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Micellar electrokinetic chromatography of monohydroxybenzo[*a*]pyrene positional isomers using γ-cyclodextrin

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Cyclodextrin-modified micellar electrokinetic chromatography was applied to the separation of benzo[*a*]pyrene and 12 positional isomers of monohydroxybenzo[*a*]pyrenes using γ-cyclodextrin together with sodium dodecyl sulfate. The optimum running conditions were found to be 20 mM phosphate–5 mM borate buffer (pH 8.5) containing 20 mM γ-cyclodextrin and 50 mM sodium dodecyl sulfate with an effective voltage of 12 kV at 20 °C. Octanol–water distribution coefficients of the compounds in the presence of γ-cyclodextrin were measured as an index of the association constants for complexes of γ-cyclodextrin with these compounds. The migration time of the analytes correlated well with their distribution coefficients, suggesting that their migration order could be correctly predicted on the basis of calculations of host–guest interaction energies. The relationship between the migration order and the structures of monohydroxybenzo[*a*]pyrene isomers is discussed.

Introduction

Benzo[*a*]pyrene (BaP) is a polycyclic aromatic hydrocarbon (PAH) that can be generated during the incomplete combustion of organic materials. As BaP is a common environmental pollutant and is carcinogenic, the processes that affect its transport and fate must be understood in order to assess the exposure risk to humans. Many studies on how BaP is metabolized have been conducted.[1–21] BaP is metabolized by mammals *via* a cytochrome P-450-dependent monooxygenase and epoxide hydrolase to produce a complex mixture of epoxides, quinones, phenols, dihydrodiols, triols and tetraols. Some of these BaP derivatives are more carcinogenic than the parent BaP.[6–8] Each of these metabolites may be further enzymatically conjugated with glutathione, glucuronide or sulfate to form even more hydrophilic compounds. These conjugates seem to be formed primarily by a detoxification pathway, but they can be toxic metabolites. BaP metabolites may be excreted either as free or as conjugated compounds in urine and feces.

There are two approaches for the determination of BaP metabolites. One is a direct determination of the conjugates.[9–11] Recently, Johnson and Greenberg[11] developed a liquid chromatographic method for the separation of five BaP sulfate and three BaP glucuronide isomers in urine samples. The other is an indirect determination that involves measuring the hydroxylated BaP metabolites after liberation of glucuronide and sulfate groups from the conjugates by acid or enzyme hydrolysis. Most studies on the BaP metabolites have been conducted by this approach using HPLC,[12–18] gas chromatography–mass spectrometric[19,20] or thin-layer chromatographic[21] techniques. Of these hydroxylated BaP metabolites, monohydroxyBaP isomers are an important group of the major metabolites. MonohydroxyBaP has as many as 12 kinds of positional isomers. However, no chromatographic methods have so far been able to achieve the simultaneous, full separation of monohydroxyBaP positional isomers.

Capillary electrophoresis (CE) has recently emerged as one of the most effective methods for achieving the separation of charged analytes. However, CE of neutral compounds cannot be performed in a free solution. This problem can be solved by using an additive to form a pseudophase. As demonstrated by Terabe *et al*.,[22,23] the introduction of micelles, which serve as a pseudophase, into the separation electrolyte results in the separation of neutral species. This separation is based on the partitioning of the uncharged analytes between the electrolyte and the micelles. This CE method, which uses introduced micelles, is called micellar electrokinetic chromatography (MEKC) or micellar electrokinetic capillary chromatography (MECC). Sodium dodecyl sulfate (SDS) has been used extensively as an electrolyte additive to form the micelles. However, PAHs, which are highly hydrophobic compounds, are strongly associated with the hydrocarbon phase and difficult to separate.[24] The PAHs spend virtually 100% of the migration period in the micelle phase, and all migrate with the same apparent mobility as the anionic micelles. Several methods have been tried for separating PAHs by CE. One method is to add tetraalkylammonium ions to the buffer solution containing a high concentration of organic solvent.[25–27] A high concentration of organic solvent seems to inhibit micellar formation of the surfactants. The interaction between tetraalkylammonium and the analytes is called solvophobic association. Subsequently, anionic surfactants, sodium dioctyl sulfosuccinate[28] and di(2-ethylhexyl) phosphate[29] were also used to separate PAHs by CE with organic modifiers. Another method is to use a buffer containing a mixture of neutral and anionic cyclodextrins (CDs).[30,31] The separation is achieved on the basis of the differential distribution of PAH components between the two CD types.

