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Extraction and high-performance liquid chromatographic separation of selected pyrene and benzo[*a*]pyrene sulfates and glucuronides: preliminary application to the analysis of smokers' urine

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Abstract

In the study of the complex mixture of urinary metabolites derived from polycyclic aromatic hydrocarbon compounds, it is desirable to simplify the analysis through separation of classes of compounds. We have developed a liquid chromatography (LC) method for the separation of selected sulfate and glucuronide conjugate isomers derived from hydroxybenzo[*a*]pyrenes (OH-BaP) and hydroxypyrenes. This LC method was utilized in the preliminary analysis of the urine of smokers by combining it with an extraction technique employing tetra-n-butyl-ammonium ion as a coupling agent to generate a 1:1 complex, extractable in chloroform at low pH prior to LC analysis.

Keywords: Pyrene; Benzo[*a*]pyrene

1. Introduction

Benzo[*a*]pyrene (BaP) is a member of the complex group of polycyclic aromatic hydrocarbons (PAH) that is formed from the incomplete combustion of carbonaceous material. Cigarette smoke is an important source of BaP and other PAH. A smoker of filter cigarettes might inhale 0.4 μg BaP per pack [1]. Several researchers have shown that there is a significant increase in the excretion of BaP and BaP metabolites in smokers' urines compared to a non-smoking control group [1,2]. BaP is an environmentally important chemical carcinogen that exerts its carcinogenic activity through metabolites which react with and modify nucleic acids and proteins. Biological systems metabolize BaP to a complex mixture of quinones, phenols, dihydrodiols, triols and tetrols [3]. These metabolites may be further enzymatically conjugated with glutathione, glucuronide or sulfate to produce even more water-soluble compounds and thus facilitate urinary and biliary excretions [4]. The formation of conjugates is primarily a detoxification pathway. However, these conjugates are potentially toxic metabolites.

1-Hydroxypyrene (1-OH-Pyr) and other hydroxy-PAHs isolated from urine are commonly used to assess human exposure to PAH [5–8]. The BaP conjugates examined in this study are selected positional isomers which are very difficult to separate by LC. Although liquid chromatography is the

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preferred separation method for BaP sulfates and glucuronides, the separation of BaP sulfate and glucuronide positional isomers is very difficult to achieve. For example, Teffera et al. were only able to separate 3-hydroxybenzo[*a*]pyrene sulfate (3-OH-BaP-S) from 1-hydroxybenzo[*a*]pyrene glucuronide (1-OH-BaP-G) and 3-hydroxybenzo[*a*]pyrene glucuronide (3-OH-BaP-G) by microbore HPLC [9]. Merrick and Selkirk separated 7-hydroxybenzo[*a*]pyrene sulfate (7-OH-BaP-S) from 1-hydroxybenzo[*a*]pyrene sulfate (1-OH-BaP-S). However, 3-OH-BaP-S, 6-hydroxybenzo[*a*]pyrene sulfate (6-OH-BaP-S) and 9-hydroxybenzo[*a*]pyrene sulfate (9-OH-BaP-S) all co-eluted with 1-OH-BaP-S [10]. Because of the difficulty in separating these sulfate isomers, the usual analytical approach for analysis of BaP conjugates has been an indirect one. It involves measuring the hydroxylated metabolites after liberation from the glucuronide and sulfate conjugates by acid or enzyme hydrolysis [9].

Typically an enzymatic and/or chemical hydrolysis of PAH conjugates (phase 2 metabolites such as sulfates and glucuronides) is used for the analysis. However, information is lost upon hydrolysis since these methods cannot differentiate between conjugated and unconjugated metabolites. Secondly, some conjugates are poor substrates for the hydrolysis process. Also, the hydrolysis approach is limited to those conjugates for which hydrolases are available [9]. Therefore, it is highly desirable to develop an HPLC method that can separate and quantify the conjugates of pyrene and BaP and possible other PAHs in the same chromatographic run. Individual conjugates can be used as biomarkers of exposure to the PAH class.

