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Separation of benzo[*a*]pyrene sulfate isomers by reversed-phase liquid chromatography

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Abstract

A liquid chromatographic (LC) method has been developed for the separation of five isomers of benzo[*a*]pyrene sulfate. This method utilizes a polymeric C_{18} column and isocratic elution with acetonitrile–water and triethylammonium acetate. Temperature, mobile phase composition, pH, type of C_{18} stationary phase and triethylamine concentration were found to influence retention and selectivity, and separations were optimized by proper choice of these parameters.

Keywords: Optimization; Benzo[*a*]pyrene sulfates; Polynuclear aromatic hydrocarbons

1. Introduction

Benzo[*a*]pyrene (BaP) is an environmentally important chemical carcinogen. Metabolites of BaP also demonstrate carcinogenic activity, possibly as a consequence of reaction with and modification of nucleic acids and proteins. BaP is metabolized to produce a complex mixture of epoxides, quinones, phenols, dihydrodiols, triols, and tetraols. [1,2]. Each of these metabolites may be further enzymatically conjugated with glutathione, glucuronide or sulfate to form even more water soluble compounds and thus facilitate urinary and biliary excretions. BaP sulfates have been found in biological fluids such as urine and stool [3].

Although liquid chromatography is the preferred separation method for BaP sulfates, the separation of BaP sulfate positional isomers is very difficult to achieve. For example, Teffera et al. [4] were only able to separate 3-hydroxybenzo[*a*]pyrene sulfate (3-OH-BaP-S) from 1-hydroxybenzo[*a*]pyrene glucuronide and 3-hydroxybenzo[*a*]pyrene glucuronide by microbore HPLC. Merrick and Selkirk [5] 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-hydroxy-benzo[*a*]pyrene sulfate (9-OH-BaP-S) all co-eluted with 1-OH-BaP-S [5]. Autrup et al. did not achieve full separation of various BaP sulfate isomers by reversed-phase HPLC [6], and Richter et al. [7] were unable to identify 1-OH-BaP-S produced by hepatocytes, because of insufficient separation. Because of the difficulty in separating these sulfate isomers, the

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usual analytical approaches for analysis of BaP conjugates have been indirect. Such approaches involve the measurement of hydroxylated metabolites after liberation from the glucuronide and sulfate conjugates by acid or enzyme hydrolysis [5,8]. We report here on a liquid chromatographic method for the direct separation of five BaP sulfates.

2. Experimental[2]

2.1. Apparatus and conditions

A Varian Model 5000 HPLC pump equipped with a Linear 206 PHD UV detector operated at 254 nm, a Rheodyne 7125 injector with a 20-μl loop and a Dionex AI 450 chromatographic data acquisition system were used.

The following commercial C_{18} columns were used in this study: Zorbax ODS (Mac Mod, Chadds Ford, PA, USA), Hypersil ODS (Keystone, State College, PA, USA) and Vydac 201 TP reversed-phase C_{18} (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. A fourth column (designated C_{18}-A) was prepared through polymeric surface modification chemistry under conditions intended to produce a densely loaded stationary phase [9,10]. 3.23 g of 3 μm YMC 200 Å spherical silica (lot 910805C, surface area 200 m^2/g) was equilibrated with moist air by aspiration. The silica was suspended in 100 ml xylene and 10 ml of octadecyltrichlorosilane was added. The slurry was stirred for 10 min, and heated to reflux for 1.5 h. The bonded silica was filtered and washed, and a 25 cm×4.6 mm I.D. column was prepared. A sample of the material was submitted for carbon analysis (Atlantic Microlab, Norcross, GA, USA). The bonded phase was determined to have a carbon loading of 16.28% (4.93 $\mu mol/m^2$).

2.2. LC conditions

Each of the four LC columns was characterized using a column selectivity test mixture developed at NIST (SRM 869). The selectivity factor $\alpha_{TBN/BaP}$ = (k' tetrabenzonaphthalene/k' benzo[*a*]pyrene) was calculated under various conditions [11,13].

