,26,29,30]. The conventional [CRE]-ratio-normalization technique is defined as the ratio, [XenoBio]/[CRE], in a given urine sample (random-spot or 24-h). Provided the renal elimination mechanisms are similar, the [CRE]-ratio-normalization technique adjusts for the dilution effect, the variable xenobiotic biomarker excretion rate and, to a lesser extent, the gender effect, i.e., body mass.

Another normalization technique that has been used to reduce the variance of determinations due to the dilution effect is SG-normalization [5,6,15,16,31–33]. In 1945, Levine and Fahy [31] demonstrated that the mass of total dissolved solids, e.g., anions, cations, ammonia, creatinine, urea, amino acids and, presumably, xenobiotic biomarkers, is inversely log (logarithm, base 10)-linearly proportional to SG minus unity (SG-1) in spot-urine samples. The "conventional" SG-normalization technique for [XenoBio] adjustment is based on the assumption that the mass ratio of the xenobiotic biomarker and total-dissolved-solids remains constant as urinary flow fluctuates throughout the day. The SG-normalization of a xenobiotic biomarker is defined as

$$[\text{XenoBio}]_s = [\text{XenoBio}]\left(\frac{1.020-1}{\text{SG}-1}\right), \quad (1)$$

where $[\text{XenoBio}]_s$ is the SG-normalized [XenoBio], and the value 1.020 [7] is defined as the mean SG of normal human urine. Unfortunately, the value for the mean SG of normal human urine is not standardized by convention and may range, arbitrarily by definition, from 1.016 to 1.024 [6,15,31].

Araki [3,34,35] demonstrated that the individual [XenoBio] is inversely log-linearly proportional to urinary flow rate, e.g., milliliters per minute (mL min^{-1}), in 24-h urine samples:

$$\log[\text{XenoBio}] = (a-b)\ \log\ \text{UrineFlow}, \quad (2)$$

where UrineFlow is the mean urinary flow rate of a timed urine sample. It may then be assumed that the same relationship is demonstrated in spot-urine samples. Thus, the xenobiotic biomarker-specific Araki b slope is defined as

$$b = \frac{\Delta \log[\text{XenoBio}]}{\Delta \log\ \text{UrineFlow}}. \quad (3)$$

The Araki method exponentially scales the [XenoBio] to its dependence on UrineFlow and normalizes urine concentrations to a standard urinary flow rate of 1 mL min^{-1}. Unfortunately, this method requires two timed, spot-urine samples to calculate UrineFlow for a given urine sample.

In 1998, Vij and Howell [7] demonstrated that SG-1 is inversely log-linearly proportional to UrineFlow and introduced the a slope, defined as

$$a = \frac{\Delta\ \log(\text{SG}-1)}{\Delta\ \log\ \text{UrineFlow}}. \quad (4)$$

By combining Eqs. (3) and (4), they introduced a new statistic, the xenobiotic biomarker-dependent Z exponent, defined as the ratio of Araki's b slope and Vij and Howell's a slope

$$Z = \frac{b}{a} = \frac{(\Delta \log[\text{XenoBio or CRE}]/\Delta\ \log\ \text{UrineFlow})}{(\Delta\ \log(\text{SG}-1)/\Delta\ \log\ \text{UrineFlow})} = \frac{\Delta\ \log[\text{XenoBio or CRE}]}{\Delta\ \log(\text{SG}-1)}, \quad (5)$$

where, simply, Z is defined as the slope of the log [XenoBio] versus log SG-1 plot. By combining the two equations, Vij and Howell eliminated the need to determine UrineFlow and, thus, the requirement of two timed, spot-urine samples. Because the exponential parameter, or Z exponent, is xenobiotic biomarker-dependent, initial empirical determinations of the relationship between SG and [XenoBio] or [CRE] are required. Once empirically-determined Z exponents are obtained, the [XenoBio] or [CRE] may be SG-normalized and Z-normalized by the "modified" SG normalization technique

$$[\text{XenoBio or CRE}]_{sz} = [\text{XenoBio or CRE}]\left(\frac{1.020-1}{\text{SG}-1}\right)^{Z}, \quad (6)$$

where $[\text{XenoBio or CRE}]_{sz}$ are the respective SG- and Z-normalized spot-urine samples and Z is the xenobiotic biomarker- or CRE-specific exponent. Consequently, the SG-normalized and Z-normalized [XenoBio] and [CRE] may be combined by the following equation to yield an SG-normalized, Z-normalized and [CRE]-ratio-normalized result

$$\frac{[\text{XenoBio}]_{sz}}{[\text{CRE}]_{sz}} = \frac{[\text{XenoBio}]((1.020-1)/(\text{SG}-1))^{Z_1}}{[\text{CRE}]((1.020-1)/(\text{SG}-1))^{Z_2}} = \frac{[\text{XenoBio}]}{[\text{CRE}]}\left(\frac{1.020-1}{\text{SG}-1}\right)^{Z_1-Z_2}, \quad (7)$$

where Z_1 is the xenobiotic biomarker-specific exponent, and Z_2 is the CRE-specific exponent.

Another technique that has been used to normalize concentrations of cotinine (COT), a major xenobiotic biomarker of NIC, and that could be used, conceivably, for other xenobiotic biomarkers, is the adjustment of [XenoBio]-to-[CRE] using

a regression-normalization technique based on the regression relationship between COT concentration, [COT], and [CRE] [36,37]. In urine, Thompson et al. [36] demonstrated a log-linear relationship between [COT] in micromoles per liter (μmol L^{-1}) and [CRE] in millimoles per liter (mmol L^{-1}) and adjusted the [COT] to the mean [CRE] for the study population of 279 men using the equation

$$\log\,[\mathrm{COT}]_c = \log[\mathrm{COT}] - 0.407(\log[\mathrm{CRE}] - 0.944), \qquad (8)$$

where $[\mathrm{COT}]_c$ is the [CRE]-regression-normalized [COT], [COT] is the observed COT concentration for each subject, [CRE] is the observed CRE concentration for each subject, 0.407 is the slope of the [COT]–[CRE] linear least squares regression and 0.944 is the log of the mean urinary [CRE] (8.8 mmol L^{-1}) for the study population. Thus, each individual [COT] is normalized to the mean [CRE] for the study population. Conceivably, this [CRE]-regression-normalization technique may be applied to any xenobiotic biomarker to improve the robustness of the relationship between xenobiotic uptake and [XenoBio].

Important xenobiotic biomarkers for tobacco smoke uptake in urine include unconjugated NIC (NIC-U), its metabolites and several other tobacco-specific and tobacco-related xenobiotic biomarkers [38]. Potential NIC xenobiotic biomarkers include the parent compound, NIC-U, unconjugated cotinine (COT-U), unconjugated trans-3′-hydroxycotinine (OHCOT-U), and liberated aglycons of nicotine-*N*-glucuronide (NIC-G), cotinine-*N*-glucuronide (COT-G) and trans-3′-hydroxycotinine-*O*-glucuronide (OHCOT-G). As a means of estimating total NIC uptake, NIC-U and the five major metabolites may be converted to molar NIC-U equivalents and summed to yield total NIC equivalents ($\mathrm{NIC_{Eq}}$-T) by the equation

$$\mathrm{NIC_{Eq}\text{-}T} = \left(\mathrm{COT\text{-}T}\frac{162.2\ \mathrm{g\,mol^{-1}}}{176.2\ \mathrm{g\,mol^{-1}}}\right) + \mathrm{NIC\text{-}T} + \left(\mathrm{OHCOT\text{-}T}\frac{162.2\ \mathrm{g\,mol^{-1}}}{192.2\ \mathrm{g\,mol^{-1}}}\right), \qquad (9)$$

where T is the analytically determined total (unconjugated and liberated aglycons) of NIC, COT and OHCOT, respectively, and 162.2 g mol^{-1}, 176.2 g mol^{-1} and 192.2 g mol^{-1} are the molecular weights for NIC-U, COT-U and OHCOT-U, respectively [39].

Studies intended for the purpose of assessing exposure to tobacco-specific and tobacco-related xenobiotics consist of three general types: (1) estimation of absolute uptake in smokers and its relationship to cigarette yield; (2) comparison of relative uptake in smokers using different products; and (3) surveillance programs. In order to produce valid and reproducible information from these studies, there must be a basic understanding of the available collection procedures, normalization techniques and nature of the resulting data. For example, the loss of time-weighted information precludes the use of single random spot-urine samples for the estimation of cigarette yields and absolute uptake in smokers; however, spot-urine samples may provide acceptable data in surveillance programs and for the comparison of relative uptake between products.

