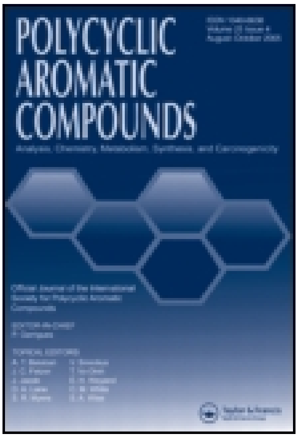

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Assessment of Exposure to PACs in Asphalt Workers: Measurement of Urinary PACs and their Metabolites with an ELISA Kit

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Assessment of Exposure to PACs in Asphalt Workers: Measurement of Urinary PACs and their Metabolites with an ELISA Kit

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An enzyme-linked immunosorbent assay (ELISA) kit made for determination of polycyclic aromatic compounds (PACs) in water was adapted for measuring PACs and their metabolites in urine. This method was then applied to a pilot asphalt worker PAC exposure study. Currently, liquid-liquid extraction with gas chromatography/isotope dilution high-resolution mass spectrometry (GC/HRMS) is the preferred method to determine urinary PAC metabolites. Although sensitive and specific, GC/HRMS is time consuming and costly.

The ELISA method had a range from 14–720 ng/ml 1-hydroxypyrene equivalents with a lower limit of detection (LOD) of 14 ng/ml urine. ELISA and GC/HRMS PAC metabolite measurements had a statistically significant correlation and the PAC ELISA results were indicative of potential asphalt exposure. PAC ELISA is promising as a more rapid and less costly routine method for determining worker exposure to PACs in asphalt emissions.

Key Words: asphalt, biomonitoring, ELISA, PAC, urine

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INTRODUCTION

Asphalt paving workers are exposed to PACs and some PACs are carcinogens (1). Among the PACs known to be present in asphalt fume are naphthalene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, chrysene, benz[a]anthracene, and a number of substituted and heterocyclic PACs (2). However, data linking exposure to asphalt with specific health effects are limited (2). Since asphalt workers have inhalation and dermal exposure routes, both air sampling and dermal sampling have been conducted (3). To overcome some of the weaknesses of air and dermal sampling, measurement of biomarkers of exposure using PAC metabolites, such as 1-hydroxypyrene in urine along with air and dermal PAC measurement, has been carried out (4–6). Other studies have also examined unmetabolized parent PACs as biomarkers of exposure (7–9). More recent studies have used both metabolized and parent PACs in urine as biomarkers of exposure (10, 11). There are a number of parent PACs and their metabolites that have been measured in urine of asphalt workers including: naphthalene and hydroxynaphthalene, phenanthrene and hydroxyphenanthrene, and pyrene and hydroxypyrene (9–11). 1-Hydroxypyrene in urine has been found to have significant correlation with airborne exposure to PACs (5, 6).

There are a number of techniques used to assess PAC metabolites in urine including liquid chromatography/mass spectrometry (LC/MS) (10), headspace solid-phase microextraction/GC (9), high performance liquid chromatography (HPLC) with fluorescence detection (5, 6), and automated liquid-liquid extraction and gas chromatography/isotope dilution high-resolution mass spectrometry (GC/HRMS) (12). Although these are highly selective and sensitive techniques, they are labor intensive and require expert knowledge to conduct and interpret the results; hence, their complexity and expense limit their usefulness for routine biomonitoring. Also, because they are usually used for a specific set of compounds that must be summed to get an overall assessment of exposure, they could miss some components that could be important. A less specific technique that responds to the overall PAC content may give a better assessment of overall exposure (13, 14).

In the work presented here, which is part of a large study to assess dermal and inhalation exposure for asphalt paving workers, an ELISA technique was developed to assess PACs and their urinary biomarkers. ELISA kits developed for PAC measurement in environmental samples such as water can be modified for use in PAC measurement in biological samples if proper changes in procedure are made (15). It is known that urine can affect the performance of the ELISA (15); therefore, the calibration curve should be prepared with a similar urine matrix as the samples and samples should be diluted to minimize the effect of urine on the ELISA. Moreover, additional steps such as treatment with ß-glucuronidase are often needed to convert metabolites into products that are similar to the parent compounds for which the ELISA was developed (16). The goals of this work were to modify an existing ELISA kit developed to measure PACs in water for use with occupational urine samples, to compare these ELISA results with automated liquid-liquid extraction and gas chromatography/isotope dilution high-resolution mass spectrometry, and to evaluate the ELISA as an overall measure of air and dermal exposure to PACs from asphalt and other sources.

