Solid Phase Extraction for 1-Hydroxypyrene as a Biomarker of Occupational Exposure to PAHs Prior to High Performance Liquid Chromatography

Shahtaheri, Sayed Jamaleddin*[+]

School of Public Health, Center for Environmental Research, Medical Sciences / University of Tehran, Tehran, I.R. IRAN

Ibrahimi, Leila; Golbabaei, Farideh; Hosseini, Mostafa; Fouladi Dehghi, Behzad

School of Public Health, Medical Sciences / University of Tehran, Tehran, I.R. IRAN

ABSTRACT: *Urinary 1-hydroxypyrene is frequently used as a major metabolite and biological indicator of the overall exposure to polycyclic aromatic hydrocarbons. In this study, solid phase extraction was appropriately conditioned with regard to sample pH, sample concentration, loading flow rate, elution solvent, washing solvent, sample volume and sorbent mass. Octadecyl silica cartridge (C_{18}) was used as solid phase adsorbent and showed to be an efficient phase in simplifying sample preparation for1-hydroxypyrene. Methanol extracted analyte from spiked urine gave a clean sample for reverse-phase HPLC-florescence. In the developed solid phase extraction conditions (sample pH: 5, sample concentration: 10 mg/l, washing solvent: distilled water, eluent: methanol, sample volume: 200 ml sample flow rate: 10 ml/min), the extraction recovery exceeded 99.96%, achieving detection limit of 0.02 mg/l. The extraction factors (sample pH, sample concentration, washing solvent , eluent , sample volume and sample flow rate), were evaluated statistically and also the procedure was validated with three different pools of spiked urine samples at low, medium , and high sample concentrations and showed a good reproducibility over six consecutive days as well as six within-day experiments. All coefficients of variations were less than 3.1%. Finally, urinary 1-hydroxypyrene of industrially exposed workers was also measured, using the appropriate conditions obtained in this study, in which, the amount of the compound of interest in the total exposed subjects was significantly higher than those of non-exposed.*

KEY WORDS: *SPE, Sample preparation, Polycyclic aromatic hydrocarbons (PAHs), 1-Hydroxypyrene, HPLC.*

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are considered as carcinogen compounds formed mainly by the incomplete combustion of organic materials such as fossil fuels [1,2], industrial activities such as aluminum

* *To whom correspondence should be addressed.*
+ *E-mail: shahtaheri@tums.ac.ir*
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electrolysis, foundries, petrochemical industries and oil refineries [3,4], diet (broiled and smoked food), smoking habits [5,6], and medication [7]. These compounds are absorbed through the respiratory and gastrointestinal tracts as well as through the skin [1]. Measurement of PAHs metabolites in urine can be used as a means of assessing recent exposure to these compounds [8-11].

In biological matrices either parent compounds or their metabolites mostly are present at a trace level, causing major problems in their determination stages [12-14]. Therefore, an essential need for sensitive and selective techniques for the analysis of trace chemicals in environmental and biological matrices have been clearly recognized [15-18]. Although the use of detection system has improved the selectivity of analytical procedures, these sensitive and selective methods require expensive equipments; moreover, they many not be available in most laboratories. Consequently, sample pre-treatment procedures which can be performed in any laboratory have been developed to simplify analytical approaches as these methods reduce expenses too [19-21].

Although many analytical methods still use liquid-liquid extraction (LLE) to perform sample clean-up [22,23], in this procedure, large volumes of organic solvents, having undesirable environmental concerns are used as well as problems associated with the technique to be automated. In addition, the recovery obtained from LLE is not often suitable and reproducible. While, solid phase extraction (SPE) methods using silica or bonded silica has proved useful in simplifying sample preparation prior to HPLC-florescence detection (FD) [24]. Isolation and purification of the compound of interest can be achieved in a short time and only low volumes of organic solvents are used during the application of this method. The use of commercially available low cost vacuum manifolds allows many samples to be processed simultaneously. Furthermore, complete automation of procedures based on SPE is now possible using commercially available instrumentations [25,26]. A wide variety of phases from many suppliers based on silica are also available including reversed phase, normal phase, ion exchange and mixed mode phases [26,27]. The phases can be selected depending on chemical nature of the analyte. This study was aimed to achieve the appropriate factors necessary for development of efficient procedure for 1-hydroxypyren

