ORIGINAL ARTICLE

HPLC Analysis of 1-Hydroxy Pyrene as a Biomarker of Polycyclic Aromatic Hydrocarbon in Urine Samples of Cigarette Smokers

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ABSTRACT

Background: High performance liquid chromatography (HPLC) with fluorescence detection was used to enhance a method for the quick and sensitive measurement of 1-hydroxypyrene in human urine. The triethylamine was added to the extracts of urine prior to liquid chromatography testing and the limit of detection was reduced. The analysis took about 16 minutes for each run in C18 column with a guard column.

Aim: This approach is used to study a HPLC method for a rapid and sensitive analysis to analyze 1-hydroxypyrene and other PAH compounds in 30 urine samples from cigarette of non-smokers, light smokers and heavy smokers who had non-occupational exposure to polycyclic aromatic hydrocarbons. cigarette smoking

Results: The average amounts of urine 1-hydroxypyrene in non-smokers were 0.13 ± 0.02 ng/ml, and for light smokers were 0.32 ± 0.06 ng/ml, and for heavy smokers were 0.96 ± 0.15 ng/ml. The correlation between urine 1-hydroxypyrene levels of cigarette smokers and non-smokers was substantial. The results shows that there is a statistically significant difference between the three groups ($p < 0.05$).

Conclusion: The findings of this study support the use of urine 1-hydroxypyrene as a biomarker for PAH exposure from cigarette smokers in humans. In future studies, include the other individual factors such as urban air pollution, occupational exposure, and food contamination will be useful to determine the all factors affecting the amount of PAH in human.

Keywords: Polycyclic aromatic hydrocarbons PAH; Biomarker; 1-hydroxypyrene; Cigarette smokers; HPLC.

INTRODUCTION

PAHs (polycyclic aromatic hydrocarbons) are chemical molecules with aromatic rings that are one of the most common organic contaminants. Some are recognized or suspected carcinogens, and they are typically produced by incomplete combustion of carbon-based fuels including wood, coal, diesel, and tobacco. They're also found in comets, meteorites, volcanoes and forest fires (1). It is necessary to apply suitable biomarkers for the quick screening of sensitive populations and to put into place suitable preventative measures in order to be able to forecast which populations are at risk from PAH exposure (2).

Biomarkers are alterations in cells or molecules that can be measured in biological media such as human tissues, cells, or fluids. These alterations might be cellular, biochemical, or molecular in nature. It is considered an indication of both healthy and disease-causing activities in biological systems in addition to being indicators of exposure, effect, or vulnerability (3). Measurement of endogenous chemicals or factors indicative of a disease process, as well as the application of pharmacodynamic and genetic markers in evidence-based laboratory medicine and therapy are all covered under biomarkers of illness(4).

Internal exposure to medicines and other substances is detected and measured using biomarkers of exposure (5). Measures of endogenous substances or parameters suggestive of pathological or physiological changes, both toxicodynamic and pharmacodynamic, occurring from exposure to medications and other chemicals are called biomarkers of response. Susceptibility biomarkers are genetic variables that affect susceptibility to drugs and other substances (6).

There are about 10.000 different polycyclic aromatic hydrocarbons compounds. The vast majority of PAHs are poorly soluble in water and hence persist in the environment by adhering to earth and sediment. Large amounts of PAHs are produced by primary metals processing, petroleum refining, and the paper and plastics industries(7). Since they come from so many different places, they may be found all over the place. Human PAH exposure may come from a variety of places, including the workplace, the environment, cigarette smoking, and food. PAHs may be absorbed by the lungs, gastrointestinal system, and skin. Most of PAH are recognized as carcinogens as pyrene (8). The following polycyclic aromatic hydrocarbons (Fig. 1) are generally regarded as being dangerous to human health in such way: benzo(a)pyrene, benzo(j)fluoranthene, anthracene, dibenzo (a, h) benzo(b)fluoranthene, and benzo(k) fluoranthene. The International Agency for Research on Cancer (IARC) identified eleven PAHs as carcinogenic in experimental animals (9).

