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Separation of polycyclic aromatic hydrocarbon metabolites by γ-cyclodextrin-modified micellar electrokinetic chromatography with laser-induced fluorescence detection

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Abstract

Using a modified micellar buffer consisting of γ-cyclodextrin (γ-CD) and sodium dodecyl sulfate (SDS), we have obtained separations of hydroxy-polycyclic aromatic hydrocarbons (hydroxyPAHs). These compounds are oxidative products of mammalian PAH metabolism. The analytes were detected with a commercial laser-induced fluorescence (LIF) detector. A number of hydroxyPAH isomers could be separated by changes in γ-CD concentration. Baseline resolution of 12 hydroxyPAHs was obtained using 30 m*M* borate, 60 m*M* SDS and 40 m*M* γ-CD. The particular site substitution of the hydroxy group can produce changes in the hydroxyPAH fluorescence spectrum, and the effect of optical filter selection was studied for the LIF detection. The mass detection limits were in the $(0.08–0.5)\times10^{-15}$ mol range. To our knowledge, this is the first report of the separation of metabolic products of PAHs (and several positional isomers) using γ-CD and micellar electrokinetic chromatography. © 1998 Elsevier Science B.V.

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1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) constitute an important class of environmental pollutants, and the US Environmental Protection Agency has classified 16 PAHs as priority pollutants [1]. While many PAHs are classified as potential carcinogens, human exposure is universal due to air pollution from automobiles, fires, cigarette smoke, and foodstuffs prepared by smoking or storing in charred barrels. Because PAHs are commonly found as complex mixtures and in low levels, they are often analyzed by techniques such as high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE), which are noted for their separation efficiency.

While we are aware of no published CE separations of PAH metabolite isomers, there has been a wide variety of native PAH separations. A few examples follow. By adding tetraalkylammonium ions as the micellar component, Nie et al. have separated PAHs by micellar electrokinetic chromatography (MEKC) with laser-induced fluorescence (LIF) detection [2]. Akbay et al. used phosphated surfactants as a micellar component in PAH separations [3]. Shi and Fritz have also obtained sepa-

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rations of PAHs using CE with an anionic surfactant, sodium dioctyl sulfosuccinate (DOSS), in a buffer containing approximately 40% acetonitrile [4]. The addition of acetonitrile acts to inhibit micellar formation, and the separation mechanism is thought to be based on the differences in the strength of the PAH–DOSS complexes. Recently, Yan et al. used capillary electrochromatography with LIF detection to obtain separation efficiencies up to 400 000 theoretical plates per meter and limits of detection from 10^{-17} to 10^{-20} mol [5]. In an approach similar to the present study, MEKC with both sodium dodecyl sulfate (SDS) and β-cyclodextrins (β-CDs) was used to separate native PAHs [6,7]. Terabe et al. have also used MEKC with CD to separate 16 PAHs [8]. The addition of CD alters the partitioning of the analyte between the micellar and aqueous–CD phases.

Since the toxicity of the native PAHs can vary between mammalian species, and even within individuals of a given species, the metabolic products of PAHs can be important markers for exposure and future health risks. Many studies have been reported on the analysis of PAH metabolites in neat solutions or in urinary excretions [9–23]. The first reports by Jongeneelen et al. isolating hydroxyPAH metabolites in urine involved enzymatic hydrolysis of the glucuronide and sulphate conjugates followed by extraction with C_{18} reversed-phase cartridges [9,10]. The extract was then separated using HPLC with fluorescence detection. Subsequent researchers have included other separation methods and detected more PAH metabolites. Ariese et al. incorporated LIF detection with HPLC to produce a detection limit of 8 ng/l for 3-hydroxybenzo[*a*]pyrene (3-OHBaP), and after chemical derivatization, they used laser-excited Shpol'skii spectrometry to achieve a 0.5-ng/l detection limit [14].

One of the challenges in the separation of PAH derivatives is the number of isomers possible from each native PAH. For BaP there are 12 commercially available monohydroxy derivatives plus several more dihydrodiols. The major metabolite of BaP is 3-OHBaP, but the low fraction of hydroxyBaPs excreted in urine makes the detection of the individual hydroxyBaP isomers quite difficult. There have been no reports on the MEKC separation of PAH metabolites; however, Copper and Sepaniak have used MEKC to separate methylated BaP isomers [24].