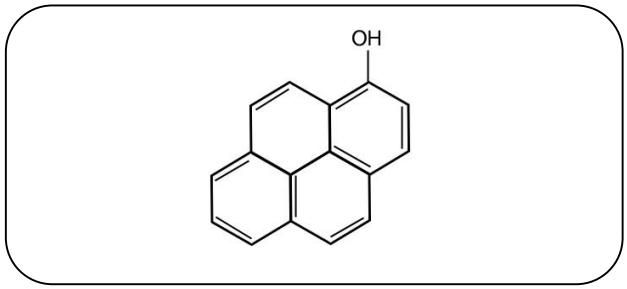

Fig. 1: Chemical structure of 1-OHP.

(Fig. 1) as the main metabolite of pyrene in urine [28,29], leading to a simple method of solid phase extraction [30] and finally facilitating assessment of occupational exposure to PAHs.

EXPERIMENTAL

Materials

1-hydroxypyrene standard was obtained from Sigma-Aldrich chemie (GmbH, Reidstr, Steinheim, Germany) methanol, ethanol and acetonitril were all HPLC grade and purchased from Merck (Darmstadt, Germany), water was double distilled and purified using the Purite system. Standard buffer solution at three pH values [4.00 $\pm$ 0.02, 7.00 $\pm$ 0.02, and 10 $\pm$ 0.02] were also purchased from Merck (Darmstadt, Germany). Octadecyl (C18) in 100 and 500 mg cartridges were obtained from Macherey-Nagel (Darmstadt, Germany) and used for solid phase extraction procedure.

Apparatus

A Vac-Elute vacuums elution system was used for retention and elution processes of C18 silica cartridges. A digital pH meter (Hanna, Singapore) was used for pH measurement. Quantitative liquid transfers were performed with pipette (Socorex, Germany). The HPLC apparatus consisted of a k-1001 single piston pump Knauer, (Socorex, Germany). The analytical column used was an RP-C18e 15×4.6 mm Merck-KuaA, (Darmstadt, Germany). Detector used was florescence RF-10AXL Knauer, (Darmstadt, Germany).

Sample preparation procedure

In this study, SPE using bonded silica (C18-100 mg) has been conditioned with regard to sample pH, sample concentration, elution solvent, elution volume, sorbent mass, sample loading flow rate and washing solvent.

The cartridges were conditioned with 6 ml methanol followed by 3 ml HPLC water. Care was taken to prevent the cartridges from drying. The samples were then passed through the column. Then, the column was washed using 3 ml of different solvents. Finally 1-hydroxypyrene was eluted from the column with 1 ml of different solvents. The extracts were then analyzed by HPLC- FD.

Chromatographic conditions

The pump was operated at 0.8 ml/min; florescence detector wavelengths for absorption and emission were set at 242 and 388 nm, respectively. Mobile phase consisted of 88% methanol and 12% water and injection volume was 20 µl. The analytical column was C18 Reversed phase 15×4.6 mm Merck-KuaA, (Socorex, Germany) and the ambient temperature was used for chromatographic system. Under these conditions, 1-OHP was eluted and detected in about 5 minutes (Fig. 2). In this study peak height was used as detector response and extraction recoveries were calculated by comparison of peak height in the chromatogram of extracts with those in the chromatogram of standard solutions prepared in the same solvent as following.