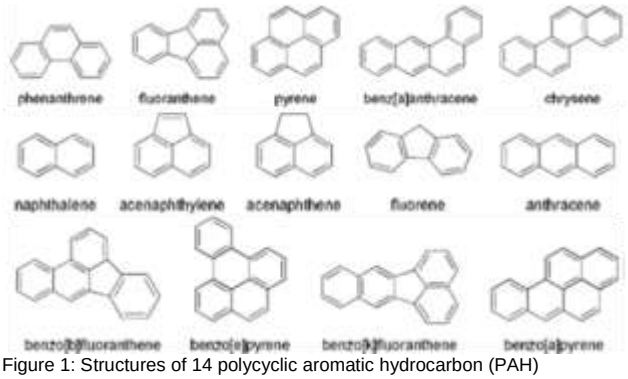

Figure 1: Structures of 14 polycyclic aromatic hydrocarbon (PAH)

The smoke from cigarettes contains around five hundred distinct PAHs(10). Cigarette smoke is known to include a range of potentially harmful components, including PAHs. The list of 93 hazardous and potentially harmful ingredients (HPHCs) found in tobacco products and tobacco smoke was compiled by the Food and Drug Administration (FDA) of the United States of America and published in the Federal Register. PAHs make up sixteen of these chemicals in total(11). In cigarette smoke, there are parents of PAHs, as well as a number of methylated PAHs with three or more fused rings such as naphthalene, acenaphthylene, acenaphthene, fluorene benzo (a) pyrene (12). Evidence shows that its application locally or inhalation causes lung cancers(13). Pyrene appears in carbon black, motor exhaust, cigarette, and is a common and typical component of ambient PAH mixes. Preceding research suggests that 1-hydroxypyrene in the urine may originate from a wide range of sources, including food, the environment, and passive smoking (14).

1-Hydroxypyrene, a metabolite of pyrene (Fig. 2), has been investigated as a most reliable biomarker and a general monitoring tool of pyrene for the determination of the degree to which humans are exposed to PAHs (15). The reason is that it appears to be a useful short-term marker and is the primary product of pyrene metabolism with 90% of its metabolites (16).

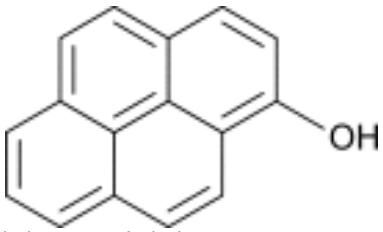

Figure 2: Chemical structure of 1-hydroxypyrene.

Depending on the cigarette brand, filtration and smoke type the PAH yields of commercially available typical American blended cigarettes are extremely variable, with the value of total PAHs in mainstream smoke varying from 0.139 to 2.6 µg per cigarette (17).

MATERIALS AND METHODS

Sample collection of urine: Adults who did not have any known PAH exposure at work provided 30 urine samples for this study before having breakfast. The participants were males' university students with the age between 18 – 26 years old. They were divided into three groups with ten samples for each group. They were non-smokers (0 cigarette per day), light smokers (1-10 cigarettes per day) and heavy smokers (11-25 cigarettes per day). The smokers had been smoking for not less than a year.

Chemicals: Concentrated HCl from Merck Ltd; Sodium chloride from Chem Center company; 1-hydroxypyrene standard from Chromsystems; Dimethyl sulphoxide (DMSO) from Carl Roth; Methanol from Sigma-Aldrich; Triethylamine from Tedia; Diethyl ether from Sigma-Aldrich. All other chemicals and reagents were of analytical grade.

Materials and method: Reverse phase HPLC well plate autosampler (Model 1100 WPALS G1367A; Agilent Technologies, Wilmington, DE) is used in this analysis. The injection loop was 20-µl with fluorescence detector: excitation (λex) 242nm and emission (λem) 389 nm.

Symmetry column C18 (3,5 µm, 150×4.6 mm i.d.), with a guard column set to 45 °C to shorten the retention time of the analyte (18).

The flow rate was set to 1.0 ml/ minute, and the column of the analysis was heated in water bath to 40 °C. Solvent A was 40% methanol in distilled water while Solvent B was 100% methanol. The analysis program of the mobile phase was gradient in which for the first three minutes, 80% of the solvent A and 20% of the solvent B run. Then the ratio of the mobile phase was changed to 20% solvent A and 80% solvent B for 12 minutes gradually, then to 100 % solvent B for 4 minutes.

