— Minireview —

Biomarkers of Exposure to Polycyclic Aromatic Hydrocarbons and Related Compounds

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Polycyclic aromatic hydrocarbons (PAHs) and nitropolycyclic aromatic hydrocarbons (NPAHs) are widespread carcinogenic compounds that arise from occupational, environmental and dietary sources. The metabolites of PAHs and NPAHs in biological fluids have been investigated as potential biomarkers for assessing human exposure to them, and, particularly, urinary metabolites are the excellent candidates due to the non-invasiveness and convenience of collecting the sample. Here we describe HPLC methods for accurately determining one type of these metabolites, monohydroxy PAHs (OHPAHs). The developed method was applied to the urine samples of non-smoker taxi drivers, traffic police officers and rural villagers of Chiang Mai, Thailand. The results showed higher urinary concentrations of OHPAHs in rural villagers, suggesting the higher respiratory exposure to PAHs contained in smoke from biomass burning. On the other hand, 1-nitropyrene (1-NP) is one of the most abundant NPAHs in diesel exhaust particulate matter (DEP). We also developed a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for determining urinary 1-NP metabolites. 1-NP metabolites were quantified in urine from healthy subjects. 6- and 8-Hydroxy-*N*-acetyl-1-aminopyrenes (OHNAAPs) and 6- and 8-hydroxy-1-nitropyrenes (OHNPs) were the most abundant 1-NP metabolites in human urine. The presence of OHNAAPs and OHNPs in human urine was demonstrated for the first time.

Key words —— biomarker, polycyclic aromatic hydrocarbon, nitropolycyclic aromatic hydrocarbon, urine, HPLC, mass spectrometry

INTRODUCTION

Lung cancer is the most common cancer and the major cause of cancer death in the world.[1, 2] Smoking is considered to be the main cause of lung cancer, while other suspected factors include automobile exhaust and ambient and indoor air pollutants.[3–6] Polycyclic aromatic hydrocarbons (PAHs) and nitropolycyclic aromatic hydrocarbons (NPAHs) are formed through the incomplete combustion of fossil fuels and are environmental contaminants widely distributed in the atmosphere, water and soil.[7–9] Additionally, several NPAHs are subsequently formed by the reaction of PAHs and NO_x in the atmosphere.[10] Many PAHs and NPAHs are well-known to be carcinogenic or co-carcinogenic compounds.[11–13] PAHs and NPAHs are taken up by humans through inhalation of cigarette smoke and polluted air (automobile exhaust), and, partly, consumption of food.[11] In order to evaluate the exposure of humans to PAHs and NPAHs from multiple routes, PAH or NPAH concentrations in the atmosphere and in inhaled particulate matter (PM) have been measured in many areas. On the other hand, a valuable tool in assessing human exposure to PAHs and NPAHs is the use of biological markers or biomarkers. The biomarker can be broadly defined as a measurable change in a biological system that is caused by exposure to an exogenous chemical.[14] Recently, a variety of PAH and NPAH metabolites in biological fluids have been investigated as potential biomarkers of exposure to these compounds. Since humans are usually exposed to quite complex mixtures of PAHs and NPAHs, it is necessary to choose suitable metabolites, besides a number of relevant parent compounds. In biological fluids, urine is an excellent candidate sample for determining the metabo-

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lites due to the non-invasiveness and convenience of collecting samples. In this paper, we briefly review analytical methods for determining urinary metabolites of PAHs and NPAHs as potential biomarkers for the exposure assessment.