Native PAHs have also been separated by MEKC adding CD to the buffer solution.[32–35] This method is called CD-modified MEKC (CD-MEKC). In CD-MEKC, a water-insoluble hydrophobic solute is partitioned between the micelles and CD. When the solute is included in the CD cavity it migrates with the

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electroosmotic velocity, and when it is incorporated into the micelle it migrates with the micellar velocity. The solutes can be separated on the basis of their differential partitioning between CD and the micelles. Accordingly, an analyte having a shorter migration time is included more strongly in the CD cavity than an analyte having a longer migration time. Jinno and Sawada[34,35] studied the relationships between capacity factors (log k') and hydrophobicities (log P) of PAHs in CD-MEKC using surface-coated capillaries. They found that, compared with the case when a surface-untreated capillary was used, C_1- and C_{18}-treated capillaries gave better linear relationships for the correlation between log k' and log P of PAHs. Recently, Jimenez *et al.*[36] reported that the separation selectivity of 20 PAHs in MEKC could be improved by using mixtures of β- and γ-CDs in the separation buffer. They also found a correlation between the logarithm of the capacity factors of the PAHs and the logarithm of their octanol–water distribution coefficients. By using MEKC with γ-CD mobile phase additives, Copper and Sepaniak were able to achieve a good separation of several BaP derivatives including six monomethylBaP isomers, and discussed the possible separation mechanism with the computational procedures.[24] Recently, Smith *et al.*[37] separated hydroxylated PAHs including four monohydroxyBaP isomers using MEKC with γ-CD.

The aim of this study was to develop a method for the simultaneous separation of BaP and 12 monohydroxyBaP isomers using CD-MEKC. Various parameters influencing the resolution and the migration time of BaP and monohydroxyBaP isomers were investigated. The elution orders in γ-CD-MEKC were found to correlate well with the octanol–water distribution coefficients in the presence of γ-CD and there was some relationship between the elution order and the position of hydroxylation in the BaP molecule.

Experimental

Reagents

Sodium dodecyl sulfate (electrophoresis purity reagent) was obtained from Bio-Rad (Hercules, CA, USA) and benzo[*a*]pyrene from Aldrich (Milwaukee, WI, USA). Twelve kinds of monohydroxybenzo[*a*]pyrene (abbreviated as 1-OH-BaP–12-H-BaP) were obtained of Chemical Carcinogen Repositories (Kansas City, MO, USA). Heptakis(2,6-di-*O*-methyl)-β-cyclodextrin and 2-hydroxypropyl-β-cyclodextrin (average degree of substitution = 7) were obtained from Sigma (St. Louis, MO, USA), hydroxypropyl-γ-cyclodextrin (average degree of substitution = 4.2) from Aldrich and ethanol (HPLC grade), β-cyclodextrin, 2,3,6-tri-*O*-methyl-β-cyclodextrin, γ-cyclodextrin and other chemicals (guaranteed grade) from Wako (Osaka, Japan).

Apparatus

Electrophoretic experiments were carried out using an HP^{3D} capillary electrophoresis system (Hewlett-Packard, Palo Alto, CA, USA). Samples were injected by applying a pressure of 5 kPa for 2 s. The separations were performed in a fused-silica bubble cell capillary of 56 cm × 75 μm i.d. (Hewlett-Packard). The capillary was kept at 20 °C. The analytes were detected at 280 nm. The power supply was operated in the constant-voltage mode at 12 kV and the substances migrated towards the negative pole.

Spectrophotometric examinations were carried out using a Hitachi (Tokyo, Japan) U-2000 spectrophotometer. The sample compartment contained a 1 cm thick quartz cell that was kept at 25 °C.