Table 1
Determined xenobiotic biomarkers and abbreviations

Biomarker	Abbreviation
Tobacco-specific	
Cotinine	COT-U
Cotinine-*N*-glucuronide	COT-G
Nicotine	NIC-U
Nicotine-*N*-glucuronide	NIC-G
Trans-3′-hydroxycotinine	OHCOT-U
Trans-3′-hydroxycotinine-*O*-glucuronide	OHCOT-G
Total molar nicotine equivalents	$\mathrm{NIC_{Eq}}$-T
4-(*N*-Methylnitrosamino)-1-(3-pyridyl)-1-butanol	NNAL-U
4-(*N*-Methylnitrosamino)-1-(3-pyridyl)but-1-yl]-β-*O*-D-glucosiduronic acid 4-(*N*-methylnitrosamino)-1-(3-pyridyl)but-1-yl]-β-*N*-D-glucosiduronic acid	NNAL-G
Tobacco-related	
S-Phenylmercapturic acid	SPMA
3-Hydroxypropylmercapturic acid	HPMA
Polycyclic aromatic hydrocarbons (surrogate)	1-OHP
Monohydroxybutenylmercapturic acids	MHBMA
1,2-Dihydroxybutylmercapturic acid	DHBMA

The major purpose of this study was to evaluate existing normalization techniques for selected tobacco-specific and tobacco-related xenobiotic biomarkers (Table 1) and to propose a sampling procedure whereby a single urine-void collection may be used in lieu of 24-h urine collection. Specific goals were to (1) verify the log-linear relationship between [CRE], [XenoBio] and UrineFlow as reported by Araki; (2) verify the log-linear relationship between SG-1 and UrineFlow as reported by Vij and Howell; (3) verify the log-linear relationship between [CRE], [XenoBio] and SG-1 and generate xenobiotic biomarker-specific *Z* exponents as reported by Vij and Howell; (4) verify the log-linear relationship between [XenoBio] and [CRE], and generate xenobiotic biomarker-specific slopes for [CRE]-regression-normalization as reported by Thompson et al.; (5) apply the [CRE]-ratio-normalization, SG-normalization, combined SG- and *Z*-normalization, combined SG-, *Z*- and [CRE]-ratio-normalization, and [CRE]-regression-normalization techniques to [XenoBio]; (6) compare spot-urine-observed and -normalized [XenoBio] to 24-h-urine-observed and -normalized [XenoBio]; and (7) compare the intra- and inter-individual variances of observed- and normalized-spot-urine and 24-h-urine [XenoBio].

2. Materials and methods

2.1. Study protocol and design

The protocol for this study was approved by an in-house human research review committee, and informed written consent was obtained from the subjects prior to participation. The study followed guidelines developed in alignment with U.S. federal regulations that address Institutional Review Boards and research conducted in humans (21 CFR Parts 50 and 56, and

45 CFR Part 46), as well as the principles of the Declaration of Helsinki. The study was designed to include two male and two female smokers in each of three tar bands (full-flavor, full-flavor low "tar" and ultra-low "tar"). Subjects were instructed to smoke their usual brand of cigarettes ad libitum throughout the study.

The study consisted of two phases and six visits in a crossover design: Phase I and, 5 weeks later, Phase II. During each phase, subjects collected a 24-h urine sample on 1 day and all individual spot urine samples on another day. Half of the subjects began Phase I with 24-h urine collection and Phase II with spot-urine collection, and vice versa (crossover). All subjects completed both phases of the study and were compensated for their participation.

2.2. Sample collection, preparation and analysis

For 24-h urine collections, subjects recorded the time of the first morning void (not collected) as "Start Time" on the collection container. Subjects collected each urine void up to and including the first morning void the next day. The time of the last void was recorded as "End Time". Subjects recorded the time of the first morning void (not collected) as "Start Time" on the first container. Subsequently, the subjects collected each spot-urine void in a separate container and recorded the "Collection Time" on each container. In the laboratory, samples were aliquoted into appropriately sized cryogenic tubes and stored at −80 °C until study completion.

The analyses for NIC-U, NIC-G, COT-U, COT-G, OHCOT-U and OHCOT-G [39], NNAL-U and NNAL-G [40], SPMA [41], HPMA [42,43] and 1-OHP [44] were performed by analyses reported elsewhere. The analyses for MHBMA and DHBMA consisted of Oasis HLB (Waters GmbH) SPE followed by gradient HPLC (2% formic acid, pH 2.0/methanol) on an Atlantis dC18 analytical column (Waters GmbH) and electrospray ionization MS/MS detection (Model API-2000, Applied Biosystems, Darmstadt, Germany). The analysis for CRE was performed with a kinetic picric acid assay based on a modification of the Jaffe method [45]. Specific gravity measurements were performed on an Atago URICON-N_E urine-specific-gravity refractometer (Fisher Scientific, Fair Lawn, NJ). Statistical analyses were performed with either Origin©software (Version 7 SR4 OriginLab Corporation, Northampton, MA) or SAS® software (Version 8.2, SAS Institute, Cary, NC).

3. Results and discussion

3.1. Summary data

A total of 215 spot-urine samples and 24 24-h urine samples were collected in the study. The individual subjects' total number of spot-urine samples (n) ranged from 4 to 14 per day with an overall mean of 9.0 ± 2.8. The overall mean 24-h urine volume (mL) was 2061 ± 1132 (males, 2426 ± 1419; females, 1696 ± 619); the overall mean total spot-urine volume (mL) was 2006 ± 1078 (males, 2379 ± 1180; females, 1559 ± 775). During the 24-h and spot-urine collection periods the volumes collected were quite variable within-subjects/between-phases and within-subjects/between-collection-type. Both 24-h and mean spot-urine SG demonstrate excellent agreement within-subjects/between-phases and within-subjects/between-collection-type. The overall mean 24-h urine total CRE output in grams per day (g day^{-1}) was 1.35 ± 0.58 (males, 1.78 ± 0.39; females, 0.93 ± 0.38). The overall mean spot-urine total CRE output in g day^{-1} was 1.60 ± 0.68 (males, 2.13 ± 0.35; females, 1.06 ± 0.46).

3.2. Biomarker and creatinine concentration/urine flow relationship

To verify the log-linear relationship between [CRE], observed [XenoBio] and UrineFlow as reported by Araki and shown in Eqs. (2) and (3), log [CRE] and log observed [XenoBio] for each of the spot-urine samples were plotted against the respective log UrineFlow. Since spot-urine sample volumes (in mL) were determined and start- and end-collection times for each spot-urine sample were recorded by the subjects, the UrineFlow for each spot-urine sample could be calculated in units of mL min^{-1}. A linear regression was performed, and the regression parameters for log observed [XenoBio] versus log UrineFlow are reported in Table 2, i.e., n, r, slope, standard error of the slope (S.E.$_{slope}$), 95% confidence interval of the slope (95% CI$_{slope}$), intercept, standard error of the intercept (S.E.$_{intercept}$), 95% confidence interval of the intercept (95% CI$_{intercept}$) and standard error of the estimate ($S_{y|x}$). NIC-G was not detected in six spot-urine samples; therefore, only 209 samples were included in the regression for that xenobiotic biomarker. For the five other xenobiotic biomarkers with $n < 215$ samples, there were insufficient spot-urine void volumes to conduct the analyses. Each log observed [XenoBio] is statistically significantly correlated with log UrineFlow, probability (p) < 0.0001. With the exception of the NIC uptake parent compound, NIC-U ($r = -0.27$), correlations range from −0.47 to −0.76 and affirm the log-linear relationship demonstrated by Araki.

3.3. Specific gravity/urine flow relationship

To verify the log-linear relationship between SG-1 and UrineFlow as reported by Vij and Howell and shown in Eq. (4), log SG-1 for each of the 215 spot-urine samples and 24 24-h urine samples was plotted separately against the respective log UrineFlow. A linear regression was performed and the regression parameters for log SG-1 versus log UrineFlow for the spot-urine samples are as follows: $r = -0.70$, $p < 0.0001$, slope = −0.61, S.E.$_{slope}$ = 0.04, 95% CI$_{slope}$ = −0.70 to −0.53, intercept = −1.86, S.E.$_{intercept}$ = 0.02, 95% CI$_{intercept}$ = −1.89 to −1.83 and $S_{y|x}$ = 0.24. The regression parameters for log SG-1 versus log UrineFlow for the 24-h urine samples are as follows: $r = -0.35$, $p = 0.09$, slope = −0.37, S.E.$_{slope}$ = 0.21, 95% CI$_{slope}$ = −0.80 to +0.07, intercept = −1.84, S.E.$_{intercept}$ = 0.05, 95% CI$_{intercept}$ = −1.95 to −1.74 and $S_{y|x}$ = 0.22. The spot-urine sample correlation affirms the log-linear relationship demonstrated by Vij and Howell.