The mobile phase was prepared as follows; reservoir A: an aqueous solution of 1% (v/v) triethylamine (TEA) adjusted to pH 4.6 with glacial acetic acid; reservoir B: acetonitrile. Separations were carried out isocratically at a flow-rate of 2.0 ml/min at various temperature and mobile phase conditions. Prior to analysis, each column was conditioned with 100% acetonitrile. Thereafter, the column was equilibrated with at least 30 ml of mobile phase.

2.3. Reagents

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

HPLC grade acetonitrile and water were purchased from J.T. Baker (Phillipsburg, NJ, USA). HPLC grade triethylamine and glacial acetic acid were obtained from Fisher Scientific (Pittsburgh, PA, USA). Standard Reference Material (SRM) 869 'Column Selectivity Test Mixture for Liquid Chromatography' was obtained from the Standard Reference Materials Program at the National Institute of Standards and Technology (Gaithersburg, MD, USA).

3. Results and discussion

Retention behavior of the BaP sulfate isomers was investigated for several different C_{18} columns. The columns were classified as monomeric or polymeric using the shape selectivity parameter $\alpha_{TBN/BaP}$, as described in the certificate of analysis for SRM 869 and in other published reports [11–13] (see Table 1).

[2]Certain commercial equipment, instruments, or materials are identified in this report to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology or the University of North Carolina, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

Table 1
Column selectivity $\alpha_{TBN/BaP}$ (smaller values indicate enhanced shape selectivity)

Temperature (°C)	Zorbax ODS	Vydac 201 TP	PAH Hypersil	C_{18}-A
23	1.66	0.56	0.50	0.38
30	1.77	0.63	0.63	0.46
40	1.82	0.80	0.78	0.62

This parameter has been shown to correlate well with a column's ability to resolve isomers and other closely related compounds based on molecular shape. This shape recognition capacity is known to vary with bonding chemistry (i.e., monomeric or polymeric surface modification) and column temperature [11,14]. Separation of the test mixture (SRM 869) under specified conditions, with subsequent determination of $\alpha_{TBN/BaP}$, enables column classification [11–13]. Shape selectivity can be further adjusted through control of column temperature.

The Zorbax ODS column exhibited properties typical for a monomeric phase, whereas the Vydac 201 TP, Hypersil PAH and C_{18}-A columns exhibited polymeric phase properties. The best separation of the five BaP sulfate standards was obtained for chromatographic systems for which $0.57 < \alpha_{TBN/BaP} < 0.63$ (Fig. 1 and Table 1). For the Vydac column, $\alpha_{TBN/BaP}=0.63$ at 30 °C while for C_{18}-A, $\alpha_{TBN/BaP}=0.62$ at 40 °C. Similar separations of the BaP sulfate standards were obtained for these two columns under these conditions. A different elution order was observed for the monomeric C_{18} column (for which $\alpha_{TBN/BaP}=1.77$). In general, the shape selectivity factor $\alpha_{TBN/BaP}$ was found to provide a useful indication of column selectivity towards the BaP sulfate isomers. Columns with similar selectivity factors such as the Vydac 201TP and C_{18}-A provided similar separations of the BaP sulfate isomers (although the latter example exhibited slightly less efficiency).

As can be observed in Fig. 1, the retention of 1-OH-BaP-S is reduced relative to 3-OH-BaP-S with increasing shape recognition of the chromatographic system (as indicated by decreasing $\alpha_{TBN/BaP}$ values). Coelution of the two components is expected for an intermediate column with $\alpha_{TBN/BaP} \approx 1.2$, with a reversal in elution order for lower values ($\alpha_{TBN/BaP} < 0.7$). The opposite trend is observed for 3-OH-BaP-S and 9-OH-BaP-S. The retention of 3-OH-BaP-S increases relative to 9-OH-BaP-S for decreasing values of $\alpha_{TBN/BaP}$, and coelution of the components occurs for $\alpha_{TBN/BaP}=0.31$ (see Fig. 5). A measure of column selectivity towards the BaP sulfate isomer is plotted as a function of the selectivity factor $\alpha_{TBN/BaP}$ in Fig. 2. The k' ratios for consecutive BaP sulfate isomers (i.e., k' ratios of 7-/6-, 1-/7-, 3-/1-, and 9-/3-BaP-S) were determined for different column selectivities resulting from different columns and column temperatures. For each of these cases, the minimum k' ratio for a separation was determined (α_{min}), and plotted as a function of the selectivity factor $\alpha_{TBN/BaP}$ (Fig. 2). The Hypersil Green PAH column ($\alpha_{TBN/BaP}=0.63$, $\alpha_{min}=1.029$) exhibited slightly different selectivity than would be predicted by its selectivity factor. The optimal column selectivity for 1-OH-BaP-S, 3-OH-BaP-S and 9-OH-BaP-S was observed for $\alpha_{TBN/BaP}=0.57$ to 0.63.