MATERIALS AND METHODS

Reagents: The PAC kits were RaPID Assay PAH Test kit (Product A00156/A00157; Strategic Diagnostics; Newark, DE) which were developed for PACs in water. The method detection limit for PACs in water as indicated by the kit insert was 0.93 ng/ml for phenanthrene, 0.27 ng/ml pyrene, 0.43 ng/ml fluoranthrene, 0.67 ng/ml benzo(a)pyrene, 2.19 ng/ml fluorene, and 86.5 ng/ml naphthalene. All components of the ELISA procedure were included with the ELISA kit including PAC-enzyme conjugate, the PAC antibody-coated magnetic particles, assay tubes, substrate and chromogen, kit standards, wash buffer, kit diluent, and a kit control. The antibody coated on the magnetic particle was a rabbit polyclonal produced against an anthracene-based immunogen. ß-glucuronidase (from E. coli K12, part number 03 707 601-001) was obtained from Roche Diagnostics (Mannheim, Germany). 1-Hydroxypyrene, 1-hydroxynaphthalene, and 2-hydroxynaphthalene were obtained from ACROS Organics (Morris Plains, NJ) and 9-hydroxyfluorene was obtained from TCI America (Portland, OR).

Equipment: The ELISA tube rack with removable magnetic base and the ELISA tube reader (model RPA-1) were obtained from Strategic Diagnostics (Newark, DE). The PAC ELISA method is pre-programmed into the ELISA tube reader that will be described later. All sample dilutions as well as production of 1-hydroxypyrene standards for the standard curve were done in 10×75 mm disposable glass tubes because some preliminary experiments indicated that some plastic tubes had an interference that produced a background with the ELISA.

Creatinine/Cotinine Determinations: Creatinine was determined using the Vitros Autoanalyzer (Ortho Clinical Diagnosis, Rochester, NY) and was used to normalize the urinary metabolite results (17). Cotinine, (Nicotine-N-oxide), was determined by NIOSH using the Immulite® 2000 (Siemens Medical Diagnosis) (17).

Worker Urine Collection and Processing: NIOSH Human Subject Review Board approval was obtained to collect urine samples from asphalt paving workers and control concrete pavers who provided informed consent. In the first part of the pilot study which was used for the data presented in this article, the asphalt workers consisted of the operator who smoked, the screedman who smoked, and a laborer who did not smoke. It is expected that the screedman and laborer would have higher relative exposure to asphalt fumes since they are nearer the asphalt as it is laid on the roadbed. The concrete pavers consisted of one worker who smoked, one who did not smoke, and one who chewed tobacco. On Monday and Thursday, pre-shift, post-shift and bedtime urine samples were collected, processed, and stored under conditions to minimize contamination, as described previously (17). In a second part of the study, three additional concrete workers and three asphalt pavers provided samples for additional PAC analyses, but GC/HRMS was not obtained for these workers. Samples from these workers were only used to examine the relationship between smoking status and PAC measurements. The collected urine samples were stored on ice in coolers in the field after they were taken and were transferred to a −20°C freezer at the end of each workday. The samples were taken to a NIOSH laboratory in Cincinnati, OH at the end of the week stored at −20°C until they were analyzed.

GC/HRMS: The GC/HRMS method was based on enzymatic deconjugation, automated liquid-liquid extraction, and gas chromatography/isotope dilution high-resolution mass spectrometry after derivatization of the OH-PACs to the trimethylsilylated derivatives (12). The metabolites included in the current method were formed from eight different parent compounds, and included 1-OH-naphthalene, 2-OH-naphthalene, 9-OH-fluorene, 2-OH-fluorene, 3-OH-fluorene, 1-OH-phenanthrene, 2-OH-phenanthrene, 3-OH-phe nanthrene, 4-OH-phenanthrene, 9-OH-phenanthrene, 1-OH-pyrene, 1-OH-benzo[*c*]phenanthrene, 2-OH-benzo[*c*]phenanthrene, 3-OH-benzo[*c*]phenan threne, 1-OH-benz[*a*]anthracene, 3-OH-benz[*a*]anthracene, 9-OH-benz[*a*] anthracene, 1-OH-chrysene, 2-OH-chrysene, 3-OH-chrysene, 4-OH-chrysene, 6-OH-chrysene, 3-OH-benzo[*a*]pyrene, and 7-OH-benzo[*a*]pyrene (12). The limits of detection were below 7 pg/ml urine when using a sample size of 2 ml, except for 1- and 2-naphthols (18 and 12 pg/ml, respectively). The recoveries of all compounds were about 70% except for 1- and 2-naphthols that have recoveries of about 50% and the relative standard deviation of repeated measurements over a 3-week period was 11% or less.

