Separation of Enantiomeric Barbiturates by Capillary Electrophoresis Using a Cyclodextrin-Containing Run Buffer

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In the last few years capillary electrophoresis (CE) has been successfully applied to a variety of analytical problems (*1*, *2*). Especially in the field of chiral separations CE has been useful in many cases. The experiment described here serves as an introduction to chiral separations by CE. The separation of enantiomers (*R*/*S*) is based on the addition of a suitable chiral selector to the buffer. Native or derivatized cyclodextrins perform as excellent selectors and have therefore been used widely (*3*). These molecules themselves are chiral. They are applied as enantiomeric pure substances and can form complexes with the chiral analyte. The compounds formed are then chemically distinguishable diastereomers. Complex formation depends on parameters such as pH, the structure of the selector and the analyte, and the concentration of the selector. A separation by CE is feasible when the two enantiomeric forms of the analyte form complexes of differing stability with the chiral selector. In the state of equilibrium the ratio of the complexed and uncomplexed forms for the *R*- and *S*-enantiomers is different. Since capillary electrophoretic separations are based on differences in charge and mass, a charged fraction of a heavier complex, for instance, has a direct impact on its migration.

Method development is facilitated and shortened by the fact that buffers as well as chiral selectors can be exchanged easily.

Barbiturates, derivatives of barbituric acid, are found in a variety of pharmaceuticals, such as sedatives, hypnotics, and antiepileptics. Some of these compounds can lead to addiction or in combination with alcohol cause serious intoxications. Most of the barbiturates are processed as racemic mixtures, since the effects of the *R*- and *S*-forms in the body differ only slightly. Very powerful separation techniques such as HPLC and CE are required to separate them and determine the subtle differences in their mechanism of action.

The separation of barbiturates in different matrices by CE has not yet been published widely (*4*, *5*). This article is intended to demonstrate the potential of CE for chiral separations. Students executing this experiment should be familiar with the separation mechanism and the CE technique; they should have some basic knowledge of stereochemistry and chiral separations. Before starting the experiment a short discussion about chiral selectors in CE, interactions of cyclodextrins with sample components, and migration behavior of the formed complexes (*6*) is recommended. Three barbiturates, two of them having a chiral center, were chosen for this separation. The influence of pH and the type and concentration of the chiral selectors on the separation is discussed. In one case the determination of the migration order of the separated *R*- and *S*-forms is described.

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The easy handling of the instrumentation and the fast and inexpensive method optimization even for challenging problems such as chiral analysis make the application of CE in general and the utilization of the described experiment in an advanced undergraduate/graduate laboratory course very attractive.

Experimental Procedure

Instrumentation

The instrument used in this work was a Beckman capillary electrophoresis model P/ACE 2100. It was equipped with a detector which has a set of fixed wavelengths in the UV region. Absorbance was monitored at 200 and 254 nm during the experiments. The columns used were uncoated fused silica capillaries with an inner diameter of 50 µm and a length of 50 cm to the detector and 57 cm to the end, respectively. The operating voltage was 15 kV. A pressure of 3.45 kPa was applied for the sample injection. The injection time was 5 s for the barbiturate standards.

In order to obtain reproducible results, the column was flushed after each run. A 5-min rinsing step with a buffer containing 20 mM borate pH 9.00 and 90 mM SDS was followed by a 5-min rinsing step with 0.1 M NaOH.

The electropherograms were recorded by an IBM/PS2 computer using the P/ACE 2000 software.

Reagents

H_3BO_3, $Na_2B_4O_7 \cdot 10\ H_2O$, and NaOH were obtained from Merck (Darmstadt, Germany). Sodium dodecylsulfate (SDS) was purchased from Riedel–de Haën (Seelze, Germany). Thiopental (5-ethyl-5-[1-methylbutyl]-2-thiobarbituric acid), cyclobarbital (5-[1-cyclohexenyl]-5-ethylbarbituric acid), and phenobarbital (5-ethyl-5-phenylbarbituric acid) were obtained from Sigma (Deisenhofen, Germany). The α- and β-cyclodextrin were purchased from Fluka (Buchs, Switzerland). All chemicals were of reagent grade. Triply distilled water was obtained from a laboratory water station.