% Recovery = (sample peak/standard peak height) ×100

RESULTS AND DISCUSSION

In order to condition SPE, several factors influencing the retention and elution process were evaluated. First, the sample pH was evaluated for extraction recovery of 1- OHP. After conditioning the C18-100 mg column with 6 ml methanol followed by 3 ml HPLC-grade water, 1 ml of 1-OHP standard solution (10 µg/l) at different pH values of 3, 4, 5, 6 and 7 was applied. The column was then washed by pure water and retained analyte was eluted by 1 ml of methanol. Table 1 shows the recovery percentage of 1-OHP obtained for samples with different pH values. From the results given in table 1, it was concluded that, the efficient recovery was obtained from C18-100 mg using sample pH=5. Therefore, this pH value was used for further experiments.

In order to evaluate the effect of sample concentration on SPE performance, different concentrations of 1-OHP ranged from 0.2 to 20 µg/l mentioned in table 2 were prepared using 1 ml HPLC grade water. Ideally, the extraction recovery should not be sample concentration dependant. In other words, in a useful and efficient method, there should not be a significant difference in recovery over the expected concentration range of the compound of interest. Table 2 gives the recoveries obtained after passing 1 ml sample at different sample concentrations followed by elution with 1 ml methanol. As can be seen, at the ranges 10 to 20 µg/l, there is no significant difference in the recovery and in both cases the recovery is in the acceptable margins. However, because exposed individuals to PAHs show high concentrations of 1-OHP (see Table 9), relative poor recoveries obtained at very low concentrations is not much critical.

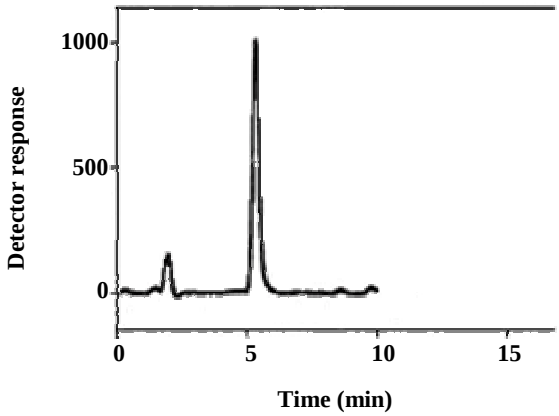

Fig. 2: Chromatogram of 1-OHP, mobile phase, 0.88% methanol/12% water; flow rate, 0.8 ml/min; analytical column, C_{18} reversed phase; flurocence detector at 242 and 388 nm; injection volume, 20 µl; ambient air.

Another stage of sample preparation process was to evaluate the effect of washing solvent type on the recovery of 1-OHP. Three solvents were screened for their ability to produce appropriate washing of the interferences from the sorbents. The washing solvents were deionized water, 1% solution of acetic acid, and 20% methanol + 80% water. The same sequence of conditioning, washing, and elution were used as in previous section. The results of this process are shown in table 3.

As can be seen, the best washing solvent is water, however, adding 20% MeOH can help to remove closely related interference compounds from the real samples. Another experiment performed during this study was evaluation of the eluent strength on 1-OHP recovery. Four solvents were screened for their abilities to produce

optimum elution of the retained 1-OHP from C18-100 mg. They were methanol 100%, methanol 80%+water 20%, methanol 70%+water 30%, and methanol 50%+ water 50%.

The same sequence of conditioning, loading the sample washing, and elution were used as the other stages. The results of these processes are shown in table 4. As shows in table 4, for different eluents, using methanol 100% have given the best results. By increasing the amount of water in eluent the recovery decreases significantly. It proves that, the analyte (1-OHP) can be well extracted using higher percentage of organic solvent. 1-OHP is a representative lipophyl metabolite of the PAH compounds, so, it is well established that, 100% methanol as a common organic solvent can elute it better [2].

In order to screen the effect of sample volume on SPE performance, different sample volumes were tested. They were 1, 10, 50, 100, 150, 200, 300, and 500 ml. The sequence of conditioning and loading the samples and washing were used. To prepare different volumes of samples, 1 ml sample at concentration of 10 µg/l was diluted in different volumes, so that, the amount of analyte in samples was kept constant. The results of this stage of the study are presented in table 5.