Preparation of the ELISA Standard Curve: Urine is known to affect the response of the ELISA. Therefore, the standard curve was prepared with a matrix similar to the samples (15), and the samples were diluted to minimize the effect of urine (15, 16). In addition, it was necessary to add ß-glucuronidase to convert glucuronide urine metabolites to parent PAC metabolites (12, 16). Urine from unexposed individuals was mixed together to create a urine pool. To 3 ml of this urine pool, 1 ml of methanol was added to produce the same methanol concentration as the worker field samples. To 250 μl of this urine pool, 20 μl of ß-glucuronidase was added; this mixture was incubated for

30 min at room temperature and the resulting treated pooled urine-methanol mixture was diluted 1/10 with kit diluent. This diluted treated urine pool was used as the diluent in the calibration standards where a 500 ng/ml stock standard of 1-hydroxypyrene was diluted serially to produce standards of 50, 25, 10, 5, 2, 1, and 0 ng/ml.

Preparation of Worker Samples: Worker samples (containing 3 ml urine and 1 ml methanol) were treated with ß-glucuronidase and diluted the same way as the pooled urine samples. This resulted in an overall dilution factor of 14.4 for the worker urine samples. This dilution factor resulted from 4/3 for methanol addition (1 ml methanol added to 3 ml urine, 270/250 for ß-glucuronidase addition (20 μl ß-glucuronidase added to 250 μl urine-methanol), and 10 for kit diluent dilution (9 parts kit diluent added to 1 part treated urine methanol) ($4/3 \times 270/250 \times 10 = 14.4$). This dilution was found to reduce the effect of urine on the assay and to give good correlation of assay response to log(1-OH pyrene concentration) as shown below.

ELISA Procedure: The ELISA technique depends on the competition of PACs in solution with the PAC-enzyme conjugate for PAC antibody binding sites on PAC antibody-coated magnetic particles. The amount of PAC enzyme conjugate bound to the antibody-coated particles is inversely proportional to the concentration of PAC in solution. Ultimately, the substrate and chromogen are converted to a colored product that is measured with a spectrophotometer. Therefore, the level of color development is also inversely proportional to the concentration of PACs in solution. Figure 1 is a schematic diagram showing

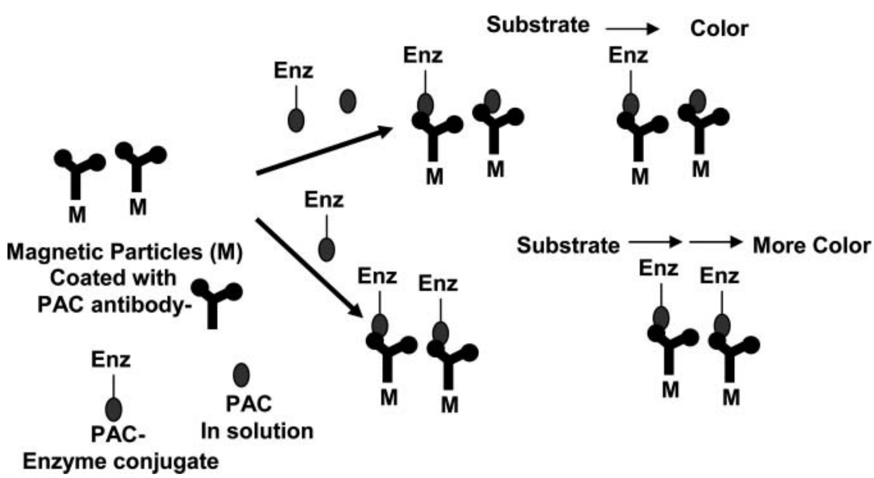

Figure 1: ELISA Principle: PAC in solution competes with PAC-enzyme conjugate for PAC antibody binding sites on the magnetic particles resulting in less conjugate binding to the magnetic particles. The binding of less conjugate results in less color being produced at higher concentrations of PAC in solution.

how the competition of the PAC in solution with the PAC-enzyme conjugate for the PAC antibody binding sites on PAC antibody coated magnetic particles results in a lower amount of color development from the substrate.