It has been recognized that 1-hydroxypyrene (1-OHpyrene) can be used as an indicator for total PAH exposure [9,10]. Several studies have shown strong correlations between airborne PAH levels and the amount of 1-OHpyrene excreted in urine [11,12,15,23]. Nevertheless, biases introduced from airborne collection and variations in the metabolic fate among individuals have indicated that further investigations for PAH markers are necessary [12,14]. Thus, if exposures to several PAHs are to be monitored simultaneously in urinary excretions, a method will be required to separate the metabolites, which may number 10 or more times greater than the individual PAHs.

With this present study, we demonstrate the first separation of 12 hydroxyPAH compounds using γ-CD-MEKC. For the detection of the analytes, a commercial CE–LIF system was modified with a UV transmitting optical fiber to transmit near-UV He–Cd laser light to the capillary detection window. Using 1-nl injection volumes, sub-femtomole detection limits were achieved with high separation efficiencies.

2. Experimental

2.1. Reagents

The running buffer consisted of 30 m*M* borate, 60 m*M* SDS (both purchased from Sigma, St. Louis, MO, USA), and 5–40 m*M* γ-cyclodextrin (Advanced Separation Technologies, Whippany, NJ, USA). The pH was adjusted to 9 with hydrochloric acid. Deionized water (resistivity greater than 10 MΩ) was obtained from a Milli-Q filter system (Millipore, Bedford, MA, USA) in combination with a Barnstead OrganicPure system (Newton, MA, USA). A sonicator was used to dissolve the γ-CD. The buffer solution was filtered through a 0.22-μm filter before use in the CE instrument. Buffer solutions were stored in a refrigerator and were disposed after 3 days. Spectrophotometric-grade acetonitrile and benzene were purchased from Baxter (Muskegan, MI, USA). HydroxyPAH metabolites (Midwest Research Institute, Kansas City, MO, USA) were initially diluted in 2–4 ml benzene. To produce stock solutions compatible with CE, 20 μl of each hydroxy-

PAH was diluted 100 times in acetronitrile–water (45:55, v/v). The dilutions produced hydroxyPAH concentrations in the 1×10^{-5} *M* range. Further dilutions were made with acetonitrile–water (45:55, v/v).

2.2. Equipment

The γ-CD modified MEKC separations were performed with a P/ACE 2200 (Beckman, Fullerton, CA, USA) which is capable of UV absorbance or LIF measurements. For the LIF measurements, a UV-transmitting fiber optic cable was used to couple the 325-nm laser light from a He–Cd laser (Model 2056–8125M, Omnichrome, Chino, CA, USA) to the detection region. The fluorescence collected from the detection region was passed through either a 400-nm interference filter (Corion, Holliston, MA, USA) or two glass cut-on filters at 366 nm (Oriel, Stratford, CT, USA). The capillary tubing had an inner diameter of 50 μm and an outer diameter of 360 μm. The total capillary length was 37 cm with an effective length of 31.6 cm. Before each injection, the capillary was rinsed with 1.0 *M* NaOH for 5 min and buffer solution for 3 min. All chromatograms were acquired at a constant temperature of 21°C.

The hydroxyBaP fluorescence spectra were recorded on a SPEX Fluorolog τ-2 spectrofluorometer (Isa Jobin Yvon, Edison, NJ, USA). The samples were placed in a quartz cuvette with a 1-cm path length for the excitation light beam. The emission monochromator scan interval was 3 nm, and the dwell time at each interval was 0.2 s.

3. Results and discussion

3.1. Fluorescence spectra

The selection of optical filters is an important detail for good limits of detection in CE–LIF. While it is obvious that the laser light scattered by the capillary walls must be efficiently rejected from the detector, the optimal bandwidth for the filter selection can vary depending on the criteria used. In a previous study of PAH separation/detection using CE–LIF, Zare and co-workers compared the change in limit of detection (LOD) using either a bandpass filter centered at 400 nm or the combination of a 280-nm longpass filter with two 600-nm shortpass filters [2]. They observed that the 400-nm bandpass filter produced better LODs for most PAHs.