Buffer and sample preparation for CE

The background electrolyte (BGE) in the electrophoretic experiments, unless stated otherwise, was 20 mM phosphate–5 mM borate buffer (pH 8.5) containing 50 mM SDS and 20 mM γ-CD and was filtered with a 0.22 μm filter before use.

De-ionized water was prepared using a Toray (Mishima, Japan) Ultra Pure Water System.

Stock standard solutions of 1 mM BaP and each hydroxyBaP were separately prepared in ethanol, stored at −20 °C and diluted to 5 μM, before use.

Determination of octanol–water distribution coefficient in the presence or absence of γ-CD by UV spectrophotometry

A 3 ml volume of octanol containing 7 μM of each BaP or hydroxyBaP was added to 3 ml of 20 mM phosphate–5 mM borate buffer (pH 8.5) with or without 50 mM γ-CD. These mixtures were incubated for 20 min at 25 °C, followed by vigorous shaking for 15 min at 25 °C and centrifugation at 3000 rpm for 1 min. Their absorption spectra (260–320 nm) in each octanol fraction and the starting octanol solutions each containing 7 μM of compound were measured. Since these compounds exhibit different absorption spectra, the octanol–water distribution coefficients were calculated from the absorption maximum of each compound.

Results and discussion

Separation of BaP and 12 monohydroxyBaPs

Effect of CD type. The effect of the type of CD on the separation of BaP and 12 monohydroxyBaP isomers was investigated by CD-MEKC using a BGE containing separately 20 mM 2,6-di-*O*-methyl-β-CD, 2,3,6-tri-*O*-methyl-β-CD, 2-hydroxypropyl-β-CD (2HP-β-CD), HP-γ-CD or γ-CD. In the case of β-CD, the separation was carried out at a concentration of 15 mM owing to its low solubility. In the absence of CD, there was no separation and the analytes were detected as a single peak [Fig. 1(A)]. The addition of β-CD to the BGE did not affect the

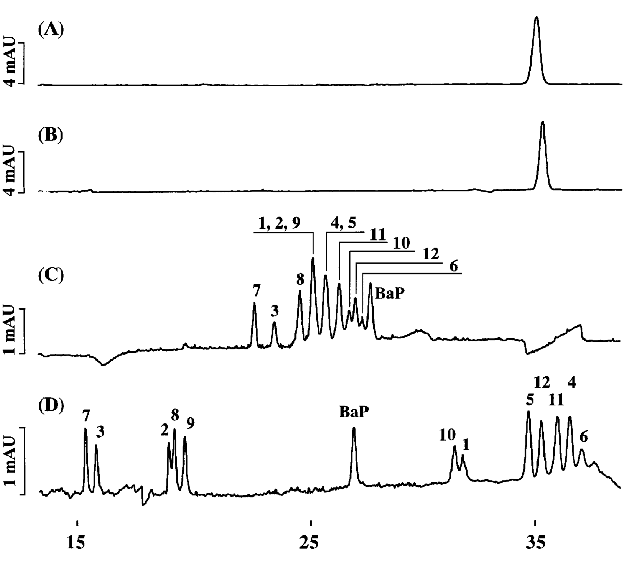

Fig. 1 Electropherograms of BaP and hydroxyBaPs obtained by MEKC using CDs. The BGE was composed of 50 mM SDS and 20 mM phosphate–5 mM borate buffer (pH 8.5) with no CD (A), 15 mM β-CD (B), 20 mM HP-γ-CD (C) or 20 mMγ-CD (D). 1–12 represent 1-OH-BaP–12-OH-BaP.