URINARY METABOLITES OF PAHS AS BIOMARKERS FOR EXPOSURE TO MULTIPLE PAHS

When metabolized, PAHs are converted via intermediate epoxides to phenols, diols and tetrols by cytochrome P450-dependent monooxygenases. Some intermediates have been shown to bind to DNA or proteins. As an example, benzo[*a*]pyrene (BaP) forms dihydrodiol-epoxide which can covalently bind to nucleophilic sites in DNA to form BaP-DNA adducts. These types of metabolites are considered as carcinogenic intermediates of BaP and general PAHs. On the other hand, the largest fraction of the metabolites, phenols and diols, is conjugated with sulfuric or glucuronic acids or with glutathione and excreted in the urine and bile.[15)]

Until now, PAH-DNA adducts in white blood cells and PAH metabolites [monohydroxylated PAHs (OHPAHs)] in urine were used as biomarkers for evaluating PAH exposure. These methods are described in several reviews.[7, 15–19)] Urinary 1-hydroxypyrene (1-OHPyr) is one of the most common markers. 1-OHPyr was first identified in 1983 as the principal metabolite of the pyrene taken from pig's urine,[20)] and an analytical method using HPLC with fluorescence detection for determining 1-OHPyr in human urine established by Jongeneelen *et al.*[21)] became the most common method. Urinary 1-OHPyr has been used in various studies as a biological indicator of exposure to PAHs.[7, 15–19)]

The use of standard addition method has been required to accurately quantify 1-OHPyr in urine samples. Consequently, use of an internal standard for the analysis of urine samples is highly recommended since the standard addition method requires that samples be run at least three times and it takes time to conduct the analysis. For this reason, we developed an HPLC method with fluorescence detection for determining 1-OHPyr that uses deuterated 1-OHPyr (1-OHPyr-d_9) as an internal standard.[22)] The analyte and the internal standard need to be separated on HPLC columns, however, the separation was not achieved by using any octadecylsilica (ODS) column. We successfully separated 1-OHPyr and the deuterated internal standard using an alkylamide-type reversed phase column (Rs = 1.45), which strongly retains phenols (Fig. 1). Moreover, the analyte and the interfering peaks originating from the urine sample were completely separated. Based on the fact that the physical properties of 1-OHPyr-d_9 were close to the analyte and 1-OHPyr-d_9 was eluted just before the analyte, it was evident that 1-OHPyr-d_9 was a superior internal standard for 1-OHPyr analysis.

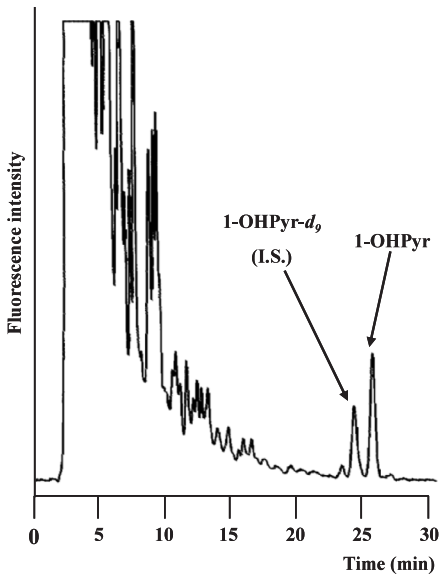

Fig. 1. An HPLC Chromatogram of a Urine Sample from a Smoker for Determining 1-OHPyr

Urinary 1-OHPyr has been established as a biomarker that can evaluate PAH exposure. However, recent findings have cast doubt on the effectiveness of evaluating exposure to all PAHs by using only the pyrene metabolite. Therefore, the use of biomarkers that measure a larger number of PAH metabolites at the same time has been gaining acceptance. A urinary metabolite of carcinogenic PAHs, especially BaP, would be more suitable as an indicator. However, BaP metabolites with five aromatic rings are mainly excreted in feces via bile,[23)] and therefore may be difficult to be detected in urine. Recently, 3-hydroxybenzo[*a*]pyrene (3-OHBaP), a major metabolite of BaP, was identified and quantified at low ng/g creatinine concentration in human urine.[24)] On the other hand, the atmospheric concentrations of the 2-, 3- and 4-ring PAHs, *e.g.*, naphthalene, phenanthrene and pyrene, which predominantly exist in the vapor phase, were significantly higher than those of PAHs with five or more rings that are primarily associated with the

particulate phase.[25–27] In particular, indoor air is a significant contributor to human exposure to PAHs, and several findings indicated that concentrations of 2–4 ring PAHs in indoor air always exceed those in ambient air.[25–27] Therefore, determination of urinary metabolites of 2–4 ring PAHs to which humans may be exposed at high concentrations may provide more comprehensive information to estimate the individual exposure to PAHs.