Enrichment of the analyte in SPE is achieved by applying large volumes of sample and eluting the analyte in a minimum volume of eluent. The eluent volume must be just sufficient to elute the compound of interest from the sorbent.

Also, loading large amount of sample has two critical aspects. On one side, compound of interest can saturate the sorbent and breakthrough will occurred. On the other side, sample itself can wash out the analytes trapped in the sorbent, resulting in the lower recoveries. So, in loading large sample volumes these points should be kept in mind. In this study, samples up to 200 ml could be safely used without significant breakthrough or washing the trapped analyte. However, it is clear that the best recoveries could be gained by loading volumes up to 150 ml. So, this method is capable of concentrating trace amounts of 1-OHP from volumes of nearly 200 ml.

The next stage of the experiment was to evaluate the effect of sample loading flow rate on the recovery of 1-OHP from C18-100 mg. Different flow rates chosen

Table 1: The recovery percentage of 1-OHP obtained at different sample pH values.

Sample pH	Recovery (%) Mean ± SD, N=5
3	15.81 ± 1.47
4	79.79 ± 3.81
5	92.06 ± 1.67
6	46.16 ± 3.5
7	52.15 ± 2.50

Table 2: The recovery percentage of 1-OHP using different sample concentration (sample volume: 1 ml).

Sample Concentration (µg/l)	Recovery (%) Mean ± SD, N=5
0.2	64.34 ± 2.17
2	68.99 ± 1.02
10	90.94 ± 1.89
20	89.30 ± 2.34

Table 3: The recovery percentage of 1- OHP using different washing solvents.

Washing Solvent	Recovery (%) Mean ± SD, N=5
Water	98.3 ± 1.11
Acetic acid 1%	60.32 ± 1.00
80% Water + 20% MeOH	91.78 ± 1.16

Table 4: The recovery percentage of 1- OHP using different eluents.

Eluent	Recovery (%) Mean ± SD, N=5
MeOH 100%	99.10 ± 0.87
MeOH 80% + H2O 20%	77.80 ± 2.31
MeOH 70% + H2O 30%	60.80±1.50
MeOH 50% + H2O 50%	54.00 ± 0.76

Table 5: The recovery percentage of 1- OHP using different sample volumes (sample concentrations: 10 µg/l).

Sample Volume (ml)	Recovery (%) Mean ± SD, N=5
1	99.92 ± 0.07
10	99.94 ± 0.05
50	98.50 ± 1.06
100	98.00 ± 1.36
150	88.93 ± 2.14
200	85.97 ± 1.31
300	43.62 ± 1.72
500	25.01 ± 1.17

were 1, 2, 5, and 10 ml/min (volume: 100 ml, concentration: 10 µg/l). The cartridge was conditioned with 6 ml methanol followed by 3 ml water, then, the sample was loaded.

The cartridge was then washed with 3 ml water and the analyte was eluted by 1 ml of methanol. The results of these experiments are shown in table 6. A proper sample loading flow rate means to give enough time to the sorbent surface and analyte to be interacted to the sorbents sufficiently.

On the other hands, preparation of a sample should be done in as short time as possible, so, the sample loading flow rate must not be too fast as it will not allow the analyte to be adsorbed to the sorbent. It is obvious that, in all applied sample flow rates of 1, 2, 5, and 10 µg/l, the recoveries are in acceptable ranges, however, the recovery trends depend on the aim of study; each desirable flow rate could be used, leading to acceptable results.

Another experiment was performed to evaluate the effect of sorbent mass on the recovery of 1-OHP from C18 sorbets. Two C18 sorbents with different sorbent masses of 100 and 500 mg were used. The results of these stages of experiments are shown in table 7. When an appropriate SPE method is used efficient recovery can be achieved for C18-100 mg. While, in case of C18-500 mg, the recovery is poor.