separation and resulted in a slightly longer migration time than that in the absence of CD [Fig. 1(B)]. As mentioned by Copper and Sepaniak,[24] it is logical that β-CD has little effect on the retention of the isomers since its cavity dimensions would permit only partial inclusion complex formation with the BaP molecule. Hence, it seems that the longer migration time results from the increased viscosity of the buffer due to the addition of β-CD. Similar results were obtained with other β-CD derivatives. BaP and the 12 isomers split into 10 peaks when HP-γ-CD was added to the separation buffer [Fig. 1(C)]. Two groups of hydroxyBaPs, 1-OH-BaP–2-OH-BaP–9-OH-BaP and 4-OH-BaP–5-OH-BaP were not resolved. On the other hand, γ-CD had a dramatic and selective effect on the retention of BaP and its isomers with migration times ranging from 15.0 min for 7-OH-BaP to 37.2 min for 6-OH-BaP [Fig. 1(D)]. Compared with γ-CD, the larger, more flexible cavity of HP-γ-CD might result in relatively uniform interactions with BaP and its isomers. In fact, the narrow range (5.4 min) of migration times with 20 mM HP-γ-CD in the buffer solution was little affected by the addition of 40 mM HP-γ-CD. Therefore, γ-CD was found to be most effective for the resolution of BaP and its monohydroxy isomers. According to Terabe *et al.*,[33] in CD-MEKC, an analyte is distributed among three phases, the micellar phase, the CD phase and the aqueous phase, although CD is not a true phase. As γ-CD is electrically neutral, it migrates at the same velocity as the bulk solution. The distribution of the analyte between the micelles and the non-micellar aqueous phase including γ-CD directly affects the resolution. Addition of γ-CD to the micellar solution decreases the partitioning of the analyte to the micelles by increasing the fraction of the analyte in the non-micellar aqueous phase. The fact that all of the analytes were totally incorporated into the SDS micelles [Fig. 1(A)] suggests that the migration order observed in Fig. 1(D) was correctly predicted on the basis of calculations of host–guest interaction energies.[24,32] This indicates that 7-OH-BaP, which migrated with the fastest velocity, formed the most stable complex with γ-CD among the BaP and its hydroxy isomers.

Effect of γ-CD concentration. The migration times of BaP and 1-, 2-, 3-, 7-, 8-, 9- and 10-OH-BaP decreased with increasing amount of γ-CD (10–30 mM, Fig. 2), suggesting that an increase in γ-CD concentration brought about a decrease in the partitioning of the analytes in the micelles. On the other hand, an increase in the γ-CD concentration caused slight increases in the migration times of 11-, 4- and 6-OH-BaP. Since these three hydroxyBaPs are included more weakly in the γ-CD cavity than the other hydroxyBaPs, the longer migration times seemed to result from mainly the increased viscosity of the BGE due to the increase in γ-CD concentration.

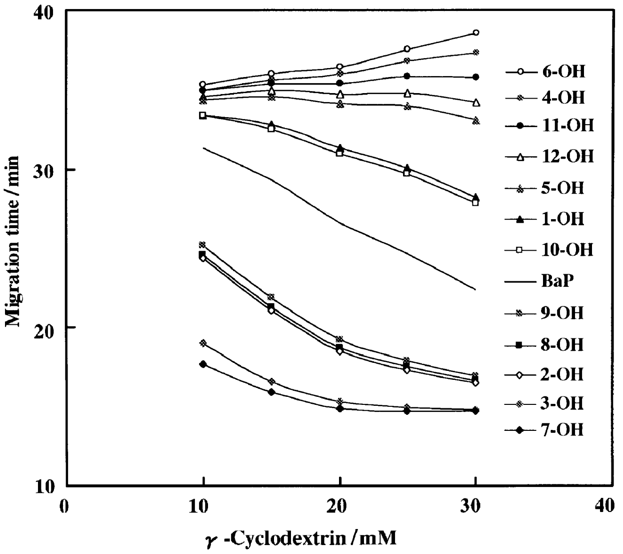

Fig. 2 Effect of γ-CD concentration on the migration time of BaP and its hydroxy isomers. The BGE was composed of various concentrations of γ-CD containing 50 mM SDS and 20 mM phosphate–5 mM borate buffer (pH 8.5).