To perform the assay, 250 μl of standard or sample, 250 μl of PAC-enzyme conjugate, and 500 μl of the PAC antibody-coated magnetic particles were added to the assay tube in the tube rack, and the mixture is incubated for 30 min at room temperature. After incubation, the assay tubes were then washed and vortexed twice with 1 ml of wash buffer to remove excess PAC-enzyme conjugate while the magnetic particles were held in the tube with the magnetic base. The substrate (hydrogen peroxide) was added along with the chromogen (3, 3′, 5, 5′-tetramethylbenzidine), and the enzyme in the PAC-enzyme conjugate converted this mixture to a blue colored product. The reaction was allowed to proceed for 20 min before the stop solution (2M sulfuric acid) was added to terminate the reaction. The absorbance for each assay tube was then read at 450 nm after blanking the tube reader with wash buffer. The PAC assay procedure is pre-programmed into the ELISA tube reader, and the program assumed the first four tubes are kit standards (0, 2, 10, and 50 ng/ml phenanthrene in water). After the first four tubes were read, the reader produced a standard curve using the absorbance values determined for the standards. The program assumed that the fifth tube was the kit control that should read 25±5 ng/ml; the reader then calculated the concentration of each sample using the determined standard curve as each tube was read, and the results were printed on the built-in printer. In addition to the kit standard curve, we produced a standard curve using 1-hyroxypyrene, as described previously, which was used to calculate the concentration of the worker samples. Therefore, all values are given as 1-hydroxypyrene equivalents.

Statistical Analysis of Data: Statistical analysis of data including curve fitting was done using Excel (Microsoft Office Excel 2007).

Standard Curve Fitting: The kit curve was fitted by the ELISA tube reader using a linear Logit (%B/Bo) vs. Ln concentration (ng/ml) function, where Logit (%B/Bo) is (Ln(%B/Bo)-Ln(100-%B/Bo)), B is the absorbance at the standard concentration, and Bo is the absorbance at zero concentration as indicated in the kit instructions. For fitting the standard curves produced with the 1-hyroxypyrene, a linear absorbance versus log of concentration (ng/ml) model was used because it gave a good fit. Logit (%B/Bo) vs. log concentration also had a good fit where Logit (%B/Bo) is (Log (%B/Bo)-Log (100-%B/Bo)), B is the absorbance at the standard concentration and Bo is the absorbance at zero concentration.

RESULTS

Response of ELISA to Several Hydroxylated PACs: Figure 2 shows the response of the assay to 1-hydroxynaphthalene, 2-hydroxynaphthalene,

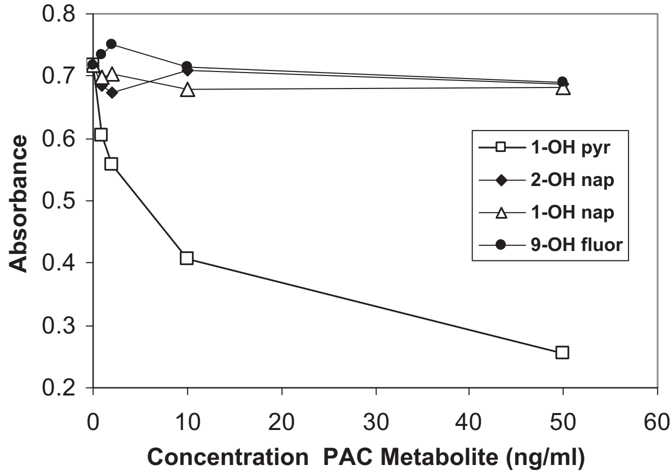

Figure 2: Response of ELISA Assay to Several Hydroxylated PACs. Response of the ELISA assay was evaluated for 1-hydroxynaphthalene, 2-hydroxynaphthalene, 9-hydroxyfluorene, and 1-hydroxypyrene over the range of 0-50 ng/ml.

9-hydroxyfluorene, and 1-hydroxypyrene. Only the 1-hydroxypyrene shows an adequate competitive inhibition with concentrations to be developed into a standard curve. This is expected since the kit manufacturer indicates good response to phenanthrene and pyrene and several other PACs of similar size but very low response to naphthalene and limited response to fluorene, which are smaller PACs.

Standard Curves: Figure 3 shows a typical standard curve fit by the tube reader using the kit standards (0, 2, 10, 50 ng/ml phenanthrene in water). A good fit was obtained for the standards and this curve was used to calculate the concentration of each sample as it is read by the reader. The range of the curve is from 0–50 ng/ml and the manufacturer has determined a limit of detection (LOD) of 0.93 ng/ml with a range of quantitation of 2.7–66.5 ng/ml. The kit curve was used mainly to assure proper performance of the assay because the 1-hydroxypyrene curve was used to calculate the concentration in the samples.

Figure 4 shows a typical curve for 1-hydroxypyrene in 1/10 treated urine-methanol kit diluent. Although these curves had the same range of concentrations as the kit curves, the curves had a smaller range of absorbance values than the curves developed from the kit standards. The range of the curve is from 0–50 ng/ml with an LOD of 1 ng/ml, thus, resulting in an LOD of 14 ng/ml and a range of 14–720 ng/ml for the assay when the dilution factor of 14.4 is taken in account.