Table 2
Spot-urine log [XenoBio] vs. log UrineFlow regression parameters for combined subjects and phases ($p < 0.0001$ for all xenobiotic biomarkers)

Biomarker	n	r	Slope	S.E.$_{slope}$	95%	CI$_{slope}$	Intercept	S.E.$_{intercept}$	95%	CI$_{intercept}$	$S_{y\|x}$
COT-U	215	−0.47	−0.31	0.04	−0.38	−0.23	0.17	0.02	0.14	0.20	0.22
COT-G	215	−0.62	−0.73	0.06	−0.85	−0.60	0.24	0.03	0.19	0.29	0.36
NIC-U	215	−0.27	−0.29	0.07	−0.43	−0.15	0.12	0.03	0.07	0.18	0.40
NIC-G	209	−0.47	−0.48	0.06	−0.60	−0.36	−0.40	0.03	−0.45	−0.35	0.35
OHCOT-U	215	−0.76	−0.94	0.06	−1.05	−0.83	0.73	0.02	0.68	0.77	0.32
OHCOT-G	215	−0.73	−0.90	0.06	−1.01	−0.78	0.24	0.02	0.19	0.28	0.33
CRE	215	−0.74	−0.86	0.05	−0.96	−0.75	1.89	0.02	1.84	1.93	0.30
NNAL-U	200	−0.65	−0.61	0.05	−0.71	−0.51	−0.83	0.02	−0.87	−0.79	0.27
NNAL-G	200	−0.75	−0.76	0.05	−0.85	−0.67	−0.44	0.02	−0.48	−0.40	0.25
SPMA	215	−0.72	−0.98	0.07	−1.11	−0.86	0.17	0.03	0.12	0.23	0.37
HPMA	215	−0.57	−0.47	0.05	−0.56	−0.38	0.16	0.02	0.12	0.20	0.27
1-OHP-T	206	−0.58	−0.77	0.08	−0.92	−0.62	−0.62	0.03	−0.68	−0.56	0.41
MHBMA	211	−0.59	−0.56	0.05	−0.67	−0.46	−1.29	0.02	−1.33	−1.24	0.30
DHBMA	211	−0.76	−0.50	0.03	−0.56	−0.44	−0.14	0.01	−0.16	−0.11	0.17

3.4. Biomarker and creatinine concentration/specific gravity relationship

To verify the log-linear relationship between [CRE], observed [XenoBio], and SG-1 as reported by Vij and Howell and shown in Eq. (5), and to generate xenobiotic biomarker-specific Z exponents, log [CRE] and log observed [XenoBio] for each of the spot-urine samples were plotted against the respective log SG-1. For the NIC and NNK xenobiotic biomarkers, the -U and -G concentrations were summed to yield -T concentrations, and a linear regression was performed on both individual and combined data sets. The regression parameters for log observed [XenoBio] versus log SG-1 are reported in Table 3 for combined subjects and phases. Each log observed [XenoBio] is statistically significantly correlated with log SG-1 ($p < 0.0001$). With the exception of the parent compound of NIC uptake, NIC-U ($r = 0.28$), correlations ranged from 0.41 to 0.94 and affirm the log-linear relationship demonstrated by Vij and Howell. In general, with the exception of NIC-U, the tobacco-specific [XenoBio] demonstrate higher correlations than the tobacco-related [XenoBio].

3.5. Biomarker concentration/creatinine concentration relationship

To verify the log-linear relationship between observed [XenoBio] and [CRE] as reported by Thompson et al. and to generate xenobiotic biomarker-specific [CRE]-regression-normalization slopes, log observed [XenoBio] for each of the spot-urine samples was plotted against the respective log [CRE]. A linear regression was performed, and the regression parameters for log observed [XenoBio] versus log [CRE] are reported in Table 4 for combined subjects and phases. Each log observed [XenoBio] is statistically significantly correlated with log [CRE] ($p < 0.0001$). The slope for COT-U (0.42) in this study is in excellent agreement with the slope for COT-U (0.407) observed

Table 3
Spot-urine log [XenoBio] vs. log SG-1 regression parameters for combined subjects and phases ($p < 0.0001$ for all xenobiotic biomarkers)

Biomarker	n	r	Z	S.E.$_{slope}$	95%	CI$_{slope}$	Intercept	S.E.$_{intercept}$	95%	CI$_{intercept}$	$S_{y\|x}$
Nic$_{Eq}$-T	215	0.87	0.91	0.03	0.84	0.98	2.77	0.07	2.63	2.90	0.17
COT-U	215	0.75	0.56	0.03	0.49	0.63	1.22	0.07	1.09	1.35	0.17
COT-G	215	0.86	1.17	0.05	1.08	1.26	2.42	0.09	2.24	2.60	0.23
COT-T	215	0.88	0.84	0.03	0.78	0.90	2.10	0.06	1.98	2.22	0.15
NIC-U	215	0.28	0.34	0.08	0.18	0.50	0.74	0.16	0.43	1.05	0.40
NIC-G	209	0.58	0.69	0.07	0.56	0.82	0.87	0.13	0.61	1.13	0.32
NIC-T	215	0.41	0.42	0.06	0.29	0.55	1.04	0.13	0.79	1.29	0.32
OHCOT-U	215	0.84	1.20	0.05	1.10	1.30	2.93	0.10	2.73	3.14	0.26
OHCOT-G	215	0.82	1.16	0.06	1.05	1.27	2.36	0.11	2.14	2.58	0.28
OHCOT-T	215	0.84	1.19	0.05	1.08	1.29	3.03	0.10	2.83	3.24	0.26
CRE	215	0.94	1.24	0.03	1.18	1.31	4.19	0.06	4.06	4.31	0.16
NNAL-U	200	0.85	0.88	0.04	0.80	0.96	0.79	0.08	0.64	0.95	0.19
NNAL-G	200	0.88	0.99	0.04	0.91	1.06	1.37	0.07	1.22	1.52	0.18
NNAL-T	200	0.89	0.96	0.04	0.89	1.03	1.47	0.07	1.33	1.61	0.17
SPMA	215	0.74	1.16	0.07	1.01	1.30	2.29	0.14	2.00	2.57	0.36
HPMA	215	0.55	0.52	0.05	0.41	0.63	1.11	0.11	0.89	1.32	0.27
1-OHP-T	206	0.54	0.80	0.09	0.63	0.98	0.83	0.17	0.48	1.17	0.43
MHBMA	211	0.78	0.85	0.05	0.76	0.94	0.28	0.09	0.10	0.47	0.23
DHBMA	211	0.82	0.63	0.03	0.57	0.69	1.01	0.06	0.89	1.12	0.15

Table 4
Spot-urine log [XenoBio] vs. log [CRE]-regression parameters for combined subjects and phases ($p < 0.0001$ for all xenobiotic biomarkers)

Biomarker	n	r	Slope	S.E.$_{slope}$	95%	CI$_{slope}$	Intercept	S.E.$_{intercept}$	95%	CI$_{intercept}$	$S_{y\|x}$
Nic$_{Eq}$-T	215	0.90	0.71	0.02	0.67	0.76	−0.27	0.04	−0.35	−0.19	0.15
COT-U	215	0.75	0.42	0.03	0.37	0.47	−0.62	0.05	−0.71	−0.53	0.17
COT-G	215	0.87	0.89	0.03	0.82	0.96	−1.43	0.06	−1.55	−1.30	0.23
COT-T	215	0.88	0.63	0.02	0.59	0.68	−0.65	0.04	−0.74	−0.57	0.15
NIC-U	215	0.32	0.29	0.06	0.18	0.41	−0.44	0.11	−0.65	−0.22	0.39
NIC-G	209	0.60	0.54	0.05	0.44	0.64	−1.42	0.09	−1.60	−1.24	0.32
NIC-T	215	0.45	0.35	0.05	0.26	0.44	−0.40	0.09	−0.57	−0.22	0.31
OHCOT-U	215	0.85	0.92	0.04	0.84	0.99	−1.02	0.07	−1.16	−0.88	0.25
OHCOT-G	215	0.86	0.92	0.04	0.85	1.00	−1.52	0.07	−1.65	−1.39	0.24
OHCOT-T	215	0.86	0.92	0.04	0.84	0.99	−0.90	0.07	−1.03	−0.76	0.24
NNAL-U	200	0.84	0.66	0.03	0.60	0.72	−2.09	0.05	−2.19	−1.98	0.19
NNAL-G	200	0.90	0.76	0.03	0.71	0.81	−1.89	0.05	−1.98	−1.80	0.16
NNAL-T	200	0.90	0.74	0.03	0.68	0.79	−1.69	0.05	−1.78	−1.60	0.16
SPMA	215	0.73	0.87	0.06	0.76	0.98	−1.50	0.10	−1.70	−1.30	0.36
HPMA	215	0.59	0.42	0.04	0.35	0.50	−0.66	0.07	−0.80	−0.51	0.26
1-OHP-T	206	0.59	0.66	0.06	0.53	0.78	−1.90	0.11	−2.13	−1.67	0.41
MHBMA	211	0.81	0.66	0.03	0.60	0.73	−2.53	0.06	−2.65	−2.41	0.22
DHBMA	211	0.87	0.50	0.02	0.46	0.54	−1.09	0.04	−1.16	−1.02	0.13

Population mean [CRE] = 87 mg dL^{-1} and population log mean [CRE] = 1.94.

by Thompson et al. (Eq. (8)). With the exception of NIC-U ($r = 0.32$), correlations ranged from 0.45 to 0.90 and affirm the log-linear relationship demonstrated by Thompson et al. The mean [CRE] for combined subjects and phases was 87 mg dL^{-1} with a log mean [CRE] of 1.94.

Given that the Vij and Howell SG-, *Z*- and [CRE]-ratio-normalization technique and the Thompson [CRE]-regression-normalization technique are implemented by fitting models to log [XenoBio], the regression models presented in Tables 3 and 4 provide a comparison of these two normalization techniques. In particular, a comparison of the correlation coefficients for each [XenoBio] indicates whether log SG-1 or log [CRE] is more highly correlated with log [XenoBio]. While no result differs by more than 0.05, the correlation of log [XenoBio] with log [CRE] is generally greater than the correlation of log [XenoBio] with log SG-1. Table 3 shows that the correlation of log SG-1 with log [CRE] is 0.94. Thus, the high correlation would lead one to expect similar performance from these two normalization techniques when they are applied to the observed concentrations. In Fig. 1, the similar performance for these two normalization techniques is demonstrated when they are applied to the observed Nic$_{Eq}$-T concentrations for all of the spot- and 24-h urine samples. In addition, the normalization techniques demonstrate an improvement in relative concentrations between and within subjects for both the spot- and 24-h urine samples.