Effect of SDS concentration. The migration times of the analytes increased with increasing SDS concentration (30–70 mM). This result is not attributable to the increase in ionic strength, because the electroosmotic flow (EOF) was not changed significantly over the whole SDS concentration range. The partitioning of the solute between the CD and the micelles depends on the SDS concentration. This suggests that the longer migration time resulted from an increase in the phase ratio of the micellar to the aqueous CD phase.

Effect of pH. The migration times slightly increased with increase in pH (7–9). This result is not related to the dissociation of the hydroxyl group of hydroxyBaPs because the migration time of BaP without a hydroxyl group also slightly increased with increase in pH.

Effect of capillary temperature. The migration times of all analytes and ethanol as an EOF marker decreased with increasing capillary temperature (15–35 °C). This result could be attributed to a decrease in the buffer viscosity.[38] However, the elution order of the analytes was not affected by the capillary temperature except that two pairs of hydroxyBaPs, 2-OH-BaP–8-OH-BaP and 10-OH-BaP–1-OH-BaP, were not resolved at higher temperatures.

Therefore, the optimum BGE conditions, *i.e.*, the conditions giving both high resolution and short migration time, were found to be 20 mM γ-CD and 50 mM SDS in 20 mM phosphate–5 mM borate buffer (pH 8.5) with an effective voltage of 12 kV at 20 °C. However, the sensitivity was insufficient for measuring real samples with a UV detector. With the usual injection volumes and a UV detector, the limits of detection (LODs) in CE are generally at the mg L^{-1} level. A more sensitive and frequently used method of detection in CE is laser-induced fluorescence, giving LODs at least an order of magnitude lower than with UV detection.[27,30,31,37] Since BaP and its hydroxy isomers are fluorescent compounds, they can be detected with this method. Moreover, on-line concentration techniques, such as sample stacking and sweeping, can improve the sensitivity.[39] Therefore, measurements of BaP and its hydroxy isomers in real samples may become possible with CD-MEKC using a combination of fluorescence detection and on-line concentration.

Consideration of the relationship between γ-CD and hydroxyBaPs

The logarithmic capacity factors (log k') of PAHs in CD-MEKC have been found to be linearly related to the logarithm of their octanol–water distribution coefficients (log P_{ow}).[34–36] The approximate capacity factor values were calculated as described by Terabe *et al.*[23] using $k' = (t_{\mathrm{R}} - t_0)/[t_0\,(1 - t_{\mathrm{R}}/t_{\mathrm{mc}})]$, where t_0, t_{R} and t_{mc} are the migration times of the bulk solution which is free from the micelles, the sample solute and the micelles, respectively. The migration time t_0 can be obtained with tracers such as an organic solvent, which appears at the detector as a solvent disturbance. Typically, a hydrophobic molecule that associates completely with the micelles can be used to measure t_{mc}. However, a hydrophobic molecule that associates completely with the micelles is difficult to detect at t_{mc} in the presence of CD, because most tracers of the micelles are partially included in CD also. For this reason, the measurement of t_{mc} was performed by injecting the analyte with a BGE containing no CD.[24,33] Jimenez *et al.*[36] used the migration time of the slowest migrating compound of PAHs (indeno[1,2,3-*cd*]pyrene) as t_{mc}. As shown in Fig. 1, the migration times of some hydroxyBaPs in the presence of γ-CD were longer than those in the absence of the CD. Therefore, t_R/t_0 was used instead of the capacity factor in this work.

The octanol–water distribution coefficients of BaP and its hydroxy isomers in the presence and absence of γ-CD are plotted in Fig. 3. In this study, 1- and 6-OH-BaPs were not used, because standard solutions of these compounds showed not only a main peak but also some minor peaks in CD-MEKC. In the absence of γ-CD, no significant differences in the logarithm of the distribution coefficients of hydroxyBaPs (log P_{OW}) were found, suggesting that the hydroxyBaPs have almost the same hydrophobicity. γ-CD was added to the water phase of the octanol–water system and the γ-CD-modified octanol–water distribution coefficients were measured as an index of the association constants for complexes of γ-CD with the analytes. For BaP and its hydroxy isomers, a strong correlation ($r^2 > 0.96$) was found between log (t_R/t_0) and the logarithm of their γ-CD-modified octanol–water distribution coefficient. This result may be related to the possibility that the migration order in CD-MEKC can be correctly predicted on the basis of calculations of host–guest interaction energies.[24,32]