In addition to the different absorptivities of the PAH and PAH metabolites at the He–Cd laser wavelength of 325 nm, fluorescence spectra vary from the smaller PAHs like naphthalene to the larger ones like dibenzo[*ghi*]perylene. These changes can be understood from the differences in molecular orbital sizes (energies). One of the added complexities of the monohydroxyPAHs is that the fluorescence spectra shifts depending on the position of the hydroxy group. These shifts can be seen for a few monohydroxybenzo[*a*]pyrenes in Fig. 1. The hydroxyBaP concentrations ranged from 1 to 10 μ*M* to produce fluorescence counts acceptable for the light detector and to avoid microcrystal growth.

The spectra from 1-OHBaP (not shown), 3-OHBaP and 9-OHBaP are very similar in that their fluorescence emission is primarily in the range from 400–500 nm. The fluorescence intensities from these three hydroxyBaPs are quite high. 7-OHBaP exhibited a different fluorescence pattern, as it fluoresced from 400 to 725 nm with two broad peaks centered at 455 and 590 nm. The extra intensity at 650 nm came from the excitation source via multiple-order transmission through the monochromator.

From a comparison of these hydroxyBaP spectra, a bandpass filter centered at 450 nm would provide

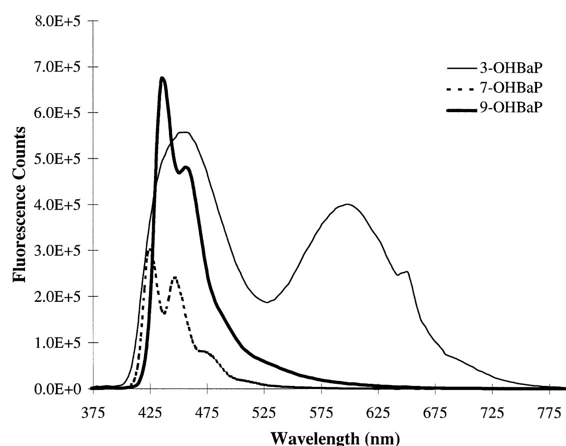

Fig. 1. Fluorescence emission spectra of monohydroxybenzo[*a*]pyrenes. Excitation wavelength is 325 nm.

favorable overlap of all spectra, but might exclude part of the 7-OHBaP intensity. Therefore, it may be desirable to choose a bandpass filter centered on emission from a single hydroxyBaP, since the detection of any BaP metabolite should be indicative of the total exposure to BaP. Alternatively, a longpass filter could be used in a general approach to establish PAH exposure. The longpass filter transmits fluorescence from the hydroxyPAHs, and the resulting electropherogram would produce a unique set of metabolite peaks that could be used as a 'fingerprint' in the confirmation of exposure to a specific PAH.

To determine the best optical filter combination for the transmission of analyte fluorescence and the rejection of scattered laser light, we tested two different types of optical filters. A bandpass interference filter centered at 400 nm with a 35-nm full width at half maximum (FWHM) produced a stable baseline and analyte LODs in the 5×10^{-7} *M* range. The second set of filters was comprised of a combination of two cut-on filters and produced better LODs. The long warm-up time for the laser was apparent as a wavy baseline in the early part of the day. To ensure the best results, the laser was turned on at least 2 h before the start of the MEKC run, and the pair of cut-on filters was used in the commercial CE instrument. Alternative methods of fluorescence signal selection, such as spectrally resolved acquisition, could provide additional peak confirmation. The present commercial CE instrument was designed with optical filters, and since replacing filters requires the shutdown of the CE instrument, filter switching was not a viable detection method.

3.2. Separation principles

When large hydrophobic analytes are separated with SDS-MEKC, the results are generally poor, with the analytes eluting very close to the micellar SDS. Organic modifiers are often used to increase the partitioning of the analyte into the aqueous phase, thus increasing the separation window. Large concentrations of organic modifier can, however, inhibit micellar formation. Several groups have examined the dynamics of CD-MEKC, and detailed information on CD-MEKC can be found therein [6,24–27]. Briefly, cyclodextrins can introduce a selectivity component that depends upon analyte size, shape, and chirality. The addition of cyclodextrins to separate large hydrophobic molecules also acts to solubilize the analytes and increase partitioning into the aqueous phase without inhibiting micellar formation.