Assessment of ELISA for Worker Exposure Assessment and Comparison with GC/HRMS: Figure 5 compares concentrations of PACs in urine as

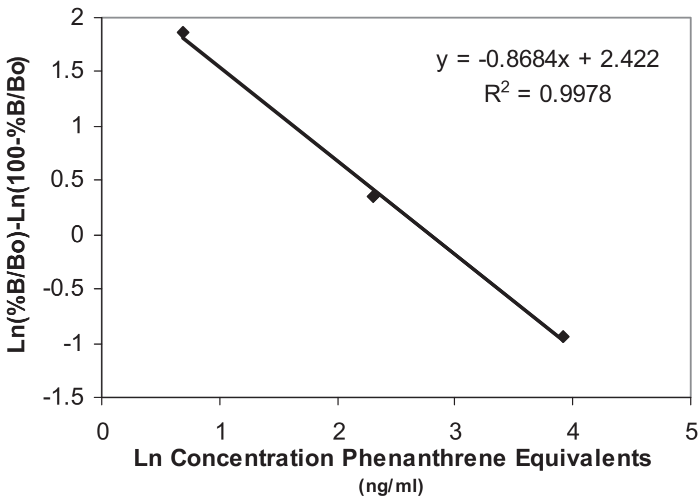

Figure 3: Kit Calibration Curve. The kit curve uses the ELISA response to kit standards (0, 2, 10, 50 ng/ml phenanthrene in water) and fits the response using Logit(%B/Bo) versus Ln concentration where Logit(%B/Bo) is (Ln(%B/Bo)-Ln(100-%B/Bo)), B is the absorbance at the standard concentration, and Bo is the absorbance at 0 concentration.

measured with the ELISA for a group of asphalt pavers with a group of concrete workers as controls over the course of a workweek. Each bar is the average concentration measured for each group of workers at a particular time in the workweek. It can be seen that the asphalt exposed workers consistently show higher levels at each time interval during the workweek. Overall, the

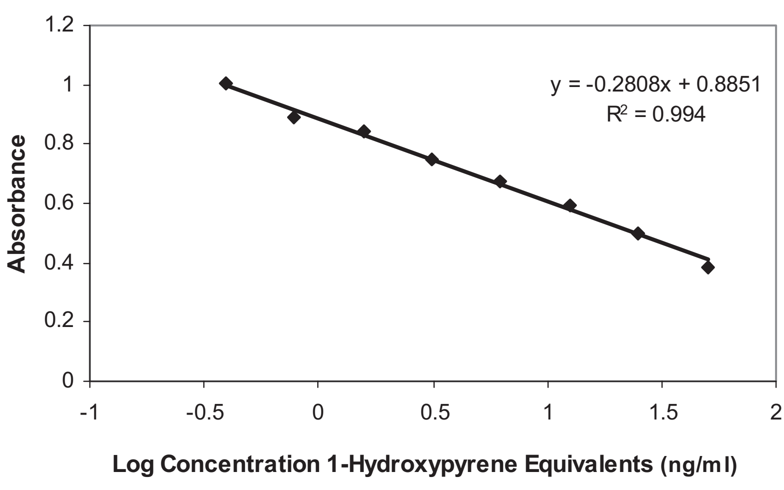

Figure 4: 1-Hydroxypyrene Standard Curve. The absorbance from the ELISA assay using 1-hydroxypyrene standards in 1/10 treated urine-methanol/kit diluent is plotted against the log of concentration over the range of 0.39–50 ng/ml.

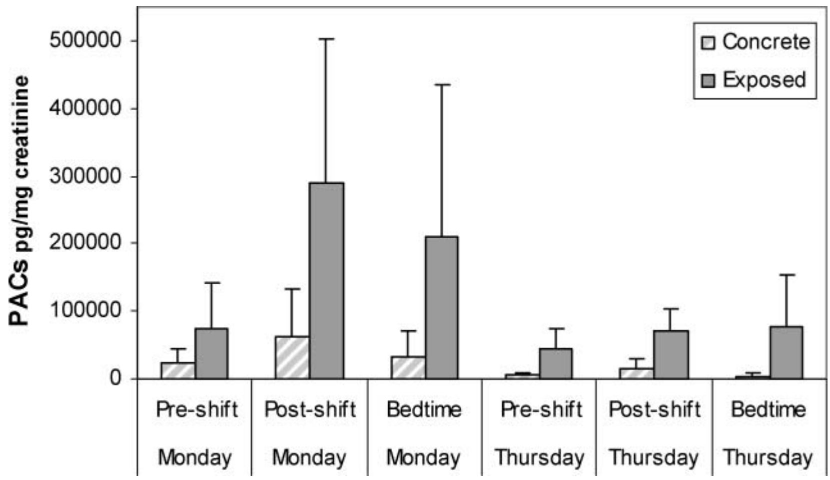

Figure 5: Comparison of PACs in Urine as Measured by the ELISA Assay for Asphalt Exposed and Controls (Non Asphalt Exposed Concrete Workers). Average PAC concentration in urine was determined by the ELISA assay for control and asphalt exposed workers at several times during the work week.