Pretreatment of urine samples: 1-Hydroxypyrene has been identified as the major phase I metabolite of pyrene. In order to hydrolyze the urine samples by acid hydrolysis, 2 mg of sodium chloride and 2 ml of hydrochloric acid were added to 20 ml aliquot of the urine sample(19). The mixture was then heated to 60 °C for one hour. Following the heating step, the shaker was used to extract the hydrolyzed sample with 20 ml of diethyl ether. At a temperature of 37 °C and using a slow-moving stream of nitrogen gas, the organic phase was allowed to completely evaporate until it was dry. Then 1 ml of MeOH was used to reconstitute the residue (20).

Blank urine samples were prepared with 200 ng/ml of 1-hydroxypyrene standard and hydrolyzed in the same way as urine samples. For the recovery study, three blank samples were prepared to the concentration of 2 ng/ml, 1 ng/ml and 0.5 ng/ml. The standard solution makes up to 2 ng/ml of 1-hydroxypyrene in dimethyl sulfoxide (DMSO). Triethylamine with 1 µl was added to 200 µl of the extract for HPLC analysis in order to prevent the secondary retention impact and improve the resolution of 1-hydroxy-pyrene and the signal response. This is because triethylamine might challenge 1-hydroxypyrene for the interaction with the column silanol mediated by hydrogen bonding and ion exchange (21).

RESULTS

The urinary 1-hydroxy-pyrene levels are classified based on the number of cigarettes smoked each day as non-smokers (0 cigarette per day), light smokers (1-10 cigarettes per day), or heavy smokers (11-25 cigarettes per day). For the non-smokers, we didn't consider if they were passive smokers or not in this study.

In order to determine the limit of detection LOD and limit of quantification LOQ, samples with known concentrations of 1-hydroxy-pyrene were analyzed, and the setting was determined to be the lowest possible level at which the concentration can be accurately detected and quantified.

The limit of detection LOD (signal to noise ratio S/N>3) for 1-hydroxy-pyrene before the addition of triethylamine was 0.13 ng/ml. However, after the addition it became 0.09 ng/ml. The limit of quantification LOQ was 0.15 ng/ml.

The mean concentration of 1-hydroxypyrene in the urine of nonsmokers were 0.13 ± 0.02 ng/ml, and for light smokers were 0.32 ± 0.06 ng/ml, and for heavy smokers were 0.96 ± 0.15 ng/ml. The results shows that 1-hydroxy-pyrene urinary levels in smokers were positively correlated with the number of cigarettes smoked per day, as stated by the smokers themselves (Figure 3).

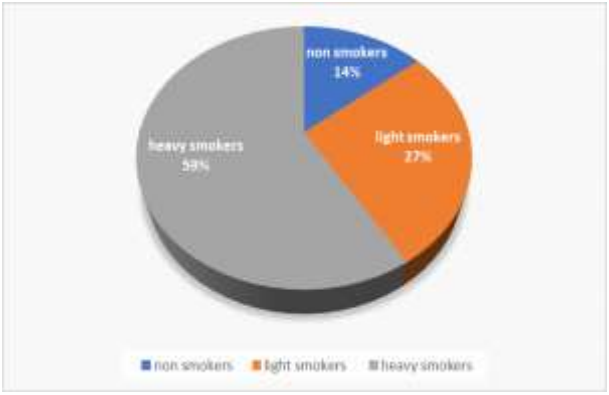

Figure 3: Correlation of urinary 1-hydroxypyrene of non-smokers, light smokers and heavy smokers with different daily cigarette consumption

The selectivity of the process was evaluated by adding a trace amount of 1-hydroxy-pyrene to a sample of blank urine and monitoring the retention time which was 10.9 min (Figure 4).

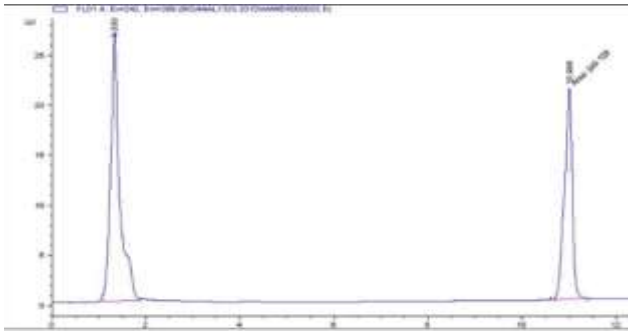
Figure 4: The chromatogram of 1-hydroxypyrene

Urinary 1-hydroxypyrene levels can be somewhat variable depending on demographic subgroup, employment, and environmental factors such as air quality.