First, we focused on fluorene, a PAH. Fluorene is one of the most abundant PAHs throughout the gas phase in the environment and, especially, in tobacco smoke. It is a component that exists at high concentrations, ranked second after naphthalene. The major metabolite of fluorene in urine is 2-hydroxyfluorene (2-OHFle) and 2-OHFle is the most abundant of OHPAHs in urine.[28] While there is a strong possibility that 2-OHFle is a good biomarker, its analytical methods have not been established. Hence, we developed a column-switching method that has a high selectivity for 2-OHFle,[29] and the urinary concentration of 2-OHFle was substantially influenced by exposure to PAHs in the vapor phase from smoking.[30] Further, we developed an HPLC method for simultaneously determining ten urinary OHPAHs, including 1- and 2-hydroxynaphthalenes (OHNaps), 2-OHFle, 1-, 2-, 3-, 4- and 9-hydroxyphenanthrenes (OHPhes), 3-hydroxyfluoranthene (3-OHFrt) and 1-OHPyr.[31] 1-OHPyr-d_9 was used as an internal standard. Figure 2 shows typical chromatograms of a standard mixture of ten OHPAHs and a urine sample from a subject. Figure 2 (a) shows that ten kinds of OHPAHs, 1- and 2-OHNaps, 2-OHFle, 2-, 3- and 4-OHPhes, 1- + 9-OHPhes, 3-OHFrt and 1-OHPyr were successfully separated on the alkylamide-type reversed phase column. The removal of substances that interfere with detection of analytes is necessary for the pretreatment of biological samples. To measure the urinary OHPAHs, we developed a pretreatment method for urine samples using two different types of Solid phase extraction (SPE) cartridge (Sep-Pak C_{18} and Silica cartridges). As shown in Fig. 2 (b), the peaks of the ten OHPAHs were free from any interfering peaks, though several interfering peaks were observed around the peaks of OHNaps.

By using the proposed method, OHPAHs were quantified and compared in urine samples of non-smoker male subjects who lived in Chiang Mai, Thailand.[31] The subjects were divided into three groups including rural villagers, taxi drivers and traffic police officers. The taxi drivers and traffic police officers lived in the urban area of the city and were expected to be continually exposed to automobile exhaust. On the other hand, the rural villagers lived in the countryside and were expected to be no source of PAHs related to automobile exhaust. The mean concentrations of OHNaps (normalized to the concentration of creatinine) were highest among the metabolites in all groups (Table 1), the urinary concentrations increased with decreasing ring number. Similarly, PAH concentrations in outdoor and indoor air increase with decreasing ring number, and especially, the concentrations of naphthalene are approximately 10–50 times higher than those of other PAHs.[25–27] Interestingly, the concentrations of all detected metabolites, except for 1-OHNap, of rural villagers were significantly higher than those of the other two groups (Table 1). The urinary OHPAH levels of the rural villagers was much higher than a proposed limit (0.24 μmol/mol creatinine for 1-OHPyr)[16] and exceeded post-shift OHPAH levels in workers exposed to diesel exhaust.[32] The high urinary levels of rural villagers are thought to be mainly due to atmospheric PAHs produced by open-burning for the agricultural purpose and by burning of biomass (wood and charcoal) for cooking. Exposure to biomass smoke including particulate matter with carcinogens such as PAHs from the combustion of solid fuels in the room without a ven-