Sample Preparation

Stock solutions of 100 mM borate pH 8.25 and 9.60 were prepared by dissolving the appropriate amount of sodium salts in triply distilled water. Separate barbiturate standards with 1 mM concentration each were prepared and stored in the refrigerator. Before the separation the three barbiturates were mixed in a 5-mL volumetric flask to give the final concentration of 0.2 mM for each component.

Each buffer (Table 1) was prepared by diluting the stock solutions to the final concentration in a 10-mL volumetric flask and adding the appropriate amount of cyclodextrin. The mixtures were carefully dissolved in an ultrasonic bath and filtered before use.

Table 1. Composition of Separation Buffers

Buffer	Composition
A	20 mM borate pH 9.60
B	20 mM borate pH 8.25, 8 mM β-cyclodextrin
C	20 mM borate pH 9.60, 8 mM β-cyclodextrin
D	20 mM borate pH 9.60, 20 mM β-cyclodextrin
E	20 mM borate pH 9.60, 5 mM α-cyclodextrin
F	20 mM borate pH 9.60, 10 mM α-cyclodextrin

Results and Discussion

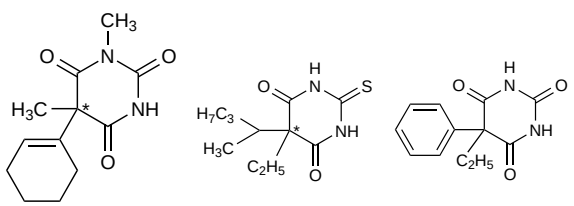
Peak Identification

Initially the mixture of standards was separated under simple conditions without the addition of cyclodextrins (Fig. 1, buffer A). Peak identification was carried out by standard addition as well as comparison of the UV spectra. To identify each peak of this three-component mixture, the addition of only one standard is necessary. In the UV region between 200 and 300 nm, maxima of absorbance for cyclobarbital are found at 200 and 241 nm, for phenobarbital at 202 and 245 nm, and for thiopental at 200 and 257 nm. Cyclobarbital and phenobarbital have their highest molar absorptivity at the low λ_{max} values. The patterns of their UV spectra are quite similar. Phenobarbital has, owing to the additional aromatic ring, a slightly higher absorptivity over the entire region. For thiopental the molar absorptivity at 257 nm is much higher than at 200 nm. If the electrophoretic separation is detected consecutively or concurrently at two wavelengths (200 and 254 nm), then based on the ratios of the peak intensities (Fig. 1) thiopental can be identified immediately. By standard addition of cyclobarbital or phenobarbital the remaining components can be identified.

When using cyclodextrin-containing buffers, the migration order of the compounds could change. Therefore random checks of the peak order are advised after changing the buffer additives.

Method Development

From a number of possible barbiturates, cyclobarbital (**1**), thiopental (**2**) and phenobarbital (**3**) were chosen for our study.



1 cyclobarbital **2** thiopental **3** phenobarbital

Phenobarbital has no asymmetric carbon atom. Although the C-5 in the heterocycle has different substituents, the ring is symmetrically built around it. Under all separation conditions it should always give a single peak only. The C-5 in the heterocycle in cyclobarbital is asymmetric. However, owing to the bulky hexene ring it is sterically hindered in undergoing complex formation. In thiopental the asymmetric carbon atom is found in the side chain of one of the substituents at the C-5 of the heterocycle. It is easily accessible

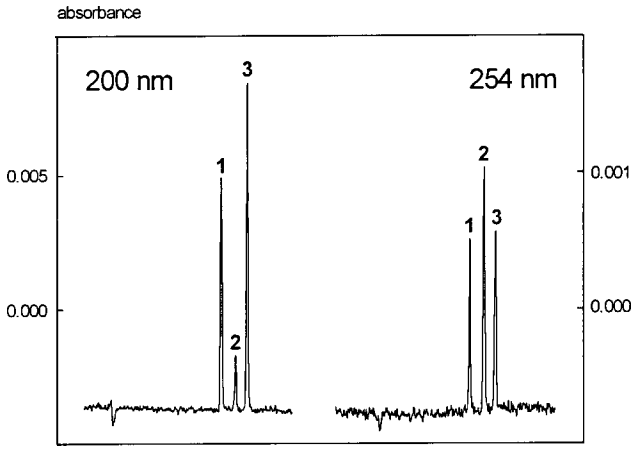


Figure 1. Separation of the standard mixture in buffer A, detection at 200 and 254 nm.

for complexation. Based on these structural differences, a distinguishable behavior of the 3 compounds during chiral separation is expected.