3.3. Effect of γ-CD concentration

Since the sizes of the hydroxyPAHs in this study vary from small naphthols to five fused ring molecules, γ-CD was chosen over α-CD and β-CD for its large cavity diameter. As is demonstrated later, γ-CD-MEKC can resolve the positional isomers of the PAH metabolites; therefore, further investigations using α-CD and β-CD were not necessary. Fig. 2 contains electropherograms of hydroxy-PAHs in which a series of borate buffers with increasing γ-CD concentration were used. The buffer was 30 m*M* borate with 60 m*M* SDS, and the applied voltage on the 37-cm total length capillary was 12 kV. γ-CD concentrations are as noted.

The separation window from the first hydroxyPAH peak to the last hydroxyPAH peak ranged from 3 to 6 min. At a zero concentration of γ-CD (not shown), there was no separation, and the analytes were detected as a single peak. We believe that most of the hydroxyPAHs have pK_a values greater than 9, thus the neutral hydroxyPAHs were associated with the micellar phase and no separation existed. When only a small (5 m*M*) concentration of γ-CD was added, the 12 analytes split into nine peaks, as shown in Fig. 2a. In studies using γ-CD-MEKC separations of *ortho*-substituted chlorinated biphenyls, even a small concentration (1 m*M*) of γ-CD produced dramatic increases in the resolution [28]. From 5 to 10 m*M* γ-CD (data not shown) all but two of the hydroxyPAHs separated to some extent, with 1-OHBaP appearing as a shoulder on the 1-OHbenz[*a*]anthracene peak. By increasing the γ-CD concentration to 20 m*M*, all analytes could be separated; the resolution of the last two hydroxyPAHs in Fig. 2b is 0.41. Raising the γ-CD concentration to 30 m*M* results in shifts in migration order, causing two components to comigrate, data not shown. At 40 m*M* γ-CD the separation efficiency increases, producing baseline resolution of the 12 hydroxyPAHs in Fig. 2c.