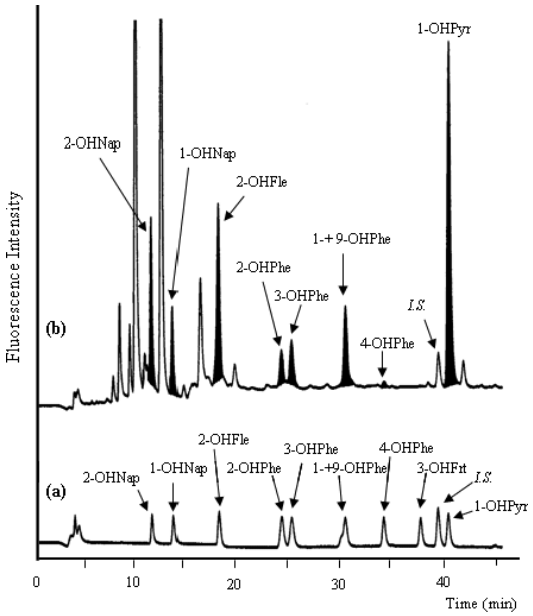

Fig. 2. Representative HPLC Chromatograms of a Standard Mixture of OHPAHs (a) and a Thai Non-Smoker Urine Sample (b)

Table 1. Urinary Concentrations (μmol/mol Creatinine) of 10 Kinds of OHPAHs

Group		1-OHNap	2-OHNap	2-OHFle	2-OHPhe	3-OHPhe
Rural villager	Mean ± S.D.	7.58 ± 4.75	12.14 ± 6.06[b]	2.62 ± 1.45[b]	0.82 ± 0.41[b]	1.15 ± 0.34[b]
(n = 10)	Range	1.69 – 14.88	2.21 – 20.17	0.4 – 5.07	0.12 – 1.28	0.48 – 1.56
	No. of nd.	—	—	—	—	—
Taxi driver	Mean ± S.D.	6.61 ± 3.79	2.28 ± 1.75	0.34 ± 0.2	0.15 ± 0.11	0.19 ± 0.11
(n = 10)	Range	1.51 – 11.96	0.51 – 4.68	0.1 – 0.62	0.04 – 0.38	0.07 – 0.45
	No. of nd.	1	—	—	—	—
Traffic police	Mean ± S.D.	4.67 ± 1.71	2.74 ± 2.33	0.37 ± 0.29	0.11 ± 0.08	0.11 ± 0.07
officer	Range	2.53 – 7.28	0.69 – 7.59	0.13 – 0.87	0.04 – 0.14	0.04 – 0.21
(n = 10)	No. of nd.	2	—	—	—	—

Group		1- + 9-OHPhe	4-OHPhe	3-OHFrt	1-OHPyr
Rural villager	Mean ± S.D.	0.51 ± 0.28[b]	0.09 ± 0.06[c]	nd[a]	1.2 ± 0.7[b]
(n = 10)	Range	0.1 – 0.88	0.02 – 0.19	nd	0.17 – 2.35
	No. of nd.	—	—	10	—
Taxi driver	Mean ± S.D.	0.1 ± 0.07	0.02 ± 0.01	nd	0.27 ± 0.19
(n = 10)	Range	0.04 – 0.24	0.01 – 0.05	nd	0.06 – 0.53
	No. of nd.	—	1	10	—
Traffic police	Mean ± S.D.	0.13 ± 0.09	0.03 ± 0.03	nd	0.18 ± 0.13
officer	Range	0.02 – 0.29	0.01 – 0.09	nd	0.1 – 0.5
(n = 10)	No. of nd.	—	2	10	—

a) Not detected, *b*) significantly different from the taxi driver and traffic policeman ($p < 0.001$), *c*) significantly different from the taxi driver and traffic policeman ($p < 0.05$).

tilation system is a serious problem in developing countries.[33] The results suggest that the proposed method is useful for the detailed analysis of urinary OHPAHs, which are related to PAH exposure.