3.6. Excretion rate/urine flow relationship

Because [CRE] and observed [XenoBio] (in mass mL^{-1}) were determined and start- and end-collection times for each spot-urine sample were recorded by the subjects, the excretion rate for each analyte could be determined as mass min^{-1}. To verify the relationship between excretion rate and UrineFlow, each of the spot-urine sample excretion rates was plotted against the respective UrineFlow. For each xenobiotic biomarker, a linear regression was performed (Fig. 2). The regression parameters for individual excretion rates versus UrineFlow are reported in Table 5 for combined subjects and phases. Boeniger et al. [8] demonstrated that the general relationship between a component's excretion rate and UrineFlow is indicative of the renal excretion mechanism. For example, a component eliminated by glomerular filtration should exhibit an excretion rate totally independent of urine flow, i.e., slope = zero, a component eliminated by active secretion should exhibit an excretion rate generally independent of urine flow, i.e., slope ≈ zero, and a component eliminated primarily by passive diffusion should exhibit an excretion rate dependent upon urine flow, i.e., slope ≠ zero. Further complicating the elimination mechanism interpretation are competitive interactions between two or more components, when they compete for the active secretion transport system [9]. Regardless of the elimination mechanisms involved, it is obvious from Fig. 2 and Table 5 that, depending upon the xenobiotic biomarker, [CRE]-ratio-normalization may lead to erroneous results due to varying urinary flow. Nine xenobiotic biomarkers (NIC-U, NIC-G, COT-U, NNAL-U, HPMA, 1-OHP-T, MHBMA and DHBMA at $p < 0.0001$ and NNAL-G at $p = 0.0007$) demonstrate a statistically significant effect of UrineFlow on excretion rate, indicating that they are affected by diuresis to some extent. CRE and four xenobiotic biomarkers (OHCOT-U, OHCOT-G and SPMA at $p > 0.10$ and COT-G at $p = 0.0922$) do not demonstrate a statistically significant effect ($p < 0.05$) of UrineFlow on excretion rate, indicating that they are minimally affected by diuresis. While [CRE]-ratio-normalization may yield reasonable results for some xenobiotic biomarkers, it also has the potential for significantly overestimating or underestimating the true xenobiotic biomarker concentration depending upon the state of hydration at the time of collection. Thus, an indiscriminate application of the classical [CRE]-ratio-normalization technique should be avoided.

Table 5
Spot-urine excretion rate vs. UrineFlow regression parameters for combined subjects and phases

Biomarker	r	p	Slope	S.E.$_{slope}$	95%	CI$_{slope}$	Intercept	S.E.$_{intercept}$	95%	CI$_{intercept}$	$S_{y\|x}$
COT-U	0.73	<0.0001	0.53	0.03	0.46	0.59	1.33	0.11	1.10	1.55	1.28
COT-G	0.12	0.0922	0.09	0.05	−0.01	0.19	2.37	0.17	2.03	2.72	1.96
NIC-U	0.79	<0.0001	0.92	0.05	0.82	1.01	0.82	0.16	0.82	1.01	1.81
NIC-G	0.40	<0.0001	0.09	0.01	0.06	0.12	0.47	0.05	0.37	0.56	0.55
OHCOT-U	−0.08	0.2499	−0.14	0.12	−0.38	0.10	7.20	0.40	6.41	8.00	4.53
OHCOT-G	−0.01	0.8869	−0.01	0.04	−0.08	0.07	2.28	0.13	2.03	2.54	1.46
CRE	0.01	0.9312	0.00	0.01	−0.02	0.03	0.96	0.04	0.88	1.05	0.49
NNAL-U	0.38	<0.0001	0.02	0.00	0.01	0.03	0.17	0.01	0.14	0.19	0.14
NNAL-G	0.24	0.0007	0.03	0.01	0.01	0.04	0.42	0.03	0.36	0.47	0.30
SPMA	−0.10	0.1264	−0.07	0.05	−0.16	0.02	2.21	0.15	1.91	2.51	1.72
HPMA	0.65	<0.0001	0.41	0.03	0.35	0.48	1.34	0.11	1.12	1.56	1.23
1-OHP-T	0.28	<0.0001	0.08	0.02	0.04	0.13	0.26	0.07	0.12	0.40	0.77
MHBMA	0.40	<0.0001	0.01	0.00	0.01	0.01	0.06	0.00	0.05	0.07	0.05
DHBMA	0.79	<0.0001	0.18	0.01	0.16	0.20	0.62	0.03	0.55	0.68	0.36

3.7. Normalization techniques and variability

The following endpoints were calculated for the 215 spot-urine and 24 24-h urine samples: observed concentration (mass/volume), excretion rate (mass min^{-1}), SG-normalized concentration Eq. (1)), SG- and *Z*-normalized concentration (Vij and Howell technique, Eq. (6)), [CRE]-ratio-normalized concentration, SG-, *Z*- and [CRE]-ratio-normalized concentration (Vij and Howell technique, Eq. (7)) and [CRE]-regression-normalized concentration (Thompson et al. technique, Eq. (8)). The xenobiotic biomarker slopes for *Z*-normalization were obtained from Table 3, and the xenobiotic biomarker slopes, mean [CRE] and log mean [CRE] for [CRE]-regression-normalization were obtained from Table 4.

To evaluate the within-subject/within-day, within-subject/between-day and between-subject variabilities of the observed and normalized spot-urine NIC [XenoBio] and non-NIC [XenoBio], a variance-component analysis was performed. Because the data were not normally distributed and demonstrated increasing S.D. with increasing mean, further statistical analyses of the data were based on log transformations. For each xenobiotic biomarker, the method of restricted maximum likelihood was applied [46]. The individual variance-component contribution to total variation was then estimated to determine the variability contribution of samples obtained from the same subject on the same day, the variability contribution of samples obtained from the same subject on different days, and the variability contribution of samples obtained from different subjects. Using these variance-components, the S.D. and 95% CI of [XenoBio] were estimated, and the results for log-transformed observed [XenoBio] were compared to the corresponding results for log-transformed normalized [XenoBio]. The variance-component analysis S.D. comparisons for log-transformed observed and normalized spot-urine NIC [XenoBio] are presented in Fig. 3, and the variance-component analysis S.D. comparisons for log-transformed observed and normalized spot-urine non-NIC [XenoBio] are presented in Fig. 4. S.D. and 95% CI values for log-transformed observed and normalized spot-urine NIC [XenoBio] and log-transformed observed and normalized spot-urine non-NIC [XenoBio] are reported in Tables 6 and 7, respectively. These quantities estimate the S.D. of values (after log transformation) that would be observed among individual spot-urine samples obtained from the same subject on the same day, from the same subject on different days, and from different subjects. For most xenobiotic biomarkers, the observed concentration and excretion rate (bars A and B) exhibit the highest within-day, between-day and between-subject variability. The normalized concentrations (bars C–G) exhibit the lowest variability, with the SG-, *Z*- and [CRE]-ratio-normalized concentrations (bar F, Vij and Howell technique) and [CRE]-regression-normalized concentrations (bar G, Thompson et al. technique) exhibiting the lowest variability overall. CRE, HPMA and DHBMA exhibit the lowest between-day variability relative to the within-day variability, while 1-OHP-T exhibits the highest between-day variability relative to the within-day variability. MHBMA exhibits the lowest between-subject variability relative to the between-day variability, and SPMA and 1-OHP-T exhibit the highest between-subject variability relative to the between-day variability. For NIC$_{Eq}$-T, COT-U, NIC-U, NIC-G, NNAL-U, NNAL-G, HPMA, 1-

Table 6
Phase, time-block and the number of samples in each block for mean spot-urine minus simulated 24-h urine log differences presented in Figs. 5 and 6

Phase	Time-block	Samples (n)
I	6:00 a.m. to 10:00 a.m.	11
I	10:00 a.m. to 2:00 p.m.	18
I	2:00 p.m to 6:00 p.m.	19
I	6:00 p.m to 10:00 p.m.	25
I	10:00 p.m to 2:00 a.m.	18
I	2:00 a.m. to 6:00 a.m.	7
I	6:00 a.m. to 10:00 a.m.	9
II	6:00 a.m. to 10:00 a.m.	15
II	10:00 a.m. to 2:00 p.m.	21
II	2:00 p.m to 6:00 p.m.	17
II	6:00 p.m to 10:00 p.m.	28
II	10:00 p.m to 2:00 a.m.	12
II	2:00 a.m. to 6:00 a.m.	4
II	6:00 a.m. to 10:00 a.m.	11