The direct assay of these conjugates in urine is difficult due to their high molecular weight (non-volatile) and the fact that they form 'fatty anions' near the pH of urine ($pK_a = 1$ for BaP sulfates and 5 for BaP glucuronide), [9] which makes them hard to separate from water. It has been demonstrated that ion-pair extraction can be used as an analytical method to extract anions from an aqueous layer into chloroform or methylene chloride [11–13]. Many anions can be extracted as ion-pairs with a large quaternary cation such as tetra-*n*-butyl-ammonium cation (TBA^+).

In this study we used tetra-*n*-butyl-ammonium hydrogen sulfate ($TBA^+HSO_4^-$) to extract pyrene and BaP sulfates and glucuronides from spiked urine and urine samples from a group of smokers. Selected conjugates of pyrene and BaP were separated by HPLC in the same chromatographic run. Very good retention time reproducibility was achieved by careful choice and control of the parameters of temperature, pH, triethylamine (TEA) and acetonitrile concentrations. However, it was not possible to positively identify the conjugates due to the lack of a suitable analytical tool such as GC–MS or LC–MS.

2. Experimental

2.1. Apparatus and conditions

Two Waters Model 501 HPLC pumps equipped with a Kratos 980 fluorescence detector (excitation/emission wavelengths were 300/389 nm), Rheodyne 7125 injector with a 20 μl loop and a Waters 840 chromatography acquisition system were used.

Column: Vydac 201 TP reversed-phase C_{18}, 5 μm particles, 25 cm×4.6 mm I.D. (Separations Group, Hesperia, CA, USA). The Vydac column was from a special bonding lot designated as high load, and has a higher bonding density than is typical for this column. The column was previously characterized using SRM 869, 'Column Selectivity Test Mixture for Liquid Chromatography' as described elsewhere [14,15].

2.2. Calculation of capacity factor (k′)

$$k'_{\text{1-OH-Pyr-G}} = (t_{\text{1-OH-Pyr-G}} - t_M)/t_M$$

where $t_{\text{1-OH-Pyr-G}}$ is the retention time of 1-OH-Pyr-G and t_M is the retention time of acetone – an unretained species (one drop acetone in 1 ml acetonitrile; 10 μl was injected).

The dead volume was determined to be 3.4 ml. k's for the other conjugates were calculated in a similar manner. k' for each conjugate was determined at different pH, temperature, %TEA and % acetonitrile.

2.3. HPLC conditions for the analysis of the smokers' urine samples

The Vydac 201 TP column was investigated for its selectivity towards the conjugate isomers of pyrene,

BaP and BaA prior to the urinary analysis. The capacity factors for the isomers were determined at different temperature, pH and TEA concentrations.

The mobile phase was prepared as follows; reservoir A: an aqueous solution of 1.8% (v/v) triethylamine (TEA) was prepared and adjusted to pH 4.2 with glacial acetic acid; reservoir B: acetonitrile. The column was eluted isocratically at 30°C at a flow-rate of 2.0 ml/min with 75% solvent A and 25% solvent B. Prior to analysis, the column was conditioned with 20 column volumes of 100% acetonitrile. Thereafter, the column was equilibrated with at least 30 ml of mobile phase.

2.4. Reagents and standards

The 1-OH-Pyr-G, 1-hydroxypyrene sulfate (1-OH-Pyr-S), 1-,3-,6-,7- and 9-OH-BaP sulfates and the 1-,3-,6-,7- and 9-OH-BaP glucuronide isomers were obtained from the National Cancer Institute Chemical Carcinogen Repository (synthesized and characterized by the Midwest Research Institute, Kansas City, MO, USA). The standards were dissolved in HPLC grade ethanol and stored protected from light at −20°C.

Acetonitrile, ethanol, glacial acetic acid and triethylamine were HPLC grade and were purchased from Fisher Scientific (Pittsburgh, PA, USA). HPLC-grade water (conductivity >18 MΩ cm) was generated from distilled water by a water purification system (Milford, MA, USA).