It seems that, volume of methanol used in conditioning stage of C18-500 mg was not sufficiently enough to make the sorbent fully conditioned, also, when the sorbent mass is increased, the volume of eluent should be increased too. So, in such research, using fewer amounts of organic solvents and the sorbent mass could be desirable as the expense as well as using hazardous organic compounds is reduced.

In order to determine the method applicability, it was necessary to be validated. To do so, day-to-day and within-day reproducibility were determined. Spiked urine can be a suitable model as it may contain interfering constituents similar to the real sample [31, 32], therefore, it was used for method validation experiments. Spiked samples of 10 ml 1-OHP were used for extraction followed by HPLC-FD determination. Linear standard curve (for extracted samples) over the range of 0-20 µg/l were obtained each day [n=6] with correlation coefficient of 0.996 and greater. The extraction procedure was reliable and reproducible from day-to-day and within-day. Table 8 shows the results obtained in method reproducibility experiments.

The method was also successfully applied to measure urinary concentration of 1-OHP in the real samples of industrially exposed workers (exposed: N=40, non-exposed: N=22). The results of these experiments are presented in table 9.

As it can be seen, the amount of urinary 1-OHP in the total exposed subjects is significantly higher than non-exposed i.e. 2.38±0.56 µmol/mol creatinine compare to 0.63±0.27 µmol/mol. Also, based on the obtained results, 1-OHP, in both smoker and non-smoker individuals are high in exposed workers compare to the industrial non-exposed workers.

However, detection of a trace residual amount of 1-OHP in non-exposed persons is most probably due to the air pollution caused by the motor vehicles incomplete combustion. The heavy city traffic system can enforce the public environmental exposure too.

CONCLUSIONS

The developed method is promising to be suitable for evaluation of PAHs metabolites and also for other closely analogue biomarkers present in biological samples. This applicability is based on the proper results obtained for CV% (less than 3.1%) in assessing both day-to-day and within-day reproducibility experiments (see table 8).

It is concluded that, appropriate conditions achieved from this study can be used in simplifying sample preparation when a trace residue analysis of PAHs metabolites is needed. Also, applicability of this appropriate method for the real sample promises that, a useful method has been developed for evaluation of workers who occupationally and industrially are exposed to PAHs.

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Table 6: The recovery percentage of 1- OHP applying different sample loading flow rates.

Flow rate (ml/min)	Recovery (%) Mean ± SD, N=5
1	97.40 ± 0.73
2	94.95 ± 0.46
5	93.23 ± 0.85
10	88.71 ± 1.19

Table 7: Recovery percentage of 1-OHP using different sorbent masses.

Sorbent mass (mg)	Recovery (%) Mean ± SD, N=5
100	99.96 ± 0.05
500	68.14 ± 2.05

Table 8: Day-to-day [D-day] and Within-day [W-day] reproducibility of 1-OHP spiked in urine, sample volume 10ml, N=6.

Concentration added (µg/l)						
Statistical Data	2		10		20	
	D-day	W-day	D-day	W-day	D-day	W-day
M±SD	1.93 ± 0.06	1.98 ± 0.01	9.86 ± 0.03	9.87 ± 0.08	19.56 ± 0.06	19.67 ± 0.34
CV%	3.1	0.5	0.3	0.81	0.3	1.73

Table 9: Urinary 1- hydroxypyrene in industrially exposed workers.

	Exposed				Non-exposed		
subjects	N (%)	Age (M±SD)	1-OHP (µmol/mol crea.) (M±SD)		N (%)	Age (M±SD)	1-OHP (µmol/mol crea.) (M±SD)
Total	40(100)	31 ± 12	2.38 ± 0.56		22(100)	29.00±10.00	0.63±0.27
smoker	11(27.5)	32 ± 7	2.86 ± 0.91		11(50)	26.50±7.50	0.89±0.21
Non- smoker	29(72.5)	27 ± 7.5	1.90± 0.48		11(50)	25.00±6.00	0.36±0.35

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