The derivatives of barbituric acid retain only little acidity (pK_a 7.5–10). To create charged analytes, buffers with a pH > 8 must be prepared. To optimize the separation, buffers having two different pH values were chosen. In one of them (Table 1, buffer B) the analytes were partially deprotonated. In other buffers (Table 1, buffers A, C–F), the compounds were fully deprotonated.

The α- and β-cyclodextrins (CD) were used as chiral selectors possessing different numbers of D-glucose subunits (6 and 7, respectively). Increasing number of subunits causes an increasing cavity in the cyclodextrin molecule. The dimension of the cavity of α-CD allows predominately the inclusion of alkyl chains; the one of β-CD can host ring systems of 5 or 6 atoms. An explanation of the CD interaction with sample molecules and the migration behavior of the formed complex (*6*) should be given by the instructor. It should give students enough information to predict an elution order of the chiral barbiturates using α- or β-CD. Students should be reminded of the position of the chiral C atom in the molecules.

The α- and β-cyclodextrins were dissolved in different concentrations in the buffer, since the concentration of the chiral selector affects the resolution of the enantiomers. Table 1 shows the composition of all buffers used in this study.

The separation of a mixture with three components in buffers containing β-CD (buffer B and C) is shown in Figure 2. The enantiomers of cyclobarbital were separated only at pH 9.60; thiopental enantiomers could not be separated by β-CD at either pH. For buffer C, a different separation pattern was observed. This is mainly due to two effects. At a high pH such as pH 9.60, electroosmosis (*1*, *2*) is increased. This leads to a faster transport of the capillary content towards the detector. At the same time the analytes are more strongly deprotonated; thus their net negative charge and electrophoretic velocity toward the inlet of the capillary increase. The latter effect is prevalent. From the sum of the velocities results a smaller net migration velocity, and the overall migration times increase. In addition, the higher charge of the analyte drastically affects complex formation with cyclodextrin. For an analyte of different charge the structure and stability of the formed complex can change. In the case of cyclobarbital, the charge increase of the mol-

ecule causes the formation of complexes of different stabilities with β-CD. The very hydrophobic cyclohexene ring of the molecule is more likely to interact with the interior of the β-CD cavity than the heterocyclic part. Additional interactions of the heteroatoms with hydroxylic groups of the upper rim of the cyclodextrin lead to the formation of stereochemically different complexes for the *R*- and *S*-forms of cyclobarbital. With 8 mM β-CD a good separation of these enantiomers was observed. For thiopental, either no stereospecific complexes are formed or the stability constants for the complexes of the two enantiomers are not sufficiently different. It is assumed that the size of the cavity of β-CD allows the side chain of the molecule with the asymmetric carbon atom to penetrate completely. No further interaction of the molecule with the hydroxylic groups of the cyclodextrin is possible. Therefore the formation of stereoselective complexes is unlikely. Phenobarbital has no asymmetric carbon atom and therefore only one peak was yielded. An increase in the concentration of β-cyclodextrin up to 20 mM (buffer D) did not improve the separation for either cyclobarbital or thiopental, but the migration time of all analytes was longer owing to increase in the viscosity of the buffers.