2.5. Liquid/liquid extraction after ion-pairing with TBA^+

Four (4) 20 ml samples of 'blank' urine were spiked with 10, 20, 40 and 60 pg of each of the BaP sulfate and glucuronide isomeric standard. Each standard was done in triplicate. One ml of 0.01M $TBA^+HSO_4^-$ was added and the pH was adjusted to 2.2 with conc. nitric acid. The samples were then extracted three times with 15 ml of chloroform. The chloroform extracts were combined and concentrated down to 1 ml in vacuo on a rotary evaporator and then evaporated to dryness under a gentle stream of N_2. The residue was reconstituted in 100 μl acetonitrile and analyzed by HPLC. Five urine samples (20 ml each) from a group of smokers and two from nonsmokers were similarly treated. The smokers (volunteers) were chosen from among the student population at the University of North Carolina at Charlotte. Urine samples from two nonsmokers were employed as blanks. However, the diets of the smokers and nonsmokers were not controlled. The urine samples were analyzed for the presence of pyrene and BaP sulfates and glucuronides by HPLC with fluorescence detection.

3. Results and discussion

Using the chromatographic conditions described in Materials and methods, 6-OH-BaP-G, 1-OH-Pyr-G and 1-OH-Pyr-S were separated from the 1-, and the 3-OH-BaP glucuronide isomers. However, the 1-OH-BaP-G co-eluted with the 9-isomer and the 3-OH-BaP-G co-eluted with the 7-isomer (Fig. 1). It proved to be extremely difficulty to separate 1-OH-Pyr-S from 6-OH-BaP-G. Indeed separation of these two conjugates is only possible by careful choice and 'fine-tuning' of the parameters of pH, temperature, TEA concentration and acetonitrile concentration as is seen in Figs. 2–4. 1-OH-Pyr-G proved to be the most polar and it eluted first with a retention time of 4 min under the chromatographic conditions given in Fig. 1. Very selective wavelengths were chosen ($\lambda_{ex}/\lambda_{em}=300/389$ nm) to optimize the detector conditions for the BaP glucuronide and sulfate conjugates. However, as seen in Fig. 1, pyrene and benz[*a*]anthracene conjugates also absorb and emit radiation at these wavelengths making their detection and measurement possible without the need for wavelength programming.

Figs. 2–4 show the effect of pH, temperature and TEA concentration on k' for BaP glucuronides and pyrene sulfate and glucuronide. Retention can be readily manipulated with careful selection of these parameters. Below room temperature, k' increases dramatically but retention is greater than 60 min resulting in peak broadening and loss of column efficiency for the sulfates as reported previously [16]. We had to utilize a higher concentration of TEA and lower pH in order to keep the retention time of the sulfates at less than 65 min. The selectivity of the glucuronides is not very sensitive to changes in pH, temperature and TEA concentration in sharp contrast to the sulfates. [16] As expected, k' for both sulfates