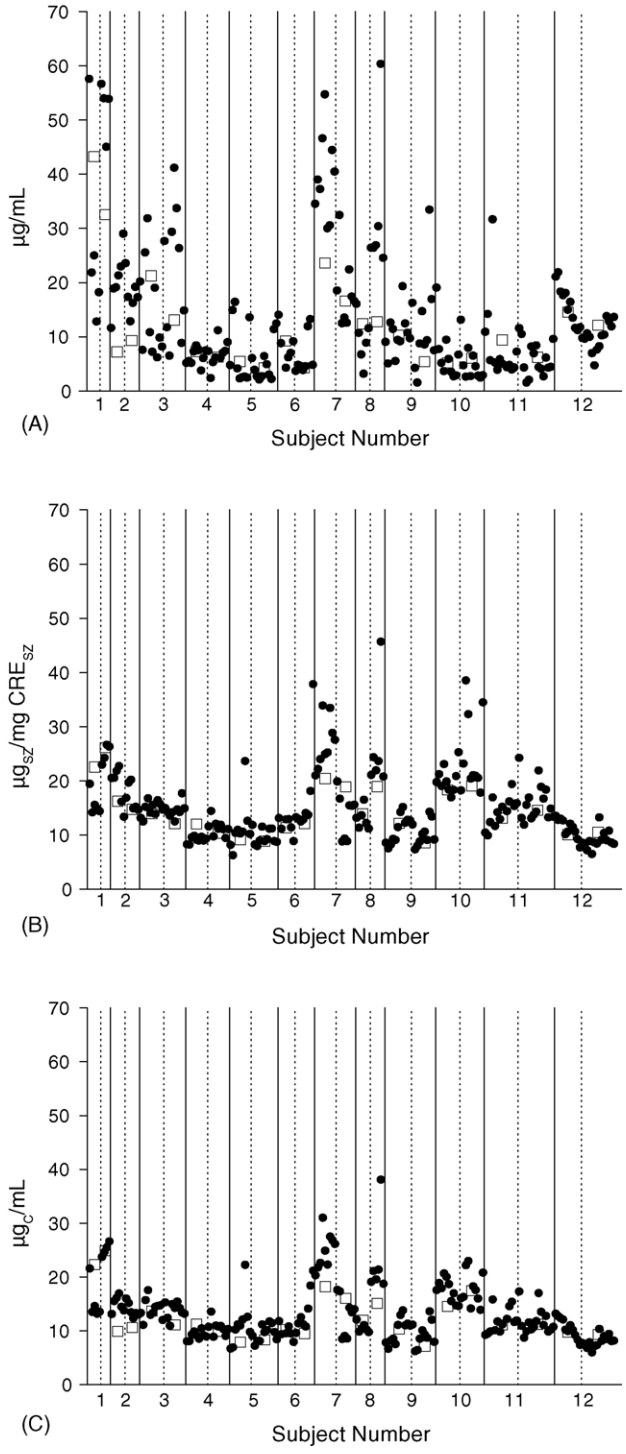

Fig. 1. Comparison of the observed concentration (graph A), the SG-, *Z*- and [CRE]-ratio-normalized concentration (graph B) and the [CRE]-regression-normalized concentration (graph C) demonstrating the similarity of the two normalization techniques on the 215 spot-urine and 24 24-h urine NIC_{Eq}-T endpoints. Solid vertical lines separate individual subjects, and dotted vertical lines separate Phases I and II samples within subjects. (●) Spot-urine samples and (□) 24-h urine samples.

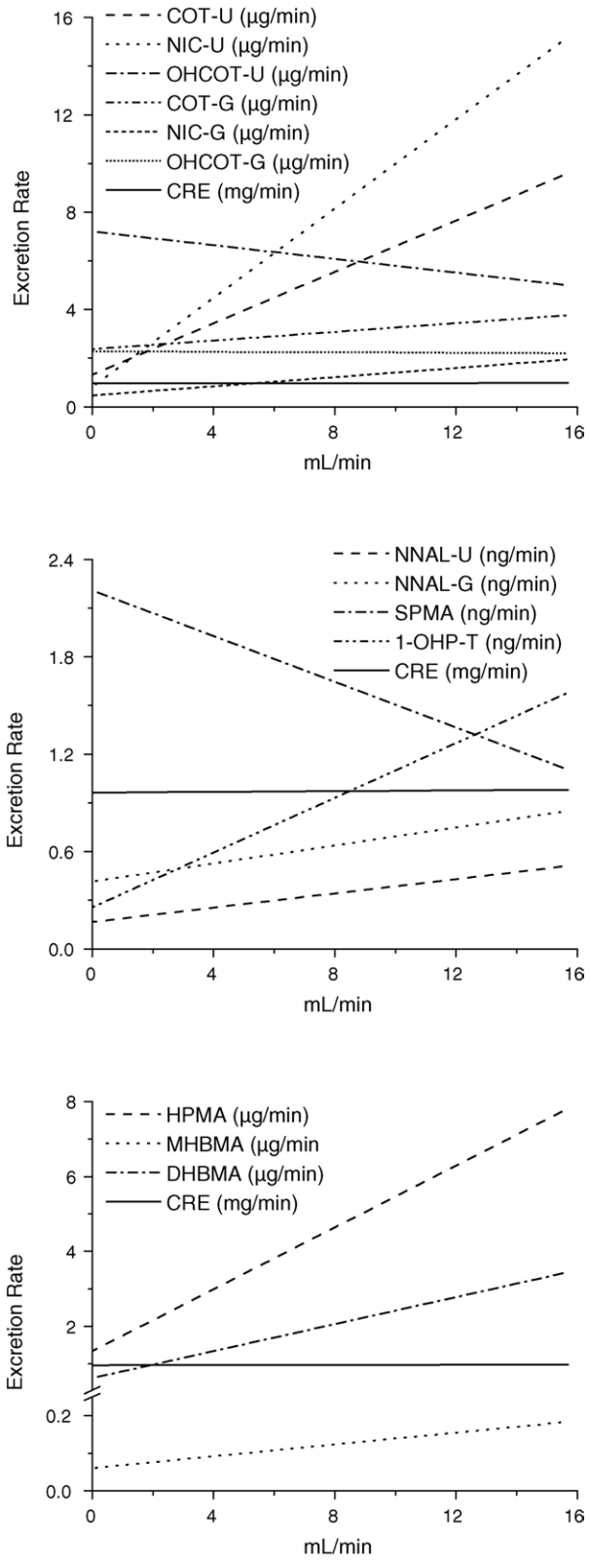

Fig. 2. Spot-urine excretion rate versus UrineFlow: linear regressions for combined subjects and phases. CRE is shown for reference on each plot as a urinary component unaffected by UrineFlow. See Table 5 for regression parameters.

OHP-T, MHBMA and DHBMA, the [CRE]-ratio-normalized concentrations (bar E) exhibit the highest between-subject variability among the normalized concentrations, and most of these xenobiotic biomarkers exhibit the highest between-day variability among the normalized concentrations. In fact, for NIC-U, HPMA and DHBMA, the [CRE]-ratio-normalized concentrations exhibit the highest between-subject variability overall, thereby confounding data interpretation when this normalization is used. The increased variability of the [CRE]-ratio-normalized concentrations is not surprising given the effect of excretion rate dependency on UrineFlow demonstrated in Table 5 and Fig. 1. Thus, [CRE]-ratio-normalization has not only the potential for overestimating or underestimating [XenoBio] relative to [CRE], but [CRE]-ratio-normalization demonstrates the highest variability among the normalization techniques as well.

3.8. Spot-urine versus 24-h urine comparisons

To determine an individual spot-urine sample's capacity to reflect same-day 24-h urine sample observed and normalized