URINARY METABOLITES OF 1-NITROPYRENE AS A PROPOSED BIOMARKER FOR EXPOSURE TO DIESEL EXHAUST

Many NPAHs are carcinogenic/mutagenic compounds, and among these compounds, 1-nitropyrene (1-NP) and dinitropyrenes have been previously reported as the main contributors of direct-acting mutagenicity of diesel exhaust particulate matter (DEP).[12,13] 1-NP is one of the most abundant NPAHs in DEP and has been proposed as a chemical marker for diesel exhaust.[34,35] Therefore, the metabolites of 1-NP are expected to be a specific biomarker of exposure to DEP because of 1-NP's strong association with diesel exhaust.[36] The metabolism of 1-NP has been studied using various tissues and species.[37–42] 1-NP is metabolized essentially through two routes; cytochrome P450-mediated C oxidation and nitroreduction. Some intermediates have been shown to bind to DNA or proteins. Urinary or fecal metabolites that have typically been observed in *in vivo* studies are hydroxy-1-nitropyrenes (3-, 6-, and 8-OHNP), hydroxy-*N*-acetyl-1-aminopyrenes (3-, 6- and 8-OHNAAP), *trans*-4,5-dihydro-4,5-dihydroxy-1-nitropyrene, *N*-acetyl-1-aminopyrene (NAAP), and 1-aminopyrene (1-AP).[37–42]

Hemoglobin adducts of 1-NP in human blood samples have been investigated as biomarkers of the exposure to DEP,[43,44] however, the difference between the adduct levels in occupational and non-occupational subjects was not statistically significant.[44] In humans, only a few studies have reported methods for determining urinary metabolites of 1-NP or other NPAHs. Although 1-AP in human urine has been measured,[45–48] 1-NP metabolites such as OHNPs and OHNAAPs, which are expected to be the major metabolites from *in vivo* and *in vitro* studies, have never been determined in human urine. Due to the expected low concentrations of 1-NP metabolites in human urine and the detection limitations, we developed a sensitive and specific liquid chromatography-tandem mass spectrometry (LC-MS/MS) method and an effective pretreatment method for the simultaneous measurement of urinary 1-NP metabolites, intended to be biomarkers of low level 1-NP (DEP) exposure in humans.[49] This report is the first to demonstrate the presence of OHNAAPs and OHNPs in

Table 2. Urinary Concentrations of 1-NP Metabolites in the Study Subjects (n = 22)

	Mean ± S.D.		Median		Range		Frequency of detection (%)
	(pmol/mol of creatinine)	(pmol/l of urine)	(pmol/mol of creatinine)	(pmol/l of urine)	(pmol/mol of creatinine)	(pmol/l of urine)	
3-OHNAAP	< DL[a)]	< DL	< DL	< DL	< DL	< DL	0
6-OHNAAP	117 ± 108	2.23 ± 2.35	54	1.29	< QL[b)]– 364	< QL– 9.11	100
8-OHNAAP	109 ± 91	1.89 ± 1.73	70	1.27	15– 300	0.30– 7.10	100
OHNAAPs (Total)	226 ± 183	4.12 ± 3.91	144	2.51	24– 648	0.49–16.20	
3-OHNP	5 ± 10	0.08 ± 0.17	< DL	< DL	< DL– 36	< DL– 0.54	18
6-OHNP	203 ± 117	3.90 ± 2.95	183	3.21	29– 420	0.8 –10.83	100
8-OHNP	137 ± 68	2.73 ± 2.07	137	2.20	28– 280	0.75– 7.30	100
OHNPs (Total)	340 ± 180	6.63 ± 4.96	344	6.06	58– 700	1.62–18.13	
1-NP metabolites (Total)	566 ± 290	10.75 ± 7.33	544	9.15	102–1348	2.71–33.70	

a) < DL; below detection limits. See the Results section, *b*) < QL; below quantification limits. The QLs were 0.30 pmol/l of urine (OHNAAPs) and 0.10 pmol/l of urine (OHNPs).