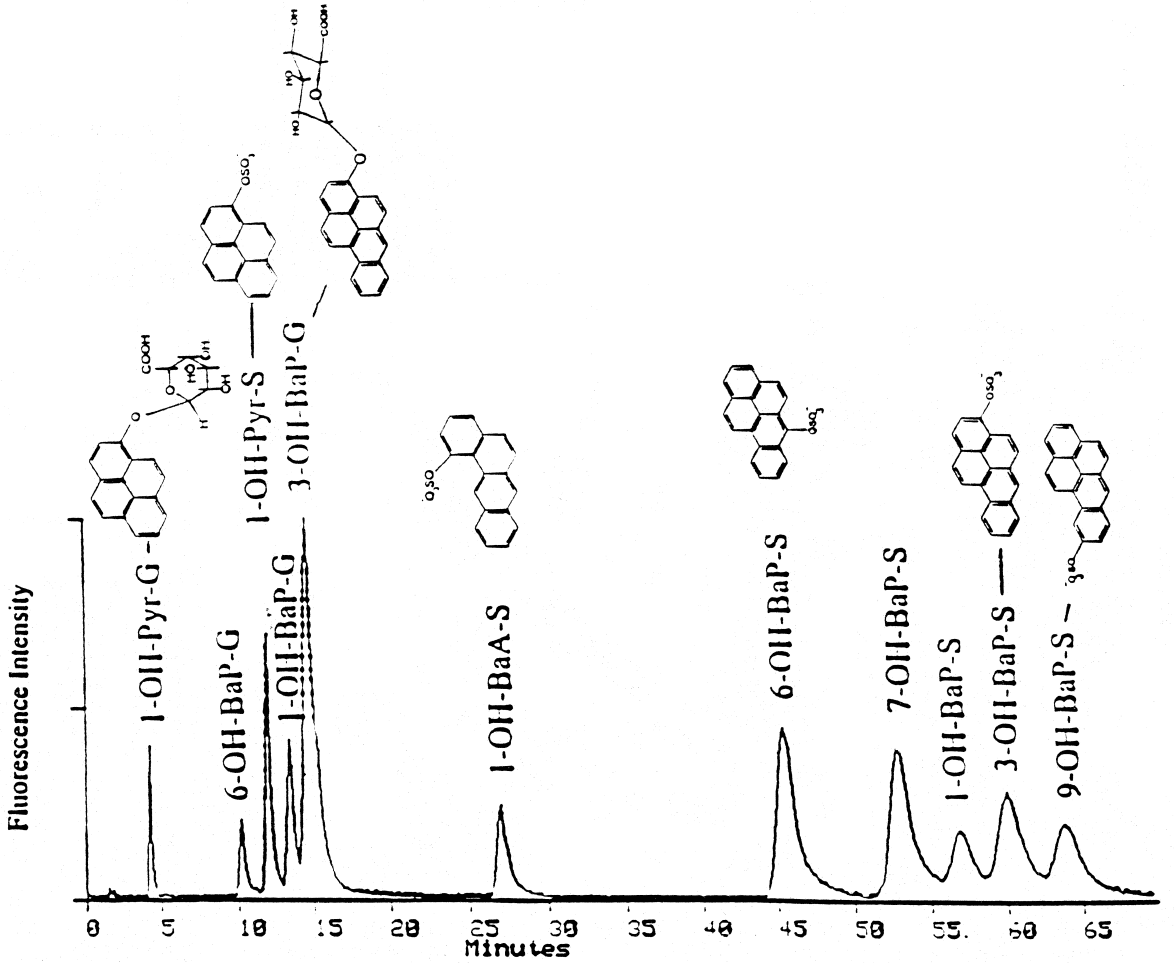

Fig. 1. HPLC separation of BaP sulfate and glucuronide isomers, 1-OH-Pyrene sulfate, 1-OH-Pyrene glucuronide and 1-OH-Benz[*a*]anthracene sulfate. Column: Vydac 201TP; mobile phase: 1.8% TEA in water (to pH 4.2 with acetic acid)–acetonitrile (75:25); 2 ml/min at 30°C.

and glucuronides is very sensitive to a change in acetonitrile concentration in the mobile phase.

Acetonitrile concentrations greater than 25% resulted in a dramatic decrease in both retention and selectivity, with 1-OH-Pyr-S coeluting with 1-OH-BaP-S. At levels less than 25% acetonitrile in the mobile phase, retention and selectivity increased resulting in good separation of the conjugates. At pH greater than 1, the BaP sulfates exist as anions ($pK_a = 1$) [9]. We hypothesize that at pH 4.6 TEA forms an ion-pair with the BaP sulfate anion to form a neutral species which is retained on the C_{18} stationary phase. At this pH, most of the BaP glucuronides exist as neutral species ($pK_a = 5$) [9]. A plot of k' vs. pH is shown in Fig. 2. Retention is constant for 1-OH-Pyr-G between pH 3.5–7. This means that pH is not an effective parameter in the manipulation of retention for 1-OH-Pyr-G. Indeed, only the parameter of acetonitrile concentration (at less than 25%) in the mobile phase proved to be useful in retention time manipulation for 1-OH-Pyr-G. There is a dramatic increase in retention and selectivity for BaP glucuronides and 1-OH-Pyr-S at a pH of 3.5. Retention then decreases at pH 5 with a rapid decrease in relative retention between 1-OH-Pyr-S and 6-OH-BaP-S with these two conjugates