The numbers of theoretical plates, *N*, from the

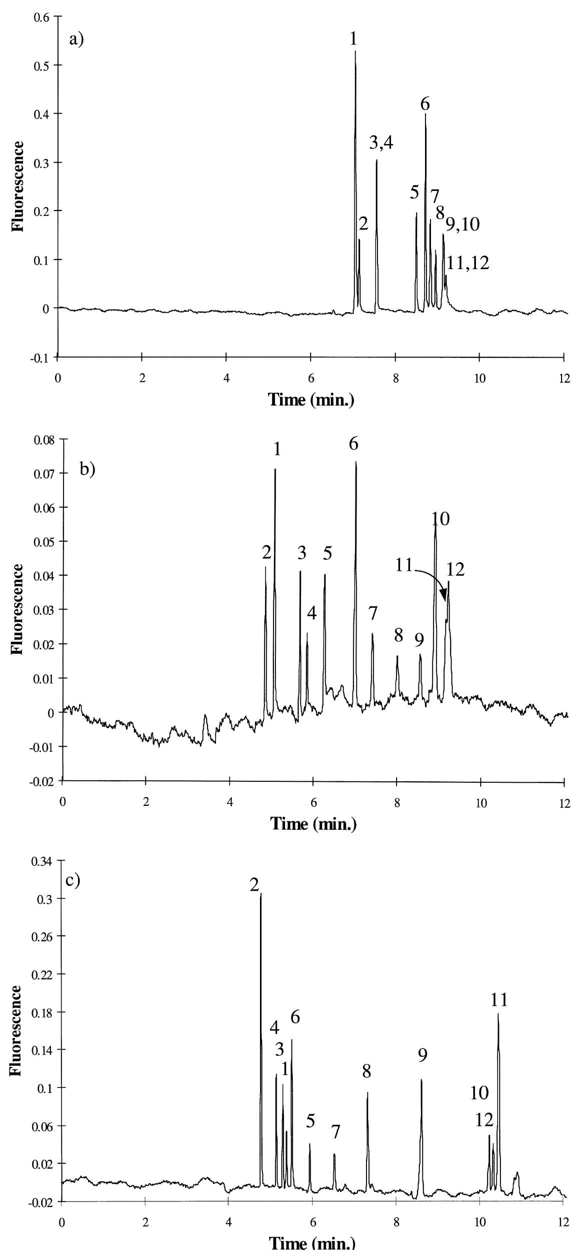

Fig. 2. Electrochromatograms showing separations of 12 hydroxy-PAHs. Separation conditions are: 37 cm×50 μm I.D. capillary, 12 kV, 30 m*M* sodium borate, 60 m*M* SDS. The peaks are identified as follows: (1) 3-OHBaP, (2) 7-OHBaP, (3) β-naphthol, (4) α-naphthol, (5) 9-OHBaP, (6) 1-OHpyrene, (7) 3-OHchrysene, (8) 3-OHbenz[*a*]anthracene, (9) 1- OHBaP, (10) 1-OHbenz[*a*]anthracene, (11) 1-OHbenzo[*b*]fluoranthene, (12) 2-OHindeno[1,2,3-*cd*]pyrene. (a) 5 m*M* γ-CD, (b) 20 m*M* γ-CD, (c) 40 m*M* γ-CD.

peaks in Fig. 2c range from 144 000 for 7-OHBaP to 484 000 for 1-OHbenz[*a*]anthracene. The equation used to calculate N was $N=5.54(t_r/w_{0.5})^2$, where t_r denotes the retention time, and $w_{0.5}$ the width at half height of the peak [26]. With LIF detection there was no peak from the analyte solvent, which is often used in UV detection to calculate the column void time, t_0. The use of UV detection to calculate capacity factors would necessitate higher hydroxyPAH concentrations. The relatively low solubilities of hydroxyPAHs in aqueous buffers at pH 9 produce unwanted effects, such as analyte dimer formation, microcrystal growth and peak shape irregularities.

By plotting the migration time versus γ-CD concentration, as shown in Fig. 3, the effect of the γ-CD on the separation window becomes apparent. From association with the γ-CD, the analytes' distribution coefficients into the micells are lowered, decreasing the migration time. Above a γ-CD concentration of 20 m*M*, the decrease in migration time levels off, except for 1-OHpyrene. The migration times of the last three PAH metabolites increase with increasing γ-CD concentration. Their complexation with γ-CD is very weak, and the lower electroosmotic flow from the buffer additives decreases the apparent mobility of these three metabolites.

We also examined the changes in the separation upon the addition of acetonitrile to the γ-CD modified buffer. The peaks broadened slightly, and the reduced electroosmotic flow increased the analysis time. While the separation window increased, we observed no further separation of co-eluting analytes at γ-CD concentrations of 5 and 20 m*M*. The lack of change in separation efficiency was expected, since the acetonitrile is not performing a different role in addition to that of the γ-CD.

3.4. Limits of detection

The ability of LIF to produce ultra-sensitive LODs for native PAH compounds has been demonstrated by workers in Zare's group [2,5]. The sensitivity available with LIF should produce similar results in analyses of hydroxyPAHs. Using the near-UV laser wavelength from a He–Cd laser, we have been able to detect 1-OHpyrene at a level of 8×10^{-8} *M* (peak-to-peak signal/noise ratio of >3). This analyte produced the best LOD. The smaller hydroxyPAHs