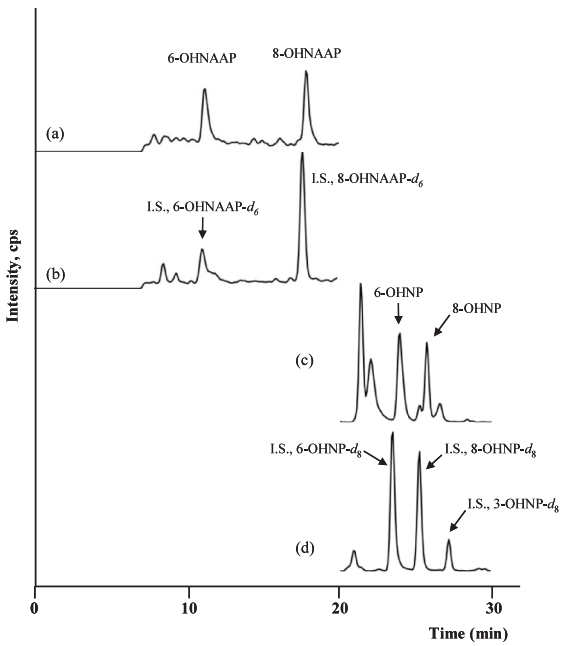

Fig. 3. Representative SRM Chromatograms Resulting from Human Urine Sample for Determining 1-NP Metabolites

(a) OHNAAPs (m/z 274 → 231), (b) OHNAAPs-d_6 (m/z 280 → 237), (c) OHNPs (m/z 262 → 232) and (d) OHNPs-d_8 (m/z 270 → 240).

human urine, in agreement with previous studies that predicted that these metabolites should be excreted into human urine. The 1-NP metabolite concentrations were determined in urine samples of healthy subjects who were non-occupationally exposed to 1-NP (DEP) and lived in Kanazawa, Japan. The use of Blue rayon, which has a high specificity for polycyclic aromatic compounds, has made it possible to selectively enrich the analytes from a large volume of urine (100 ml). Figure 3 shows typical Selected reaction monitoring (SRM) chromatograms of a subject's urine. The physiological components of the urine did not interfere with the identification and quantification of the analytes in the chromatograms. The concentrations of OHNPs and OHNAAPs found in spot urine samples are presented in Table 2. 6-, 8-OHNAAPs and 6-, 8-OHNPs were abundant in human urine, while 3-OHNAAP and 3-OHNP were hardly detected in the urine samples. Further studies are needed to examine the correlation between urinary 1-NP metabolites and personal exposure to PM and 1-NP in non-occupationally and occupationally exposed subjects.

CONCLUSION

Exposure to PAH and NPAH can be effectively evaluated by measuring urinary metabolites of these compounds in addition to atmospheric monitoring. We developed HPLC methods for determining OH-PAHs in human urine. The use of deuterated compounds as internal standards on HPLC with fluorescence detection allowed the PAH metabolites to be accurately and precisely quantitated. The developed method was used to estimate the exposure to PAHs from multiple routes in Thai subjects. Excretion of OHPAHs in rural villagers was significantly higher than that in taxi drivers and traffic police officers, and was comparable to that in occupationally exposed workers in advanced countries. This suggests that the rural villagers experience high-exposure to biomass smoke that includes PM with carcinogens such as PAHs. The urinary profile of OHPAHs can serve as biomarkers for multiple PAHs which reflect

the exposure to PAHs from the environment and human activities. In the future, it will be necessary to investigate the current status of PAH exposure in developing countries. On the other hand, much attention has focused on the development of biomarkers for NPAH exposure, especially for 1-NP as a proposed marker for DEP exposure. A highly specific and sensitive analytical method using LC-MS/MS was developed to determine urinary 1-NP metabolites. Our demonstration that 1-NP metabolites are present in human urine and our development of a method for detecting 1-NP metabolites should accelerate studies of the assessment of human exposure to DEP, 1-NP metabolism in human and polymorphisms of 1-NP metabolic enzymes.

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