concentration of PACs in the asphalt workers is significantly different from the concrete workers ($p < 0.05$). However, due to the small sample number there is large variation in each worker group for each individual time period, as shown by the large error bars in Figure 5.

Figure 6 presents data from the PAC ELISA assay and GC/HRMS determinations of PACs in urine for several controls (non-asphalt-exposed concrete workers) and asphalt-exposed paving workers at several times during the workweek. Although the absolute value of the concentrations measured by the two procedures is different, the same general trends are observed, i.e., when the GC/HRMS concentrations are high the ELISA concentrations are high. The GC/HRMS method required summing of the individual metabolites measured to obtain a total response; thus, GC/HRMS underestimates the total metabolites present, while the ELISA method provides a more inclusive result for the PAC metabolites present in the urine sample.

Figure 7 shows the correlation between GC/HRMS and ELISA assay measurements for PACs in urine for all samples taken during the pilot study. Again, the GC/HRMS data required summing of concentrations of the metabolites present to obtain a total response. There is good correlation between the two measurements ($R^2 = 0.79$, $p = 0.004$) even though the absolute values of the concentrations measured are different, possibly, in part for the reasons stated above.

Cotinine Measurements and 1-OH Pyrene Equivalents: Cotinine levels and 1-OH pyrene equivalents were measured for concrete workers (4/6

