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New insights into urine-based assessment of polycyclic aromatic hydrocarbon-exposure from a rat model: Identification of relevant metabolites and influence of elimination kinetics☆

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ABSTRACT

A gas chromatography tandem mass-spectrometry method dedicated to the analysis of 50 metabolites of polycyclic aromatic hydrocarbons (OH-PAHs) was applied to urine specimens collected from female Long Evans rats under controlled exposure to a mixture of PAHs (at 7 doses ranging from 0.01 to 0.8 mg/kg, by gavage, 3 times per week for 90 days). On four occasions (day 1, 28, 60 and 90), urine samples were collected over a 24 h period. Among these 50 OH-PAHs, 41 were detected in urine samples. Seven additional OH-PAHs were identified for the first time: 1 corresponding to metabolite of pyrene and 3 of anthracene.

Strong linear dose *versus* urinary concentration relationships were observed for 25 of the 41 OH-PAHs detected in rat urine, confirming their suitability for assessing exposure to their respective parent compound. In addition, some isomers (e.g. 1-OH-pyrene, 3-OH-/4-OH-chrysene, 10-OH-benz[*a*]anthracene, 8-OH-benzo[k]fluoranthene, 11-OH-benzo[*b*]fluoranthene and 3-OH-benzo[*a*]pyrene) that were detected starting from the lowest levels of exposure or even in controls were considered particularly relevant biomarkers compared to metabolites only detected at higher levels of exposure. Finally, on the basis of the excretion profiles (on days 1, 28, 60 and 90) and urinary elimination kinetics of each OH-PAH detected at days 1 and 60, this study highlighted the fact that sampling time may influence the measurement of metabolites in urine.

Taken together, these results provide interesting information on the suitability of the analysis of OH-PAHs in urine for the assessment of PAH exposure, which could be taken into consideration for the design of epidemiological studies in the future.

1. Introduction

On the assumption of relationships between exposure to Polycyclic Aromatic Hydrocarbons (PAHs) and several health disorders such as respiratory and cardiovascular diseases, neurobehavioral impairments as well as different types of cancer, several recent research studies have focused on the assessment of human exposure to these ubiquitous pollutants (Appenzeller and Tsatsakis, 2012; Maitre et al., 2002, 2003; Thai et al., 2016). As part of these research efforts, numerous analytical techniques were used for the determination of urinary PAH metabolites, including HPLC with fluorescence detection, LC–MS/MS, GC–MS, GC–HRMS and GC–MS/MS (Hagedorn et al., 2009). In the last decades, several PAH metabolites have been analyzed in urine, with a special focus on 1-OH-pyrene and 3-OH-benzo[*a*]pyrene (3-OH-B[*a*]P) which have sometimes been proposed as the most representative biomarkers of global exposure to PAHs (Chien and Yeh, 2010; Marie et al., 2010). In fact, the measurement of 1-OH-pyrene in urine, which is the pyrene's most abundant metabolite, has for a long time been suggested as an indicator of global exposure to PAHs, due to the ubiquitous distribution of pyrene into the atmosphere under particulate phase and also to the easiness of its analysis. Nevertheless, recent research focusing on the internal dose of PAHs in smokers demonstrated that the urinary concentration of 1-OH-pyrene failed to correlate with the atmospheric concentration of the 16 PAHs listed as priorities for their toxicity by international agencies (e.g.

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US-EPA), including B[*a*]P (Aquilina et al., 2010). In parallel, urinary 3-OH-B[*a*]P, one of the most abundant metabolites of B[*a*]P, presented significant correlation with biologically-active internal dose (DNA levels in lungs) in animal models (Barbeau et al., 2011; Marie-Desvergne et al., 2010) and thus seemed to be more relevant than 1-OH-pyrene. However, the very low amounts of 3-OH-B[*a*]P excreted in urine and its highly variable concentration limit its relevance as a reliable biomarker of exposure (Ariese et al., 1994; Chien and Yeh, 2012; Payan et al., 2009). Since people are generally exposed to mixtures of PAHs, more comprehensive methods covering a larger number of chemicals were developed (Buratti et al., 2007; Chetiyanukornkul et al., 2006; Hollender et al., 2000; Li et al., 2008; Romanoff et al., 2006; Scinicariello and Buser, 2014; Tsumura et al., 2011). In addition to the monitoring of the light PAHs (up to four fused benzene rings) which constitute a major component in a large number of exposure sources, particular attention has therefore been paid to the heavy PAHs (more than four fused benzene rings) which are more stable and more toxic (Yu, 2015). To achieve this objective, several authors have recommended the direct analysis of parent PAHs in urine, based on the assumption that the measurement of unmetabolized species in urine, could be complementary to the analysis of metabolized species (Campo et al., 2011, 2014). This recommendation was supported by the fact that some parent PAHs, especially the light ones, contribute significantly to the total of PAH+OH-PAHs excreted in urine. For instance, phenanthrene and pyrene made up 42% and 56%, respectively, of the total PAH+OH-PAH concentration measured in urine of 9 volunteers under controlled dietary conditions (Motorykin et al., 2013). The analysis of parent PAHs is however accompanied by several drawbacks such as the limited number of these compounds excreted in urine since faeces appears as the main route of excretion of heavy PAHs (Bouchard and Viau, 1998; Ramesh et al., 2004), the possibility of external contamination of the biological specimens and loss of the most hydrophobic compounds which can adsorb on material used to collect and store specimens (Ramirez et al., 2011; US-EPA, 1999). Nevertheless, in 2011, Campo et al. developed a very specific method for the analysis of 13 PAHs in urine, which takes into account all possible sources of bias. The latter appears particularly suitable for the quantification of carcinogenic 4- to 6-ring PAHs in the urine of subjects with and without occupational PAH exposure (Campo et al., 2011).