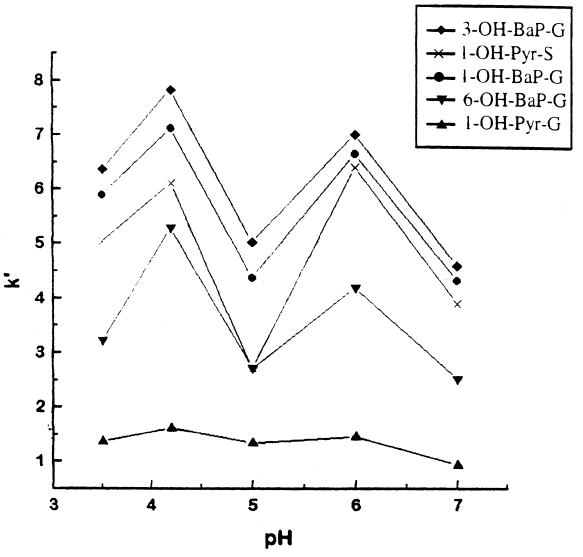

Fig. 2. Influence of pH on the capacity factor (k') of BaP glucuronide isomers, 1-OH-pyrene glucuronide and 1-OH-pyrene sulfate. Column: Vydac 201TP; mobile phase: 1.8% TEA in water–acetonitrile (75:25); 2 ml/min at 30°C.

coeluting. Retention and selectivity then increase once again but the relative retention between 1-OH-Pyr-S and 1-OH-BaP-G decreases.

The influence of the concentration of TEA was also studied, and a plot of k' vs. TEA concentration is shown in Fig. 4. Retention increases with increasing concentration of TEA and then decreases at concentrations greater than 1%. The decrease is more gradual for the pyrene conjugates. Changes in the relative separation of the BaP sulfate isomers were not observed as a function of either pH or TEA concentration.

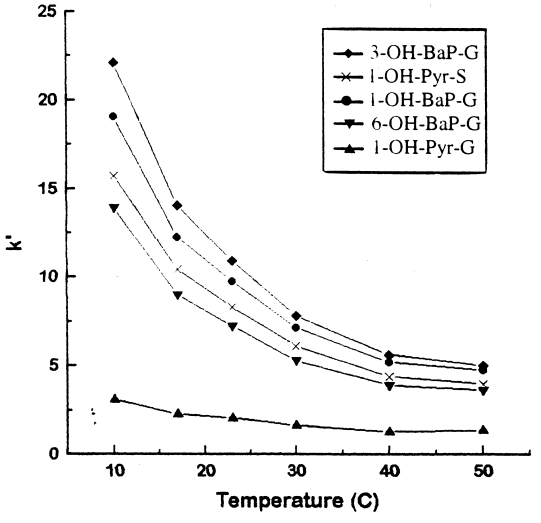

Fig. 3. Influence of temperature on the capacity factor (k') of BaP glucuronide isomers, 1-OH-pyrene glucuronide and 1-OH-pyrene sulfate. Column: Vydac 201TP; mobile phase: 1.8% TEA in water (to pH 4.2 with acetic acid)–acetonitrile; and 2 ml/min flow-rate.

Fig. 1 also shows that the retention of 1-OH-BaA-S is such that it elutes long after pyrene sulfate and glucuronide and the BaP glucuronides. In addition, it elutes much earlier than the BaP sulfates. This makes BaA sulfate conjugate an excellent choice as a potential biomarker since small changes in pH, temperature, TEA and acetonitrile concentrations in the mobile phase will not result in it coeluting with any of the other conjugates studied. Optimum separation was achieved with the Vydac 201 TP column with 1.8% TEA in water (to pH 4.2 with glacial acetic acid)–acetonitrile (75:25) at 30°C and 2 ml/min.

