Development of HPLC determination method of urinary hydroxy polycyclic aromatic hydrocarbons (OHPAHs) as biomarkers to estimate human exposure to PAHs

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学位授与の題目	Development of HPLC Determination Method of Urinary Hydroxy Polycyclic Aromatic Hydrocarbons (OHPAHs) as Biomarkers to Estimate Human Exposure to PAHs（ヒトの多環芳香族炭化水素(PAHs)曝露評価を目的とした尿中水酸化体(OHPAHs)をバイオマーカーとするHPLC分析法の開発）
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学位論文要旨

ABSTRACT

Several polycyclic aromatic hydrocarbons (PAHs) are toxic ubiquitous compounds, which are mainly formed through imperfect combustion of organic matters. Humans are daily exposed to PAHs from diesel exhaust particulate, cigarette smoke and many industrial processes. Human exposure to PAHs is a risk factor for the carcinogenicity as well as endocrine disruption, which is connected with their metabolism. To estimate the exposure amount of PAHs, it is therefore necessary not only to characterize distribution and occurrence of PAHs in the environment but also to quantify available and effective PAHs in human body because PAHs have multiple exposure routes. Thus, the biological monitoring of urinary metabolites of PAHs is useful to evaluate the exposure to PAHs, since OHPAHs are mostly conjugated as glucuronides and sulfates to be excreted. Single urinary 1-hydroxypyrene was popularly used as a biomarker.

First, I have developed two HPLC-FL methods, for the determining urinary 1-OHPyr and 2-OHFle as biomarker. By using this methods, the significant differences of urinary level

of two markers were found between non-smoker and smoker, Japanese and Thai group. For example, 25 times different were observed for 1-OHPyr in smoker (Thai > Japanese) and 15 times different in non-smoker (Thai >Japanese). 2-OHFle was a better marker of exposure to PAHs from cigarette smoking ($P < 0.01$; Japanese, $P < 0.001$; Thai). Next, multi-OHPAHs metabolites were selected to provide more comprehensive profile of exposure to PAHs.

An HPLC simultaneous determination method of ten OHPAHs, 1-, and 2-hydroxynapthalenes (1- and 2-OHNap), 2-hydroxyfluorene (2-OHFle), 1-, 2-, 3-, 4- and 9-hydroxyphenanthrenes (1-, 2-, 3-, 4- and 9-OHPhe), 3-hydroxyfluoranthene (3-Frt) and 1-hydroxypyrene (1-OHPyr) has been developed. The sample treatment involved enzymatically hydrolysis, following by the modification of double (Sep-Pak C_{18} and silica) solid phase extraction, elution with hexane/ethyl acetate (9:1, v/v) and finally separation on HPLC coupled with fluorescence detection. Deuterated 1-OHPyr was first synthesized by CYP450 and selected as a suitable internal standard. All OHPAHs were separate well on an alkylamide type reverse phase column, except for coeluted analytes (1- and 9-OHPhe). The method was precise and accurate for application study.

The developed method was applied to Japanese subjects lived Kanazawa city and Thai subjects lived Chiang Mai province (rural villagers, red-taxi drivers and traffic-policemen). The results (mean concentrations of each 10 OHPAHs) indicated that Thai subjects exposed to PAHs at the higher level than Japanese. And their exposure levels also higher in other countries (Canada, The Netherlands, Sweden).

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are major environmental pollutants, which are the risk factor for carcinogenicity and endocrine disruption in human. After PAHs enter the body through several routes (inhalation, digestion and dermal penetration) they are

readily and predominantly metabolized to hydroxy PAHs (OHPAHs) as well as glucuronides and sulfates.

The biological monitoring of the human exposure to PAHs is very important to reflect the exposure level. Therefore, this thesis proposed urinary hydroxyl PAHs having 2-4 rings in human urine as useful biomarkers. At the beginning, two HPLC-FL methods have been developed to quantify urinary 1-OHPyr and 1-OHFle. The method used deuterated 1-OHPyr and 1-OHFle, respectively, as internal standard. Deuterated 1-OHPyr and 2-OHFle were separated well prior to their non-deuterated ones on alkylamide-type reversed phase columns (Chapter 1 and 2). The methods were used to determine the amount of them in urine of Japanese and Thai subjects (smokers and non-smokers) (Chapter 3). Finally, an HPLC-FL method for simultaneous determination of 10 OHPAHs having 2-, 3- and 4-rings in urine has been developed. Multi-biomarkers provides more comprehensive profiles data of PAHs' exposure. Applications were examined in Thai subjects (rural villagers, red-taxi drivers and traffic-policemen) (Chapter 4).

RESULTS AND DISCUSSION

The detection limits of 1-OHPyr and 2-OHFle by the developed HPLC-FL determination methods were 1.0 and 0.03 nmol/L (S/N = 3), respectively. The conjugates of PAHs in urine sample were completely hydrolyzed to free OHPAHs for 2 h with β-glucuronidase/ aryl sulfatase, then OHPAHs were cleaned up solid phase extraction using a Sep-Pak C_{18} cartridge. The methods were applied for the determination of urinary 2-OHFle and 1-OHPyr of smokers and non-smokers who lived in Japan and Thailand not occupationally exposed to PAHs. The mean concentrations of 2-OHFle and 1-OHPyr of smokers (0.46 and 1.32 μmol/mol creatinine) were significantly higher than those of non-smokers (0.13 and 0.41 μmol/mol creatinine). For Japanese subjects, the difference

between smokers and non-smokers was more significant of 2-OHFle than of 1-OHPyr, suggesting the larger intake amount of PAHs in the vapor phase. 2-OHFle was not closely linked with smoking in the Thai subjects, possibly because the background level of the exposure to PAHs was relatively high in Thai subjects. However, the large variations in individuals, both non-smoker and smoker were found (Table 1). Finally, 10 OHPAHs were separated on an alkylamide-type reversed phase column, except for 1- and 9-hydroxyphenanthrenes which were coeluted (Figure 1). The developed method showed good linearity of calibration curves (r^2 ranged from 0.996 to 0.999). Intra and inter assays of urine samples treated enzymatically showed good reproducibility (RSD < 17%). The limits of detection (S/N = 3) were in the range from 0.05 to 32 μg/L. This method was usefully applied to the determination of urinary ten OHPAHs in Thai subjects and the result indicated the differences of exposure to PAHs in individual and between the countries.