When the median 1-hydroxy-pyrene levels of nonsmokers, light smokers, and heavy smokers were examined, there were statistically significant differences between these groups. In addition, the median of 1-hydroxy-pyrene excretion of heavy smokers was significantly higher than that of nonsmokers and light smokers ($p<0.05$) (Table 1).

Table 1: Concentration of 1-hydroxy pyrene in urine samples of non-smokers, light smokers, and heavy smokers according to smoking habits for ten samples of each type.

	Non Smoker (ng/ml)	Light Smoker (1-10 cigarettees / day) (ng/ml)	Heavy smokers (1-25 cigarettees / day) (ng/ml)
Mean	0.13	0.32	0.96
SD	0.02	0.06	0.15
Median	0.13	0.31	0.9
Min	0.08	0.23	0.82
Max	0.17	0.42	1.2
RSD %	18 %	19 %	15 %
Confidence Level(95.0%)	0.017	0.044	0.108

The reliability was determined using the addition method of standard. One-milliliter duplicates of the control urine sample were spiked with different concentration of 1-hydroxy-pyrene 0.05, 0.25, 0.5, 1 and 1.5 ng/ml. The original concentration of 0.06 ± 0.01ng/ml of 1-hydroxy-pyrene was used as the baseline. The repeatability results of 1-hydroxypyrene were achieved with good values. In order to check the linearity of our results, a standard curve calibration was performed. There is a linear relationship between different concentration of 1-hydroxy-pyrene. Further, the average regression equation was obtained by running a series of standard solutions that were prearranged in a blank urine. The average regression equation was $y = 731.62x + 144.93$ with $R^2 = 0.999$ (Figure 5).

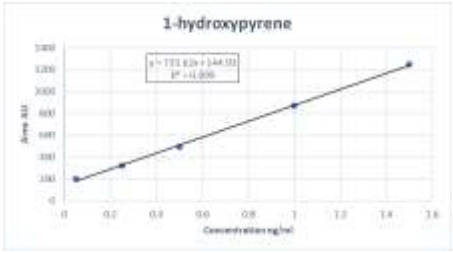

Figure 5: Chromatogram of 1 hydroxy pyrene

When compared to non-smokers, the level of 1-hydroxypyrene in the urine of light smokers was 2 times higher than that of non-smokers, while for heavy smokers was 7 times higher than that of non-smokers. These findings demonstrate a robust dose-response relationship between urine 1-hydroxypyrene concentration and the quantity of cigarettes smoked by the non-occupationally exposed group to PAH (r^2 =0.999, Confidence interval: 95.00%).

The physiological components of the urine did not interfere with the 1-hydroxypyrene peak in the chromatogram. Other PAHs, such as pyrene, benzopyrene, chrysene and anthracene, did not interfere with the analysis either (Figure 6).

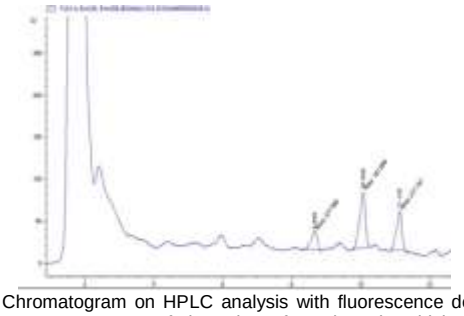

Figure 6: Chromatogram on HPLC analysis with fluorescence detection of different PAH components of the urine of smokers in which 1-hydroxy-pyrene is not overlapped with other PAH component

CONCLUSION

An improved HPLC technique for a quick and sensitive determination of urine 1-hydroxypyrene is presented in this paper. This sensitive technology makes it possible to obtain data that can be used to evaluate the baseline values of PAH as well as differentiate between different exposures such as secondary smoking. When using genuine samples, a distinct dose-response association between cigarette smoking and urine 1-hydroxypyrene excretion was identified. Specifically, this relationship was observed to be positive. The findings of this research provide credence to the concept of using 1-hydroxypyrene in urine as a human biomarker for PAH exposure.

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