In the second phase of this work, the isolation and LC methods were utilized for preliminary urinary assay of the isomers of BaP and pyrene sulfates and glucuronides in smokers' urine. The urine samples from the five smokers gave HPLC peaks that had retention times similar to authentic standards of 1-OH-Pyr-S, 1-OH-Pyr-G,1- and 9-OH-BaP-G, 3- and 7-OH-BaP-G and 1-, 3-, 6- and 7-OH-BaP-S. Tentative peak assignments were made for the conjugates based on similar retention times. Serious quantitation efforts were only attempted for 3-OH-BaP-S (Fig. 5b). Based on these tentative peak assignments we were able to quantify 3-OH-BaP sulfate in two of the urines by comparison with a standard curve (Fig. 6) generated from spectral peak heights of authentic 3-OH-BaP-S standard. This is what is expected since 3-OH-BaP is known to be the most prominent phenol in mammalian systems and the sulfate is formed from the phenol during the phase 2 metabolic process. Levels of 67.3 and 42.1 pg/ml of 3-OH-BaP sulfate were detected in the urines of two smokers. The detection limit (signal-to-noise$>$4) of 3-OH-BaP-S was 0.25 pg/ml. The standard curve showed no significant deviation from linearity and had correlation coefficients of 0.99. The y-intercept of the curve was not significantly different from zero at the 95% confidence level. It was not possible to

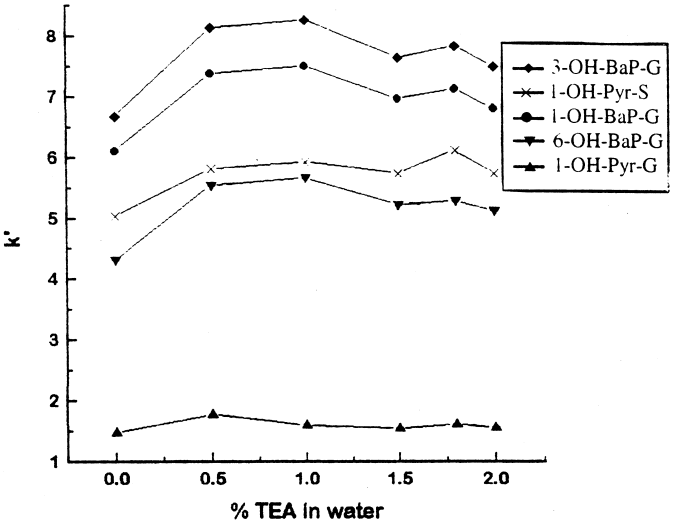

Fig. 4. Influence of triethylamine concentration on the capacity factor (k') of BaP glucuronide isomers, 1-OH-Pyrene glucuronide and 1-OH-Pryene sulfate. Column: Vydac 201TP; mobile phase: TEA in water (adjusted to pH 4.2 with acetic acid–aceotonitrile (75:25); 2 ml/min.

positively identify and quantify the other conjugates since they co-eluted with other compounds. No BaP sulfates or glucuronides were detected in nonsmokers urine. The fluorescence detector conditions were set and maximized to measure BaP sulfate and glucuronide conjugates ($\lambda_{ex/em}=300/389$ nm). The pyrene fluorophore ($\lambda_{ex/em}=296/360$ nm) is a smaller conjugated system compared to BaP and so has a much smaller absorptivity and fluorescence at $\lambda_{ex/em}=300/389$ nm. Wavelength programming would have yielded better results but this feature is not found in the Kratos 980 fluorescence detector used in this work.

Fig. 5 shows chromatograms of blank (nonsmokers) urine, urine from a smoker, blank urine spiked with the five BaP sulfates and five BaP glucuronides, and a mixture of BaP sulfates and glucuronides and benz[*a*]anthracene-1-sulfate. At pH 4.2, the sulfates exist as anions ($pK_a=1$) while the glucuronides exist as neutral species ($pK_a=5$). Previously, we hypothesized that at pH 4.2, TEA ion pairs with the BaP sulfate anions to form a neutral species which is retained on the C_{18} stationary phase [16]. The separation that was achieved for the sulfates and glucuronides is superior to what was achieved previously by other workers [9,10]. Base line separation of the BaP sulfates was first reported in connection with the current study [16]. We have not detected any endogenous compounds at the ppt level that fluoresce ($\lambda_{ex/em}=300/389$ nm) and elute at a t_R between 35 and 60 min, the region in which the BaP sulfates elute.