As expected, retention of the isomers decreased with increasing concentration of acetonitrile in the mobile phase. At acetonitrile concentrations greater than 30% there is a dramatic decrease in both retention and selectivity, and at levels of 40% and greater acetonitrile, the BaP sulfates eluted in the void volume. At a pH greater than 1, the BaP sulfates exist as anions ($pK_a \approx 1$) [4]. 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. A plot of k' vs. pH is shown in Fig. 3. Retention is constant for pH<4.6, and at higher pH, retention decreases markedly. 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 1.5%. Changes in the relative separation of the BaP sulfate isomers was not observed as a function of either pH or TEA concentration.

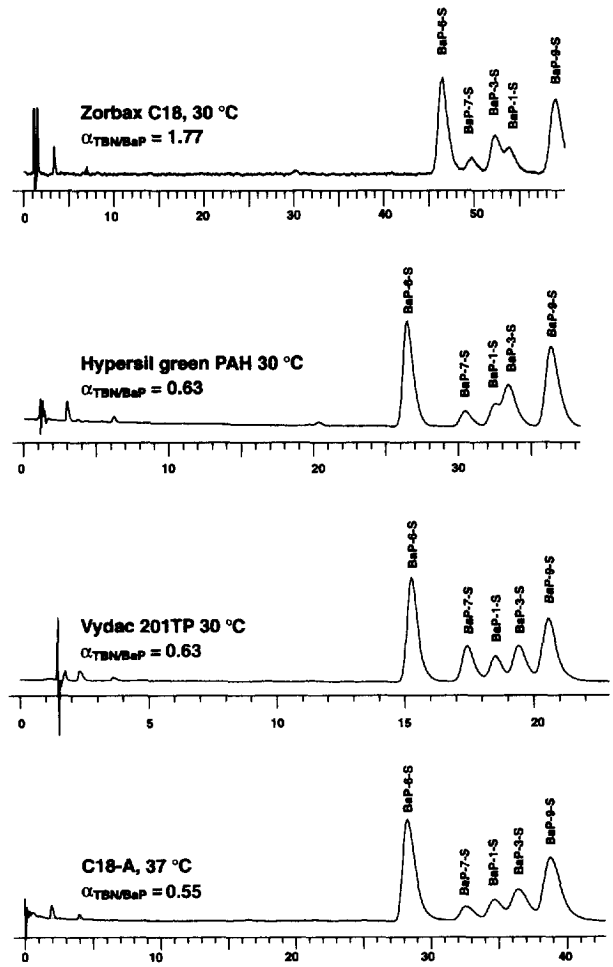

Fig. 1. Separation of BaP sulfate standards on commercial and custom C_{18} columns. See Section 2.2 for an explanation of $\alpha_{TBN/BaP}$.

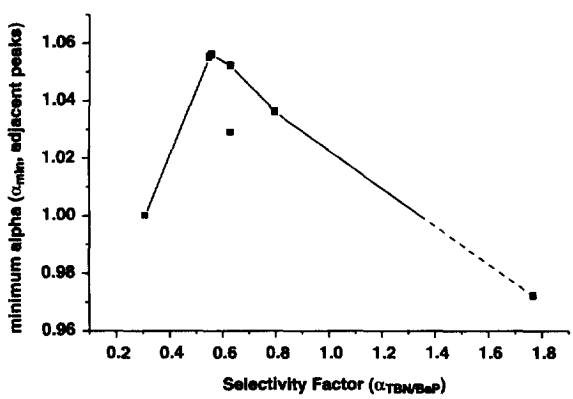

Fig. 2. Correlation of column selectivity toward BaP-sulfate isomers with the column selectivity factor $\alpha_{TBN/BaP}$. The dotted line indicates a reversal in elution order for 1- and 3-OH-BaP-S.