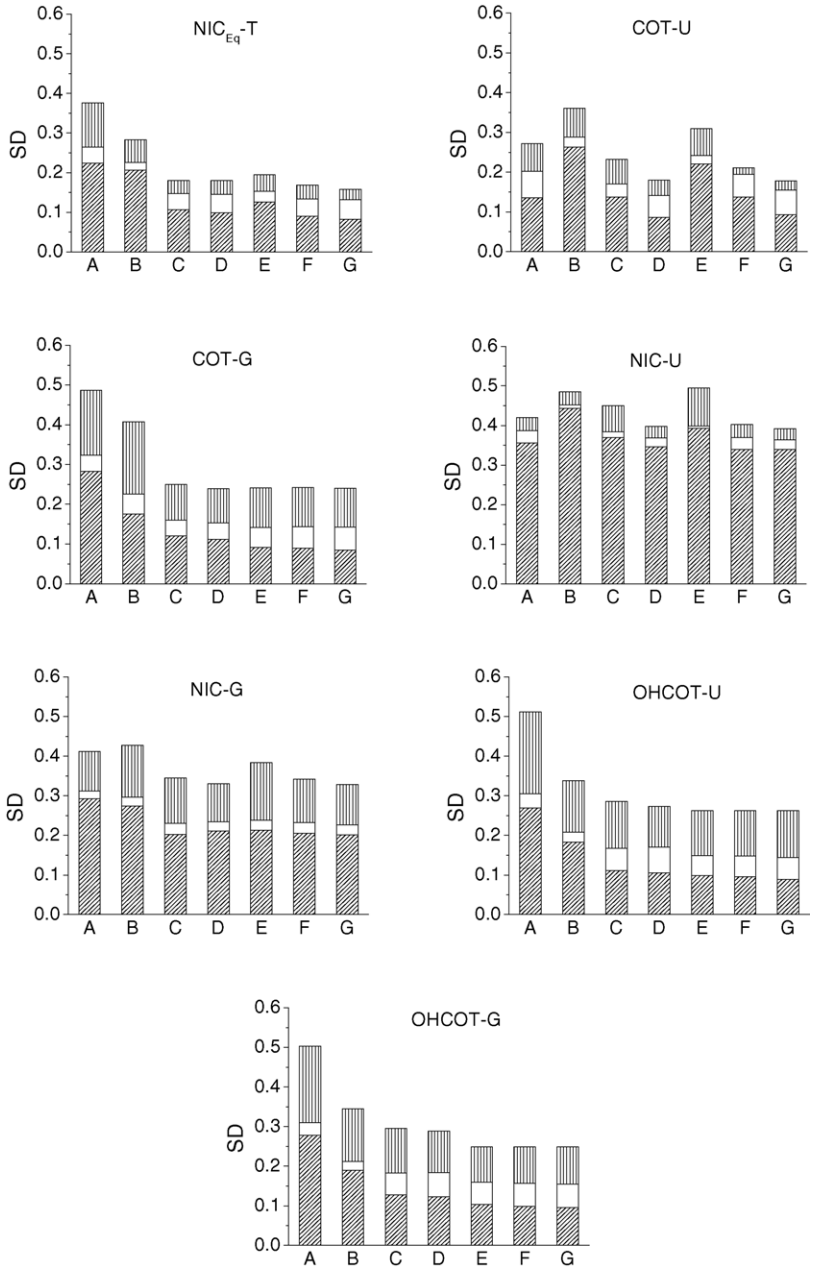

Fig. 3. Stacked-bar chart variance-component analysis (S.D. scale) of each log-transformed spot-urine NIC [XenoBio]: observed concentration (bar A), excretion rate (bar B), SG-normalized concentration (bar C), SG- and *Z*-normalized concentration (bar D), [CRE]-ratio-normalized concentration (bar E), SG-, *Z*- and [CRE]-ratio-normalized concentration (bar F) and [CRE]-regression-normalized concentration (bar G). Slanted-line-shaded bars represent same subject, within-day S.D.s; non-shaded bars represent same subject, between-day S.D.s; and vertical-line-shaded bars represent between-subject S.D.s.

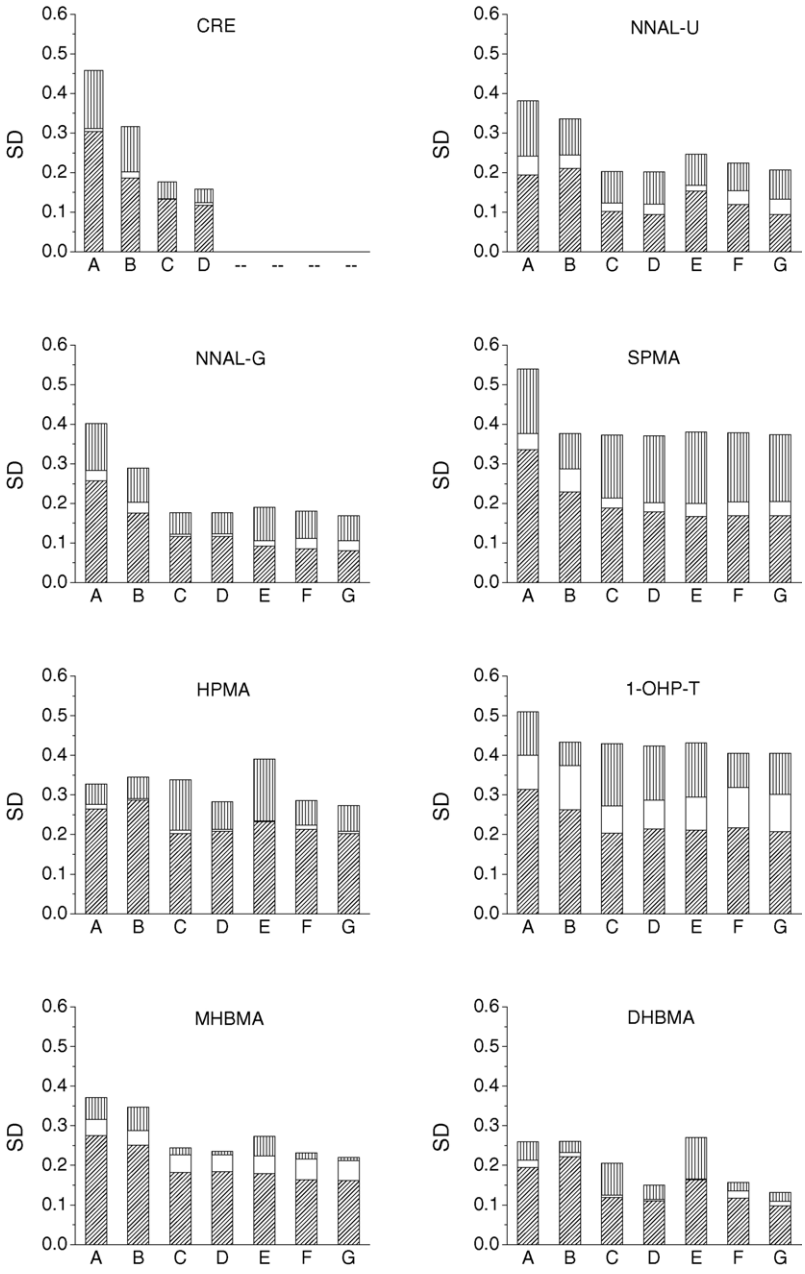

Fig. 4. Stacked-bar chart variance-component analysis (S.D. scale) of each log-transformed spot-urine non-NIC [XenoBio]: concentration (bar A), excretion rate (bar B), SG-normalized concentration (bar C), SG- and *Z*-normalized concentration (bar D), [CRE]-ratio-normalized concentration (bar E), SG-, *Z*- and [CRE]-ratio-normalized concentration (bar F) and [CRE]-regression-normalized concentration (bar G). Slanted-line-shaded bars represent same subject, within-day S.D.s; non-shaded bars represent same subject, between-day S.D.s; and vertical-line-shaded bars represent between-subject S.D.s.

concentrations, each subject's daily spot-urine sample results were mathematically pooled to calculate a simulated 24-h urine sample result for Phases I and II collection periods. Then, the individual spot-urine samples were compared to the simulated 24-h urine sample. Equations for mathematically pooling a subject's daily spot-urine sample results to yield a simulated 24-h urine sample result for a given xenobiotic biomarker are described below. For observed concentration (mass/volume), the volume weighted average concentration is defined as

$$[\text{XenoBio}]_{(\text{pooled})} = \frac{\sum v_i[\text{XenoBio}]_i}{\sum v_i}, \tag{10}$$

where $[\text{XenoBio}]_{(\text{pooled})}$ is the simulated 24-h urine concentration, v_i is the individual spot-urine volume and $[\text{XenoBio}]_i$ is the individual observed [XenoBio]. For excretion rate (mass min^{-1}), the time-weighted average excretion rate is defined as

$$\text{excretion rate}_{(\text{pooled})} = \frac{\sum v_i[\text{XenoBio}]_i}{\sum t_i}, \tag{11}$$

where excretion $\text{rate}_{(\text{pooled})}$ is the simulated 24-h urine excretion rate, and t_i is the individual spot-urine sample time. For SG-normalized concentration, the volume- and SG-weighted

average concentration is defined as

$$[\text{XenoBio}]_{\text{s (pooled)}} = [\text{XenoBio}]_{\text{(pooled)}} \left(\frac{1.020 - 1}{(\sum v_i \text{SG}_i / \sum v_i) - 1} \right), \quad (12)$$

where $[\text{XenoBio}]_{\text{s (pooled)}}$ is the simulated 24-h SG-normalized urine concentration, 1.020 is the mean SG of normal human urine and SG_i is the individual spot-urine SG. For SG- and *Z*-normalized concentration, the volume-, SG- and *Z*-weighted average concentration is defined as

$$[\text{XenoBio}]_{\text{sz (pooled)}} = [\text{XenoBio}]_{\text{(pooled)}} \left(\frac{1.020 - 1}{(\sum v_i \text{SG}_i / \sum v_i) - 1} \right)^{Z}, \quad (13)$$

where $[\text{XenoBio}]_{\text{sz (pooled)}}$ is the simulated 24-h SG- and *Z*-normalized urine concentration, and *Z* is the respective xenobiotic biomarker *Z* exponent. For [CRE]-ratio-normalized concentration, the volume- and [CRE]-weighted average concentration is defined as

$$\left(\frac{[\text{XenoBio}]}{[\text{CRE}]} \right)_{\text{(pooled)}} = \frac{\sum v_i [\text{XenoBio}]_i}{\sum v_i [\text{CRE}]_i}, \quad (14)$$

where $([\text{XenoBio}]/[\text{CRE}])_{\text{(pooled)}}$ is the simulated 24-h [CRE]-ratio-normalized concentration and $[\text{CRE}]_i$ is the individual observed [CRE]. For SG-, *Z*- and [CRE]-ratio-normalized concentration, the volume-, SG-, *Z*- and [CRE]-weighted average concentration is defined as

$$\begin{aligned} \left(\frac{[\text{XenoBio}]_{\text{sz}}}{[\text{CRE}]_{\text{sz}}} \right)_{\text{(pooled)}} &= \frac{[\text{XenoBio}]_{\text{(pooled)}} [(1.020 - 1)/((\sum v_i \text{SG}_i / \sum v_i) - 1)]^{Z_1}}{[\text{CRE}]_{\text{(pooled)}} [(1.020 - 1)/((\sum v_i \text{SG}_i / \sum v_i) - 1)]^{Z_2}} \\ &= \frac{[\text{XenoBio}]_{\text{(pooled)}}}{[\text{CRE}]_{\text{(pooled)}}} \\ &\times [(1.020 - 1)/((\sum v_i \text{SG}_i / \sum v_i) - 1)]^{Z_1 - Z_2}, \end{aligned} \quad (15)$$

where $([\text{XenoBio}]_{\text{sz}}/[\text{CRE}]_{\text{sz}})_{\text{(pooled)}}$ is the simulated 24-h SG-, *Z*- and [CRE]-ratio-normalized concentration, $[\text{CRE}]_{\text{(pooled)}}$ is the simulated 24-h [CRE], Z_1 is the xenobiotic biomarker-specific exponent, and Z_2 is the CRE-specific exponent. For [CRE]-regression-normalized concentration, the volume- and [CRE]-weighted average concentration is defined as

$$[\text{XenoBio}]_{\text{c (pooled)}} = [\text{XenoBio}]_{\text{(pooled)}} \left(\frac{\overline{[\text{CRE}]}}{[\text{CRE}]_{\text{(pooled)}}} \right)^{m}, \quad (16)$$

where $[\text{XenoBio}]_{\text{c (pooled)}}$ is the simulated [CRE]-regression-normalized concentration, $\overline{[\text{CRE}]}$ is the mean urinary [CRE] for the study population and m is the slope of the respective log [XenoBio]–log [CRE] linear least squares regression.