A recovery study was conducted for 6-OH-BaP-S and 6-OH-BaP-G on triplicate spiked urine (nonsmokers) at four different concentrations (5,10,15 and 20 pg/ml). Recoveries (mean±S.D.) were 87.0±2.6%, 83.1±6.5%, 84.6±3.2% and 82.9±3.3% respectively for the sulfates and 89.4±1.3%, 83.1±4.3%, 76.7±4.7% and 85.3±4.9% respectively for the glucuronides. The standard curves showed no significant deviation from linearity and had correlation coefficients of 0.98 for the sulfates and 0.95 for the glucuronides. The *y*-intercepts of the curves were not significantly different from zero at the 95% confidence level. In all of the recovery studies, a mixture of standard was used (i.e., 1-, 3-, 6-, 7- and 9-OH-BaP sulfates and 1-, 3-, 6-, 7, and 9-OH-BaP glucuronides). The recovery of 9-OH-BaP-S was low compared to the other sulfates. Unfortunately, it was not possible to quantify the recovery of 9-OH-BaP-S, but a comparison of the chromatograms of the spiked urine samples with that of the standards suggests poor recovery. This probably means that the TBA^+ extraction protocol is not

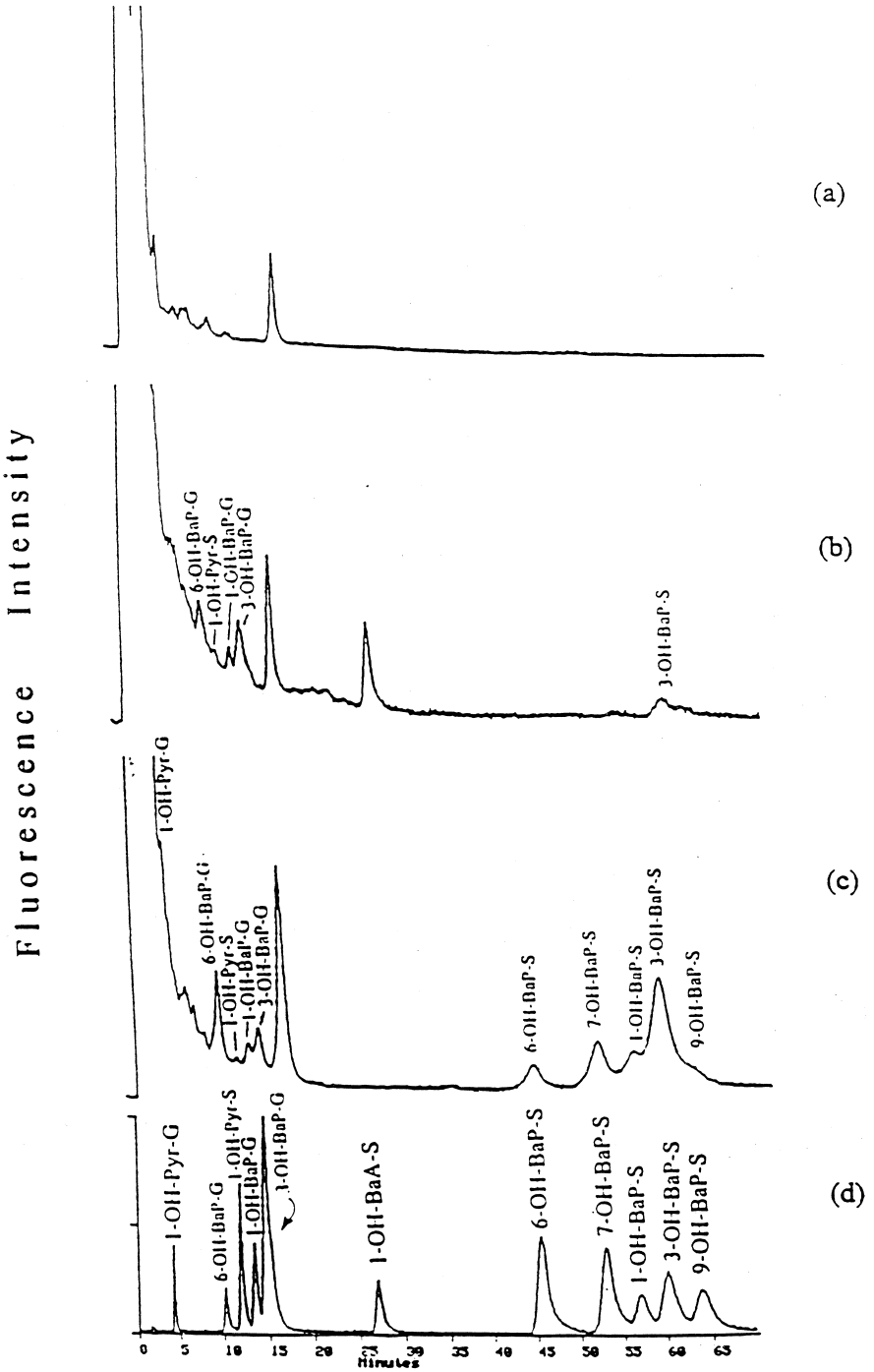

Fig. 5. Reversed phase HPLC chromatograms of (a) urine from a nonsmoker, NSI (b) urine from a smoker, SM5 (c) spiked nonsmokers' urine and (d) mixture of conjugate standards of pryene, B[*a*]A and BaP. Column: Vydac 201 TP; mobile phase: 1.8% TEA in water (to pH 4.2 with acetic acid)–acetonitrile (75:25); 2 ml/min at 30°C. Details of sample preparation are given in the Experimental Section.