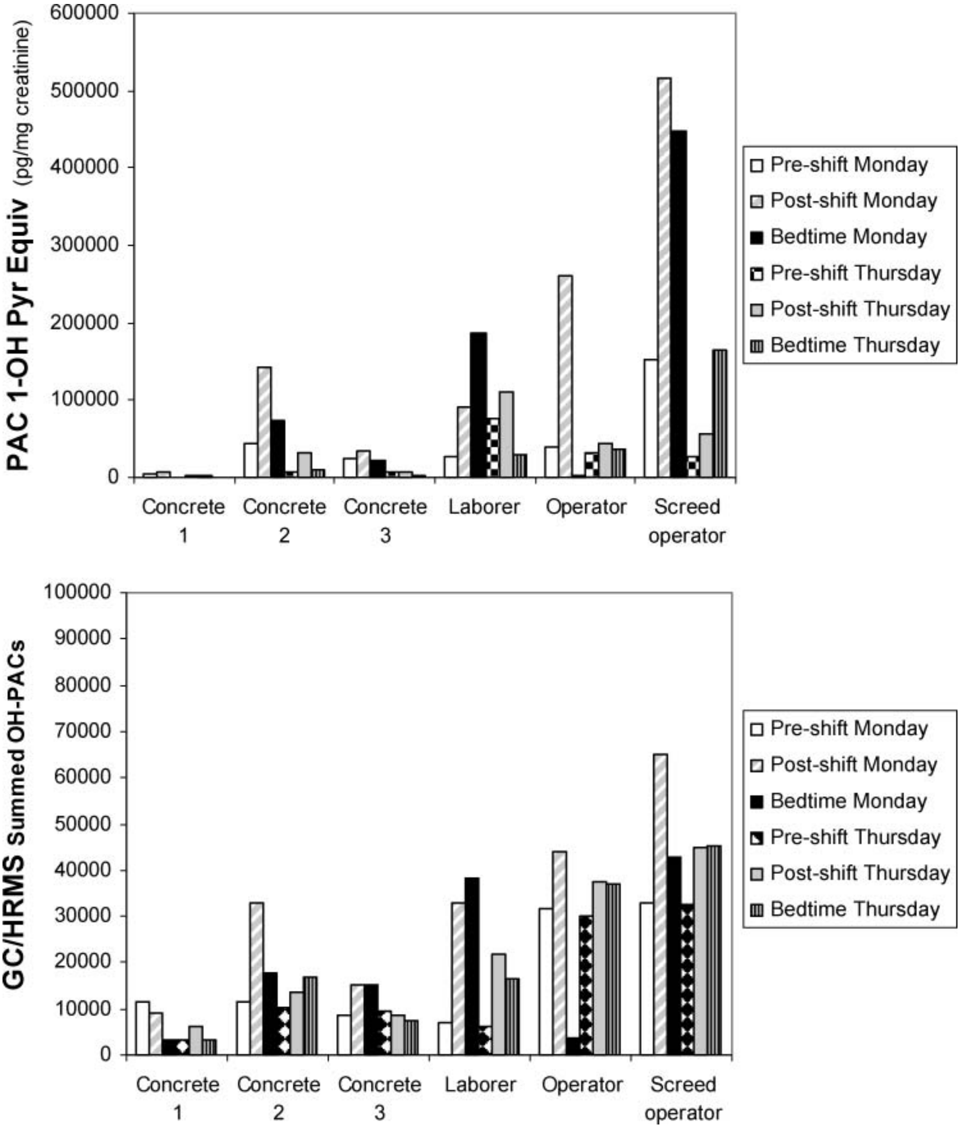

Figure 6: Comparison of PAC Concentrations in Urine from GC/HRMS and ELISA. The sum of the PAC metabolites measured by GC/HRMS (described in 'Materials and Methods') is compared to the ELISA PAC measurements for a number of workers sampled at various times during the work week.

smokers) and asphalt pavers (2/6 smokers). Each measurement at different times during the workday/workweek was plotted for each worker (except for the concrete worker that regularly cleaned his equipment with diesel) (Figure 8). Table 1 shows the mean concentration of cotinine and 1-OH pyrene equivalents for each worker group and the p values for the difference in mean

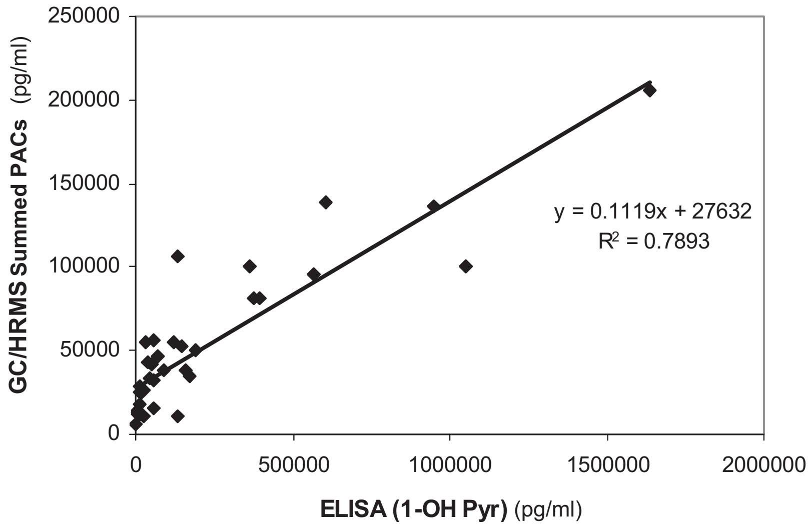

Figure 7: Correlation of GC/HRMS and PAC ELISA Measurements of PACs in Urine of Asphalt Pavers and Controls (Non Asphalt Exposed Concrete Workers). The sum of select GC/HRMS PAC urine metabolites (naphthalene, fluoranthene, phenanthrene, and pyrene) was correlated to measurements of PACs in urine using the PAC ELISA for asphalt exposed and control concrete workers during the pilot study.

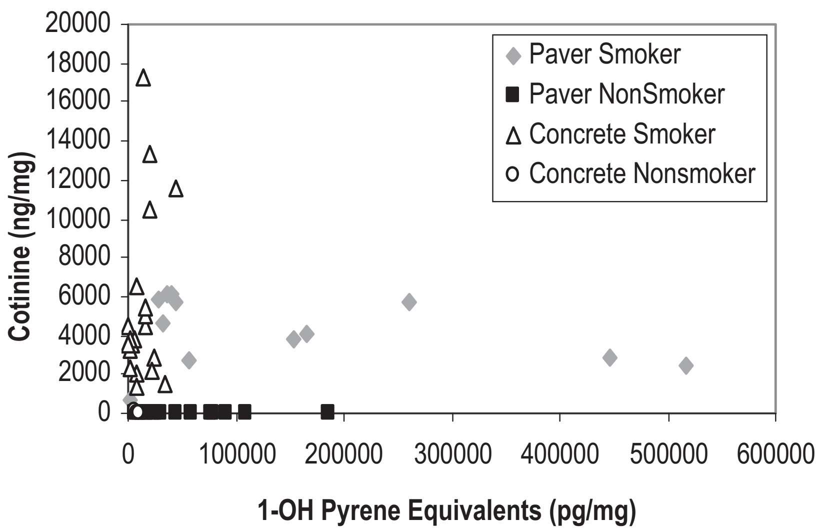

Figure 8: Examination of Cotinine Measurements and ELISA 1-OH Pyrene Equivalents. Cotinine levels and 1-OH pyrene equivalents were measured for concrete workers and asphalt pavers. Each measurement at different times during the workday/workweek was plotted for each worker.