In parallel, several studies still tend to support metabolites as the most relevant biomarkers in that they provide information on the different phases of metabolism. Such an approach was for instance used in the NHANES cohort, in which 22 OH-PAHs urinary metabolites were analyzed in approximately 2800 volunteers from US between 1999 and 2000 (Li et al., 2008). On top of highlighting the wide distribution of OH-PAHs concentration in urine samples (spanning three to four orders of magnitude), the authors pointed out that children presented the highest levels, thereby suggesting higher environmental exposure. In addition, concentration levels of major PAH metabolites, especially combined concentration of the metabolites from the same parent PAHs, were highly correlated with each other, suggesting common sources of exposure to different parent PAHs and similar metabolic pathways (Li et al., 2008).

In the last decades, the importance of sampling time for the analysis of OH-PAHs in urine was clearly established (Biotox, 2016; Li et al., 2010). For instance, the analysis of urinary 1-OH pyrene conducted on coke oven workers exposed to a wide range of PAHs highlighted, despite high intra-individual differences (ranging from 40% to 62%), a significant relation between the concentration in the first morning voids and in the 24-h urinary collections (in ng/ml urine). Similar correlations were also noticed for 8 other OH-PAHs measured in urine collected from non-occupationally exposed subjects (n = 427) (Li et al., 2010). More recently, in order to properly assess occupational exposure to PAHs with urinary metabolites, the French Institute of Research and Security (INRS) has therefore recommended 3 different sampling times: at the start of the shift, after 48 h without any sort of exposure, so as to evaluate residual concentration; - At the beginning of the second day of exposure, to evaluate the exposure of the day before;- and finally at the end of the fifth day, to evaluate cumulative exposure (Biotox, 2016). Finally, the importance of taking into consideration the variability in OH-PAHs biological half - life in urine was recently highlighted by Lutier et al. (2016). In fact, they demonstrated that by reason of the delay observed for the maximum urinary excretion rates of 3-OH-B[*a*]P in humans, the sampling should be performed in the morning following exposure; unlike 1-OH-pyrene for which post-shift sampling is recommended. No consensus has been reached yet on the ideal time of urine sampling and further information is still needed.

In this study, a method based on gas chromatography tandem mass spectrometry was developed for the analysis of 50 urinary metabolites of 2- to 6-ring PAHs. This method was then applied to urine collected from rats exposed to 7 doses of PAHs for 90 days in order to assess the excretion of the targeted PAH metabolites in this matrix and to confirm that the method was sufficiently sensitive to highlight low levels of exposure and therefore to use them as biomarkers. On day 90, the association between the dose of exposure and the level of concentration of hydroxylated metabolites in urine was evaluated to assess the suitability of each metabolite for the evaluation of the exposure to their respective parent compound. Finally, the influence of sampling time was investigated on the basis of the excretion profile of urinary OH-PAHs at different time points (days 1, 28, 60 and 90) and of the elimination kinetics of each mono-hydroxylated-PAH detected at days 1 and 60.

2. Material and methods

2.1. Chemicals

The sixteen PAHs naphthalene (napht), fluorene (fluo), acenaphthene, acenaphtylene, anthracene, phenanthrene (phen), fluoranthene (fluoranth), pyrene, benz[*a*]anthracene (B[*a*]A), chrysene (chry), benzo[*b*]fluoranthene (B[*b*]F), benzo[k]fluoranthene (B[k]F), benzo[*a*]pyrene (B[*a*]P), benzo[g,h,i]perylene (B(g,h,i)P), indeno[1,2,3-*c*,*d*]pyrene (I[1,2,3-*c*,*d*]P) and dibenz[a,h]anthracene (DIB[a,h]A) used for the animal experiment model were purchased from Sigma Aldrich (Bornem, Belgium). With the exception of B[*a*]P and dibenz[a,h]anthracene which presented purity rates of 96% and 95%, all the other parent compounds were above 98%. Standard solutions of 1-OH- and 4-OH-phenanthrene were supplied at 10 mg/L by Dr Ehrenstorfer (Augsburg, Germany). Stable isotope labeled analogues 1-OH-pyrene-D_9 and naphthol-D_7 were obtained from Chiron AS (Trondheim, Norway) and Medical isotopes inc. (Pelham, USA) respectively. 3-OH-fluoranthene-$^{13}C_6$, 1-OH-benz[*a*]anthracene-$^{13}C_6$ and the 50 OH-PAHs investigated in this study were purchased in powder form from MRI-Global (Kansas City, Missouri, USA). The purity rate of the latter metabolite standards was above 98%, except 2-OH-chrysene, 9-OH-benzo[*a*]anthracene, 11-OH-benzo[*a*]anthracene and 8-OH-indeno[1,2,3-*c*,*d*]pyrene which presented purity rates ranging from 91% to 96%. The level of purity was taken into account for the preparation of the standards solutions. ENVI-Chrom P solid-phase extraction columns (sample volume 1 mL and stationary phase 100 mg) and derivatization reagent N-*tert*-Butyldimethylsilyl-N-methyltrifluoroacetamide, 1% TBDMCS (MtBSTFA, ≥ 97% purity) were purchased from Sigma-Aldrich (Bornem, Belgium). A stock solution