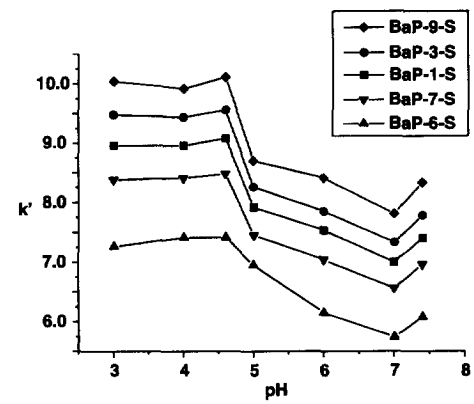

Fig. 3. Influence of pH on capacity factor (k') of BaP sulfate isomers. Column: Vydac 201TP; mobile phase: 1% TEA in water–acetonitrile (7:3) at 30 °C and 2 ml/min.

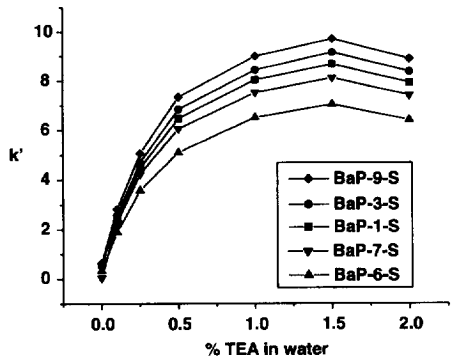

Fig. 4. Influence of triethylamine concentration on the capacity factor of BaP sulfate isomers. Column: Vydac 201TP; mobile phase: TEA in water (pH adjusted with acetic acid)–acetonitrile (7:3); 2 ml/min.

Fig. 5 illustrates changes in retention and selectivity that occur with changes in temperature. The retention of 3-OH-BaP-S varies significantly relative to the other isomers as a function of temperature. With the Vydac 201 TP column at temperatures less than 15 °C, 3-OH-BaP-S coelutes with 9-OH-BaP-S, whereas at 50 °C and above, 3-OH-BaP-S coelutes with 1-OH-BaP-S. Optimum separation was achieved with the Vydac 201 TP column at 30 °C (Figs. 1 and 5).

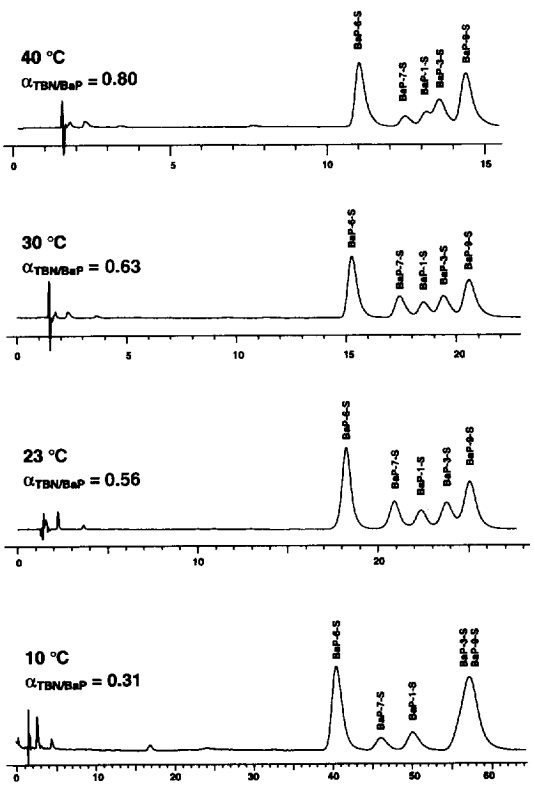

Fig. 5. Separation of BaP sulfate standards on Vydac 201TP column at various temperatures.

4. Conclusions

Retention and selectivity of BaP sulfate isomers can be controlled by appropriate choices of pH, temperature, TEA concentration, and type of stationary phase. The best separation of the BaP sulfate standards was obtained with a polymeric C_{18} column operated at 30 °C ($\alpha_{TBN/BaP} \approx 0.63$). This separation, when combined with fluorescence detection, offers the potential for the determination of BaP sulfate isomers with high sensitivity and specificity.

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