Table 1: Cotinine and 1-OH-Pyrene equivalents for smoking and nonsmoking asphalt pavers and concrete workers

Mean concentrations of cotinine and 1-OH-Pyrene equivalents for each worker group			
worker group	cotinine (pg/ml)	OH-pyrene (pg/ml)	
concrete smokers	5.44E+06	1.33E+04	
paving smokers	4.23E+06	1.49E+05	
concrete nonsmokers	5.73E+04	8.47E+03	
paving nonsmokers	7.55E+03	4.80E+04	
1-OH Pyrene Equivalents (pg/mg) p values for difference of means for worker groups			
	concrete nonsmokers	concrete smokers	paving nonsmokers
concrete smokers	0.105		
paving nonsmokers	0.004*	0.009*	
paving smokers	0.017*	0.021*	0.073
Cotinine (pg/mg) p values for difference of means for worker groups			
	concrete nonsmokers	concrete smokers	paving nonsmokers
concrete smokers	2.289E-05*		
paving nonsmokers	0.347	2.049E-05*	
paving smokers	5.130E-06*	0.280	4.862E-06*

*indicates significance at 95% confidence.

values for the groups. Figure 8 and Table 1 show that 1-OH pyrene equivalents in the concrete workers, nonsmokers and smokers, tend to be lower than the 1-OH pyrene equivalents in the asphalt pavers. In addition, smokers, both concrete workers and pavers, have higher cotinine levels than nonsmokers.

DISCUSSION

Biomarkers of PAC exposure in asphalt workers are useful because they allow measurement of multiple routes of exposure, rather than exclusively dermal or inhalation exposure. There is work examining the relationship between the different routes of exposure and production of various biomarkers although there are still questions about these relationships. Many of the techniques used for analysis of PAC exposure biomarkers are highly sensitive and specific but require expertise and are labor intensive and time consuming; therefore, they are not practical for routine biomonitoring and assessment of control strategies.

In this work, we examined the use of an ELISA kit as a supplement to a GC/HRMS procedure and as a possible routine monitoring method for PACs and their metabolites in urine. For the GC/HRMS procedure the response for the range of compounds measured were summed to give a total response. The ELISA and total GC/HRMS total response correlated well (see Figure 7), but the ELISA also correlated with potential exposure to asphalt for the workers examined (see Figure 5). Costs associated with the ELISA would be about $20 per samples as opposed to the GC/HRMS that would cost about $300 per sample. When measurement of exposure to specific PACs is needed for health assessment purposes, the GC/HRMS can be employed to measure these specific PACs, but when a more general technique that responds to a range of PACs is needed the ELISA can provide a lower cost alternative. This is true of routine monitoring to assess possible routes of exposure. More work could be done to compare the GC/HRMS and ELISA in other matrices such as water/methanol to investigate the differences in the measurements made by the two techniques in worker urine.

A perceived weakness of the ELISA assay may be its low response to smaller PACs such as fluorene and naphthalene. Although naphthalene and its alkylated isomers are the most abundant PACs in asphalt, smoking contributes to the naphthalene and other PACs contained in the exposure. Therefore, the use of these PACs or their metabolites in urine as a biomarker of exposure to asphalt may be confounded by smoking (11). However, because the results for naphthalene and its metabolites appear to be more affected by smoking than phenanthrene and pyrene and their metabolites, the latter might be more reflective of PAC exposure to asphalt (11). The data shown in Figure 8 supports this conclusion even though the low sample number necessitates further investigation. Since the ELISA gives a total response to a wide range of PACs including both parent PACs and metabolites, it may give a better assessment of total exposure than a more specific technique that measures a limited set of compounds (13, 14, 18).

CONCLUSION

This preliminary work indicates that the PAC ELISA kit is a promising method for routine assessment of PAC biomarkers in the urine of asphalt paving workers and should be useful for assessing PAC exposures for any worker exposed to PACs. If specific metabolite information is needed, then one of the chromatographic methods must be employed. However, if interested in total exposure the ELISA may offer a distinct, low-cost alternative. Further examination of the relationship of the ELISA response to worker exposure and to other more specific biomonitoring techniques is warranted to further assess its utility.

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