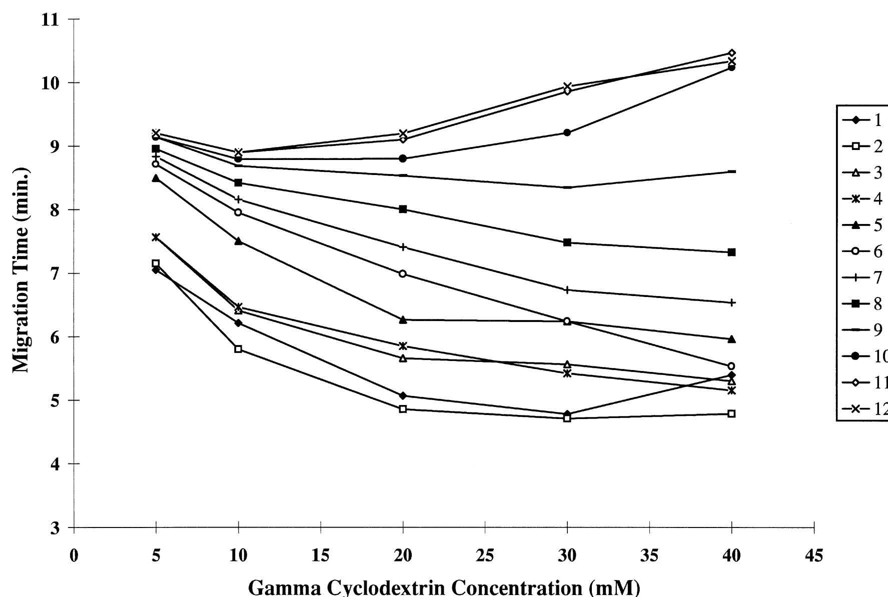

Fig. 3. Effect of γ-CD concentration on migration time for the 12 analytes identified in Fig. 2.

had LODs near 3 μ*M*, and the larger ones absorbed more of the laser light to produce detectable fluorescence signals in the 0.5-μ*M* range. These results are comparable to native PAH LODs on a similar commercial CE instrument [7]. Our efforts to improve the LOD included increasing the laser power from 4 to 8 mW, but doing so only increased the baseline noise.

Previously, it has been noted that buffer additives may have an impact upon analyte fluorescence, thus changing the detection sensitivity [5]. Examination of the total peak signals did not reveal a significant change, but the baseline noise generally increased. This effect arises from the increase in laser light scatter due to the increase in γ-CD concentration. Light scattering and buffer additive fluorescence are quite common with deep-UV and near-UV LIF detectors, but light scattering lessens dramatically for analyte systems that can be excited with visible laser light. The use of SDS also produced a baseline noise increase. Separations using capillary electrochromatography with LIF detectors have a definite advantage since the lack of buffer additives produces lower laser light scattering and background fluorescence. It is possible to study the effects of buffer additives on analyte fluorescence spectra with a fluorometer, and this is perhaps a meritorious investigation for future work.

When the LODs are compared between the hydroxyPAHs in this study and the native PAHs from Ref. [5], there is approximately a factor of 100 change to higher amounts in the hydroxyPAH LODs. The most likely cause for the difference comes from the detection optics. The optics in the P/ACE 2200 have an ellipsoidal reflector placed directly behind the capillary excitation window. This reflector collects analyte fluorescence, capillary fluorescence, and laser light scattered from the capillary walls. The light reflected towards the photomultiplier tube passes through a set of optical filters, which must discriminate against the large laser light scatter.

The mass limits of detection can be determined from the amount of sample injected into the capillary column. Typical injections lasted for 1 s using 0.5 p.s.i. of N_2 (1 p.s.i.=6894.76 Pa). The volume corresponding to the injection was approximately 1 nl. The amount injected on-column was 80 amol for 1-OHpyrene.

3.5. Conclusions

Using a combination of SDS and γ-CD, efficient

separations of PAH metabolite isomers were effected for the first time. This work presents the possible CD-MEKC separation and analysis of biomarkers for human exposure to PAHs. While the moderately large number of isomers may produce inconclusive results from mass spectral assignments, the successful separation of isomers (such as 1-OH, 3-OH, 7-OH and 9-OHBAP) to produce a unique separation pattern, can be important for exposure identification. Characterization of the photophysical properties of the hydroxyPAHs is important for optimizing the LIF detection system. The detection limits in the sub-femtomole range can be used in many commercial applications, and further improvement in LOD should be possible with confocal optical excitation/detection. We are in the process of adapting a commercial CE instrument to a confocal LIF module and will test this new system with PAH metabolite extractions from human urine.

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