Two simulated 24-h urine sample results (Phases I and II) were generated for each subject and each xenobiotic biomarker and compared to the individual spot-urine sample results. To improve the detection of differences between the spot and simulated 24-h samples and to reduce the effect of daily outliers, the data were log-transformed. Utilizing log-transformed data to evaluate differences enabled a meaningful combination of results from different subjects and different collection days. For each xenobiotic biomarker and each subject, the log differences (spot-urine concentration minus simulated 24-h urine concentration) were calculated for each xenobiotic biomarker. Since spot-urine samples were collected ad libitum, the sample-collection times varied for each subject and each phase and complicated the evaluation of time trends within a day. To address this problem, results from samples collected in 4-h time-blocks were combined. Based on the subject-reported "Collection Time", i.e., the time that the spot-urine sample was collected, the mean log differences were placed into appropriate 4-h time periods or blocks for Phases I and II. Since some subjects did not collect their first daily spot-urine sample until after the first 4-h period, an additional 4-h block was added at the end of each phase so that their last "Collection Time" sample could be included in the analysis. Then, the mean log differences from Phases I and II were combined by weighting the means from each phase for the number of samples in each phase. Phase, time-block and the number of samples in each block are reported in Table 6. The mean log differences for each NIC [XenoBio] and each endpoint are presented in Fig. 5, and the mean log differences for each non-NIC [XenoBio] and each endpoint are presented in Fig. 6.

It is obvious from Fig. 5 that NIC-U exhibits a diurnal variation distinctly different from the other NIC xenobiotic biomarkers. For the concentration endpoint (graph A), NIC-U is out of phase with the other NIC [XenoBio] and exhibits increased amplitude of variation relative to the other NIC [XenoBio]. During morning and early afternoon hours, 6:00 a.m. to 2:00 p.m., spot-urine NIC-U exhibits concentrations much less than simulated 24-h pooled concentrations. From afternoon to late evening hours, spot-urine NIC-U concentrations more closely reflect simulated 24-h pooled concentrations, although the dinner-hours, 6:00 p.m. to 10:00 p.m., tend to show higher spot-urine concentrations than simulated 24-h pooled concentrations probably due to increased after-work smoking activity. However, during early morning hours, 2:00 a.m. to 6:00 a.m., spot-urine NIC-U concentrations are again much less than simulated 24-h pooled concentrations. Seemingly, this diurnal variation is attributable to sample collections prior to the establishment of daily "steady state" NIC-U concentration conditions and to the relatively short half-life of NIC-U [39,47]. Thus, spot-urine samples collected during time-blocks other than 2:00 p.m. to 2:00 a.m. have the potential for underestimating the true 24-h NIC-U uptake. Conversely, spot-urine samples collected during the 2:00 p.m. to 2:00 a.m. time-blocks, where "steady state" conditions have been established, are more likely to reflect the true 24-h NIC-U uptake. For the other NIC xenobiotic biomarkers, including NIC_{Eq}-T, the concentration and excretion rate endpoints (graphs A and B) exhibit the greatest variability or deviation from the simulated 24-h output. With the exception of NIC-U, the five normalized concentration endpoints (graphs C–G) demonstrate less deviation from the simulated 24-h concentrations. The SG-, *Z*- and [CRE]-ratio-normalized concentration

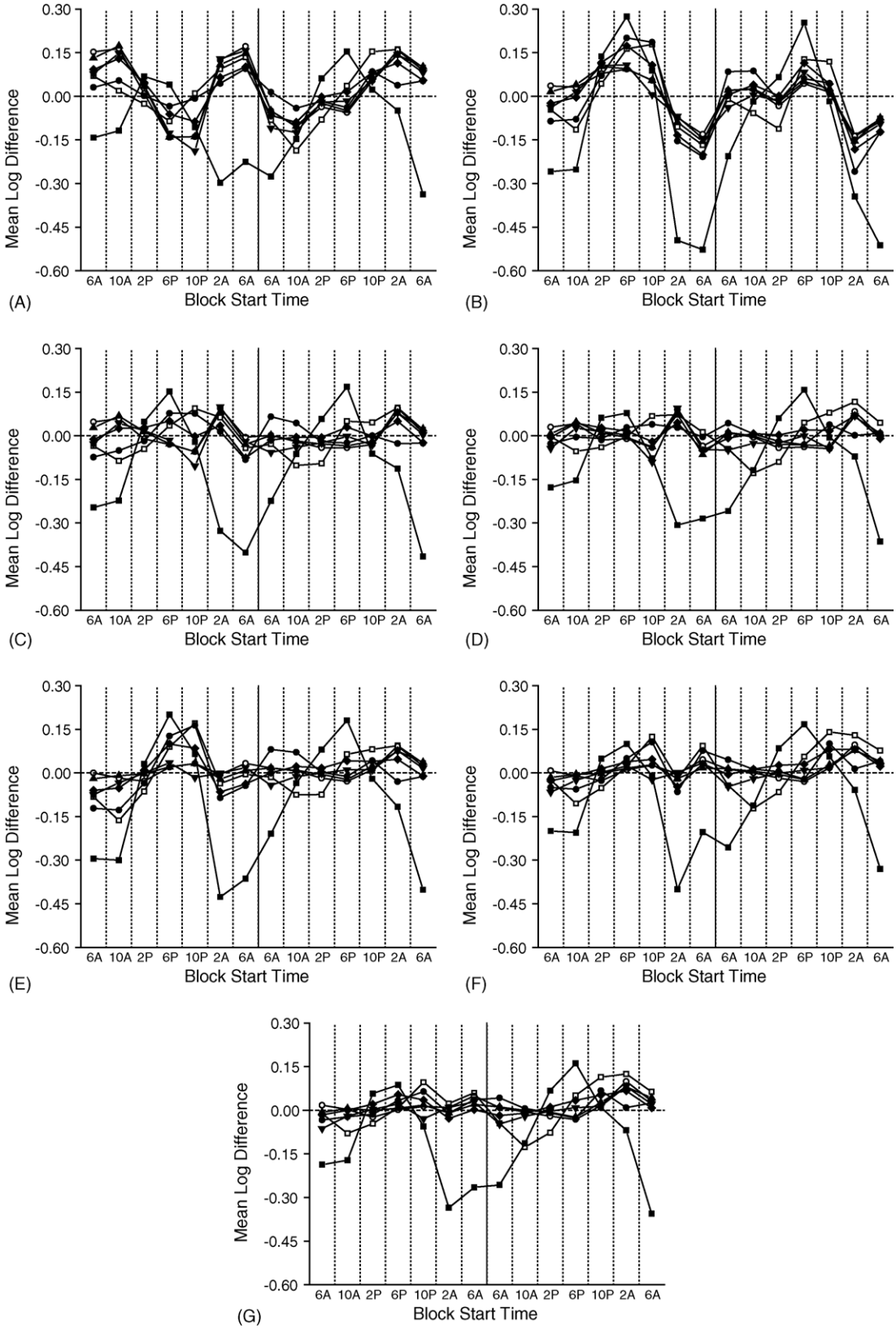

Fig. 5. Comparison of Phases I and II log differences (mean spot-urine minus simulated 24-h urine) for each NIC [XenoBio]: observed concentration (graph A), excretion rate (graph B), SG-normalized concentration (graph C), SG- and *Z*-normalized concentration (graph D), [CRE]-ratio-normalized concentration (graph E), SG-, *Z*- and [CRE]-ratio-normalized concentration (graph F) and [CRE]-regression-normalized concentration (graph G). Dotted vertical lines separate the 4-h time-blocks. Solid vertical lines separate Phases I and II. Dashed horizontal lines represent a zero log difference. (♦) NIC_{Eq}-T, (●) COT-U, (○) COT-G, (■) NIC-U, (□) NIC-G, (▲) OHCOT-U, (▲) OHCOT-G.