Table 1 Urinary concentrations of 2-OHFle and 1-OHPyr (μmol/mol creatinine) in Japanese and Thai subjects

Characteristic	Smoker		Non-smoker		Ratio (Smoker/Non-smoker)	
	Japanese	Thai	Japanese	Thai	Japanese	Thai
Number	10	7	7	6		
(Male)	(10)	(5)	(4)	(3)		
Age,	48	54	33	34		
Mean (Range)	(23-69)	(32-76)	(22-51)	(26-47)		
2-OHFle,						
Mean±S.D.	0.26 ± 0.16**	0.75 ± 0.15***	0.04 ± 0.02	0.22 ± 0.15	6.5	3.4
CV (%)	61.5	20	50	68		
Range	0.06 - 0.51	0.50 - 1.01	0.01 - 0.06	0.05 - 0.46		
1-OHPyr,						
Mean±S.D.	0.12 ± 0.13	3.03 ± 1.91*	0.06 ± 0.08	0.91 ± 0.59	2.0	3.3
CV (%)	108	63	133	65		
Range	0.02 - 0.45	1.00 - 5.76	0.01 - 0.22	0.20 - 1.72		
Ratio (2-OHFle/						
1-OHPyr)	2.2	0.25	0.67	0.24		

* Significantly different from non-smoker ($P < 0.05$), ** Significantly different from non-smoker ($P < 0.01$),
*** Significantly different from non-smoker ($P < 0.001$)

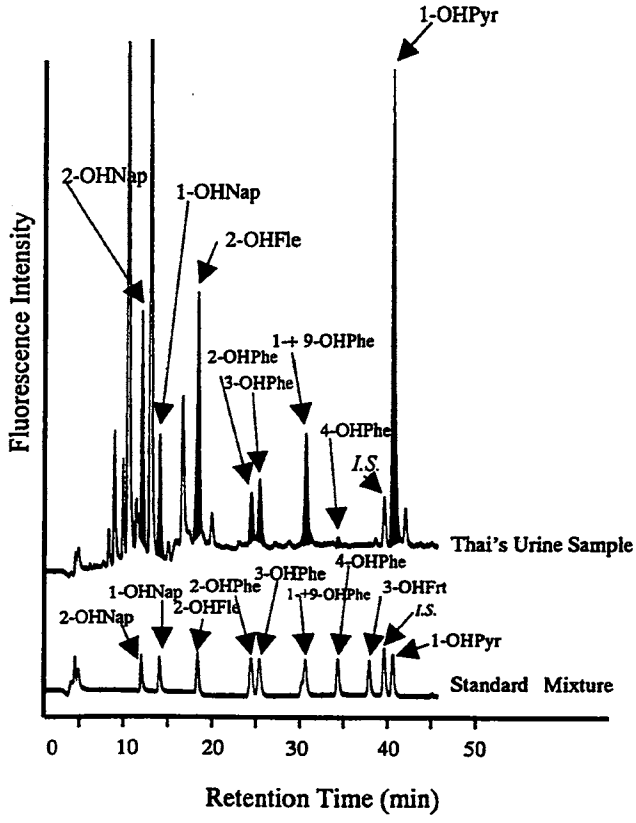

Figure 1 HPLC chromatograms of a Thai subject and a standard mixture (10 OHPAHs)

学位論文審査結果の要旨

[審査経過]平成16年1月28日の第1回審査委員会で審査方針を決定した。基礎学力を確認し、各委員による面接と諮問を行い、2月2日に口頭発表（最終試験）を行った。終了後に開催した最終審査委員会において協議した結果、次の結論を得た。

[審査結果]今日の大気汚染は、ぜん息や肺がんの増加を招き、内分泌撹乱作用との関連も疑われる等、深刻化している。その原因物質の一つとして、石炭や石油等の化石燃料の不完全燃焼で発生する多環芳香族炭化水素（PAH）類が指摘されている。本研究は、PAHが体内で水酸化体（OHPAH）、更にグルクロン酸や硫酸抱合体に代謝されて、尿や胆汁中に排泄されることに着目し、尿中の一連のOHPAHをバイオマーカーとするHPLC分析法を開発した。これにより、自動車排ガス、工場や暖房の排煙、タバコ煙、食物等の多様な発生源に対応した個人のPAH曝露を正確に測定できるようになった。次いで、開発した方法を応用して、喫煙のバイオマーカーには1-ヒドロキシピレンより2-ヒドロキシフルオレンの方が適すること、日本の都市住民よりタイの山間部住民のPAH曝露量が著しく多く、これは後者が住居内で使用する薪の燃焼煙に由来すると推定されること等の事実を明らかにした。本研究は、有用なPAHの個人曝露評価法を提供しており、多方面でその活用が大いに期待できる。よって、本審査委員会は博士（薬学）に値すると判定した。