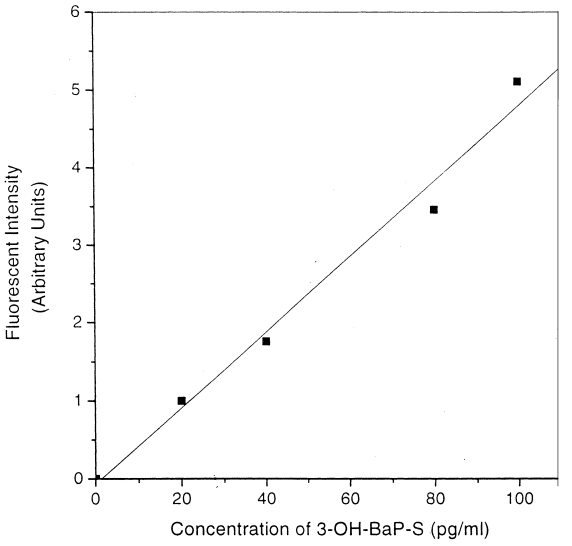

Fig. 6. Standard curve showing effect of 3-OH-BaP-SO4 concentration on fluorescence yield (absorption–emission wavelengths at 300/389 nm).

very efficient for 9-OH-BaP-S. The detection limit (signal-to-noise$>$4) of urinary BaP glucuronides was 0.14 pg/ml and for BaP sulfate it was 0.25 pg/ml.

The extraction efficiency with chloroform after ion-pairing with TBA^+ is better than 80% for both the sulfates (except for 9-OH-BaP-S) and glucuronides. However, the extraction technique gave consistently higher recoveries for the glucuronides at pH 2.2 (data not shown). At pH 4.6, with no ion-pairing with $TBA^+HSO_4^-$, there was negligible recovery of the sulfates and less than 20% recovery of the glucuronides. Therefore, this method shows promise for the quantitation of BaP sulfates and glucuronides in urine and possibly other biological fluids if a suitable analytical technique is utilized. These conjugates can potentially be used as biomarkers of exposure to BaP.

This method avoids the use of the enzymatic and/or acid hydrolysis technique which is the usual approach in the analysis of the sulfate and glucuronide conjugates in biological fluids. Selected sulfates and glucuronides can be separated in a single HPLC run in 1h.

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