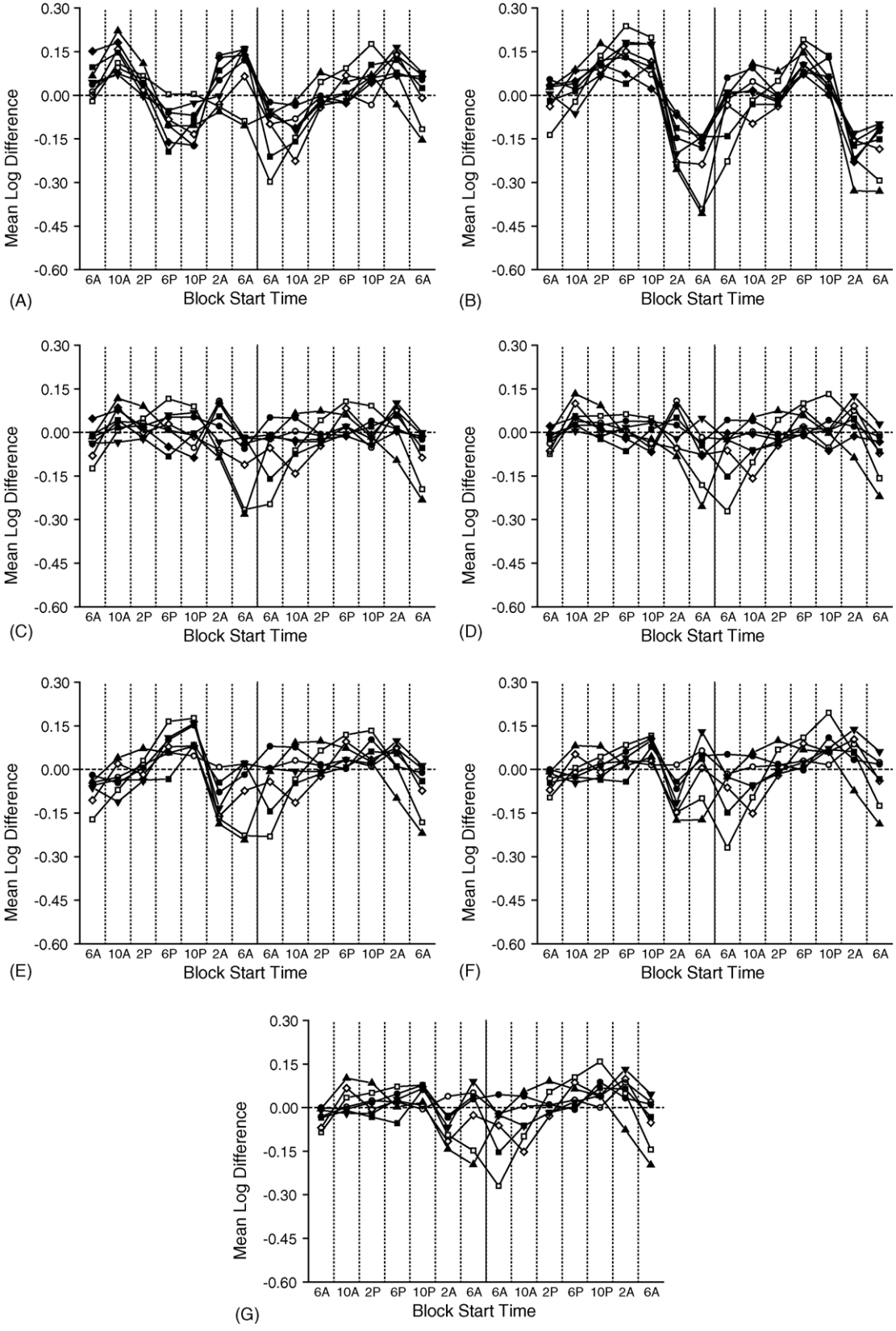

Fig. 6. Comparison of Phases I and II log differences (mean spot-urine minus simulated 24-h urine) for each non-NIC [XenoBio]: observed concentration (graph A), excretion rate (graph B), SG-normalized concentration (graph C), SG- and *Z*-normalized concentration (graph D), [CRE]-ratio-normalized concentration (graph E), SG-, *Z*- and [CRE]-ratio-normalized concentration (graph F) and [CRE]-regression-normalized concentration (graph G). Dotted vertical lines separate the 4-h time-blocks. Solid vertical lines separate Phases I and II. Dashed horizontal lines represent a zero difference. (♦) CRE, (●) NNAL-U, (○) NNAL-G, (■) SPMA, (□) HPMA, (◊) 1-OHP-T, (▲) MHBMA, (▼) DHBMA.

endpoint (graph F) and the [CRE]-regression-normalized concentration endpoint (graph G) demonstrate less spot-urine sample deviation from the simulated 24-h concentrations. Regardless of the endpoint, a sample-collection time-block where the mean log difference is nearest to zero has the potential for providing a spot-urine sample collection that most closely reflects the 24-h uptake. In general, the non-NIC [XenoBio] (Fig. 6) demonstrate diurnal variations similar to each other and to the NIC [XenoBio] (Fig. 5) except NIC-U. Again, throughout the day, the concentration and excretion rate endpoints exhibit the greatest variability or deviation from the simulated 24-h output. Although not as dramatic as before, the SG-, *Z*- and [CRE]-ratio-normalized concentration endpoint and the [CRE]-regression-normalized concentration endpoint demonstrate less spot-urine sample deviation from the simulated 24-h concentrations. Overall, for the NIC and non-NIC [XenoBio], the 2:00 p.m.–2:00 a.m. time-blocks with the SG-, *Z*- and [CRE]-ratio-normalized concentration or the [CRE]-regression-normalized concentration endpoints provide smaller mean log differences between the spot-urine samples and the simulated 24-h urine samples. Thus, a spot-urine sampling regimen during the 2:00 p.m. to 2:00 a.m. window appears adequate for accurately reflecting a 24-h urine sample concentration provided the appropriate normalization techniques (SG-, *Z*- and [CRE]-ratio-normalized concentration or the [CRE]-regression-normalized concentration are applied. Any study that utilizes solely NIC-U concentrations may provide confounded and inaccurate information especially if an inappropriate time point for sample collection is selected; however, if all NIC [XenoBio] are combined to yield molar nicotine equivalents (NIC_{Eq}-T), the NIC-U diurnal variation effect is minimized.

4. Conclusions

A review of the current literature was conducted to identify potential normalization techniques that could be used to adjust random spot-urine sample observed concentrations in order to accurately reflect 24-h urine sample concentrations. SG, [CRE] and [XenoBio] (eight tobacco-specific and five tobacco-related) were determined in 215 spot urines and 24 24-h urines from six male and six female smokers across the full range of "tar" category products (full-flavor, full-flavor low "tar", and ultra-low "tar"). In addition, NIC [XenoBio] were converted to molar nicotine equivalents and summed to yield NIC_{Eq}-T concentrations. Statistically significant log-linear relationships were observed for all xenobiotic biomarkers with regard to [XenoBio]-urine flow, [XenoBio]-specific gravity, and [XenoBio]-[CRE]. A statistically significant log-linear relationship was observed for most xenobiotic biomarkers with regard to excretion rate and urine flow; however, CRE demonstrated a zero-excretion-rate/urine-flow slope suggesting no urine flow effect on CRE excretion rate. Seven endpoints were identified and determined or calculated for further investigation: observed concentration (mass/volume), excretion rate (mass min^{-1}), SG-normalized concentration, SG- and *Z*-normalized concentration (Vij and Howell technique), [CRE]-ratio-normalized concentration, SG-, *Z*- and [CRE]-ratio-normalized concentration (Vij and Howell technique) and [CRE]-regression-normalized concentration (Thompson et al. technique). To evaluate the within-subject/within-day, within-subject/between-day and between-subject variability of the observed and normalized spot-urine NIC [XenoBio] and non-NIC [XenoBio], variance-component analyses were performed. For most xenobiotic biomarkers, the observed concentration and excretion rate endpoints exhibited higher within-day, between-day and between-subject variability while the normalized concentrations exhibited lower variability. The SG-, *Z*- and [CRE]-ratio-normalized concentration (Vij and Howell technique) and [CRE]-regression-normalized concentration (Thompson et al. technique) exhibited the lowest variability overall. For most xenobiotic biomarkers, the [CRE]-ratio-normalized concentrations exhibited the highest between-subject variability among the normalization techniques evaluated. Therefore, [CRE]-ratio-normalization has the potential for misestimating [XenoBio] relative to [CRE] due to potentially different excretion-rate/urine-flow relationships. To determine an individual spot-urine sample's capacity to reflect same-day 24-h urine sample observed and normalized concentrations, each subject's daily spot-urine sample results were mathematically pooled to calculate a simulated 24-h urine sample result for Phases I and II collection periods. An ANOVA indicated that, overall, for the NIC and non-NIC [XenoBio], a 2:00 p.m. to 2:00 a.m. spot-urine collection window utilizing the SG-, *Z*- and [CRE]-ratio-normalized technique or the [CRE]-regression-normalized technique provides the most accurate reflection of the true 24-h urine concentration. These two random spot-urine collection and normalization techniques can provide useful information for studies where the intended purpose is to compare relative uptake in smokers using different products or in surveillance programs. However, the loss of time-weighted information precludes the use of random spot-urine collection for the estimation of absolute uptake in smokers and cigarette yield without the application of further assumptions or normalization, e.g., the mean daily CRE output or the mean daily volume of human urine. In conclusion, renal excretion mechanisms are xenobiotic-specific and require a thorough understanding of the relationship between the xenobiotic biomarker concentration and excretion rate, urine flow, specific gravity and creatinine concentration to avoid the application of normalization techniques that may be inappropriate.

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