The formation of an inclusion complex between the guest molecule and the CD cavity is dependent upon the geometry, size and physico-chemical properties of the molecule. Although hydrophobic interactions predominate in the cavity, these interactions can act in concert with polar or hydrogen-bonding interactions that occur with hydroxyl groups located on the outer lips of the cavity. The relationship between the elution order of hydroxyBaP isomers in γ-CD-MEKC and the position of the hydroxy group in the BaP molecule may be related to the ring structure of the BaP, which is shown schematically in Fig. 4. As shown in Fig. 1(D), five isomers (7-, 3-, 2-, 8-, and 9-OH-BaP) migrated faster than BaP. These isomers have a hydroxyl group on either ring A or D in Fig. 4. Therefore, this result may be ascribed to hydrogen-bonding interactions between the hydroxyl group of these isomers and hydroxy groups of γ-CD in addition to the hydrophobic interactions. Five other isomers (4-, 5-, 6-, 11- and 12-OH-BaP) migrated later in γ-CD-MEKC. Since these isomers have a hydroxy group that is located at the center of the BaP molecule (rings B, C and E in Fig. 4), it seems likely that γ-CD will permit only partial inclusion complex formation with these isomers. Each of three groups of hydroxyBaPs, 7-OH-BaP–3-OH-BaP, 2-OH-BaP–8-OH-BaP–9-OH-BaP and 10-OH-BaP–1-OH-BaP, have a hydroxy group on ring A or D and migrated closely together in γ-CD-MEKC. When the structural model of hydroxyBaP folds in two at the broken line (Fig. 4), C1, C2, and C3 on ring A overlap with the nearby C10, C9/C8 and C7 on ring D, respectively. That is, all the middle points linked between C1 and C10, between C2 and C9, between C2 and C8 and between C3 and C7 are located nearby on the broken line. Therefore, the probability that ring A is the first to enter the cavity of γ-CD appears to be almost the same as the probability that ring D is the first to enter the cavity.

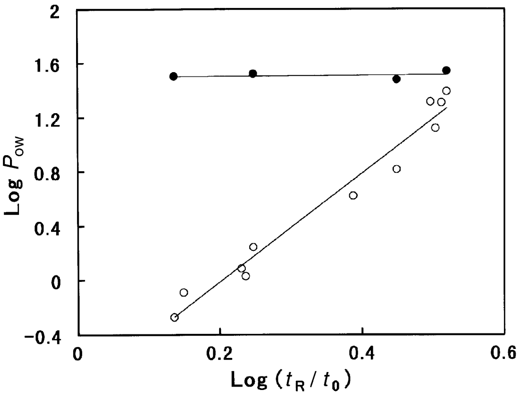

Fig. 3 Relationship between log (t_R/t_0) and log P_{OW}. Octanol–water distribution coefficients were measured in the presence (open circles) or absence (closed circles) of 50 mM γ-CD.

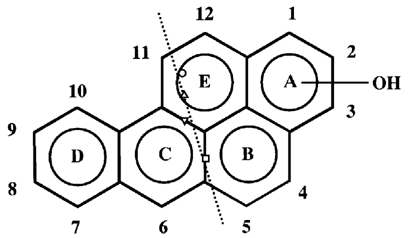

Fig. 4 Conformational relationship of monohydroxyBaP isomers. ○, △, ▽ and □ represent the middle points of C1 and C10, C2 and C9, C2 and C8, and C3 and C7, respectively.

In conclusion, a full separation of BaP and 12 mono-hydroxyBaPs was performed by using γ-CD-MEKC. For BaP and its hydroxy isomers, good correlations were found between the log (t_R/t_0) and the logarithm of their γ-CD-modified octanol–water distribution coefficients. This result suggests that the migration order in CD-MEKC can be correctly predicted on the basis of calculations of host–guest interaction energies.

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