The effect of the concentration of α-CD on the separation is shown in Figure 3. At a concentration of 5 mM α-CD (buffer E), thiopental was almost completely separated into its *R*- and *S*-forms; cyclobarbital, however, was separated only partially. It must be pointed out that the migration order of thiopental and cyclobarbital has changed with respect to β-CD. This is caused by other interaction modes of the sample molecules with α-CD owing to the smaller cavity. The substituents of the thiopental at the C-5 are optimal in size and structure to fit into the cavity of α-cyclodextrin. Along with interactions between parts of the molecule and the hydroxylic groups at the rim of the CD, this leads to the formation of stereochemically different complexes. They can be separated very well even at low concentrations of α-CD. For cyclobarbital, a partial inclusion of the cyclohexene ring or the methyl group at the C-5 of the heterocycle into the cavity of the α-CD is probable. This brings the asymmetric carbon atom close to the cyclodextrin ring and facilitates the stereoselective discrimination via interactions with hydroxylic groups. The structural fit of cyclobarbital and α-CD is not as good as with thiopental. Therefore the separation with low cyclodextrin concentration was less efficient.

Higher concentrations of cyclodextrins (buffer F) slowed the entire separation. While the resolution of thiopental was not affected by a doubled concentration of α-CD, the resolution for cyclobarbital improved significantly. This is based on two effects. The slower separation allows more time for the analyte to interact with the chiral selector. In addition, the cyclodextrin concentration is part of the equilibrium of the complex formation. In many cases, higher CD concentrations improve the resolution of enantiomeric compounds. In some cases, however, the resolution as a function of the CD concentration goes through a maximum and with further increase in concentration of the chiral selector a negative effect on resolution is observed.

The differences in structure of the cyclodextrins are the basis for the formation of different complexes for thiopental and cyclobarbital. This also caused a change in the migration order for these two compounds (buffers C and E). When using concurrent or consecutive detection at two wavelengths (Fig. 4), it was shown that in a β-CD containing buffer cyclobarbital is detected first. In an α-CD containing buffer thiopental passed the detector first. The absorption maximum for thiopental is at 257 nm. Therefore, at 200 nm a considerably lower signal for this compound was detected.

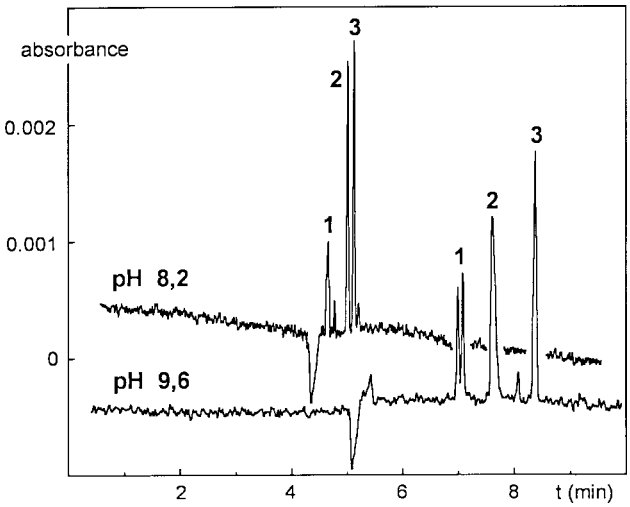


Figure 2. Separation of the standard mixture at different pH values using buffers B and C.

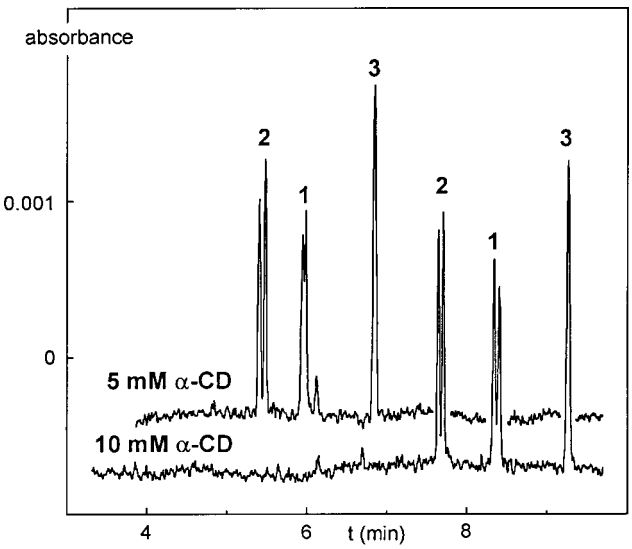


Figure 3. Separation of the standard mixture with different concentrations of α-cyclodextrin using buffers E and F.

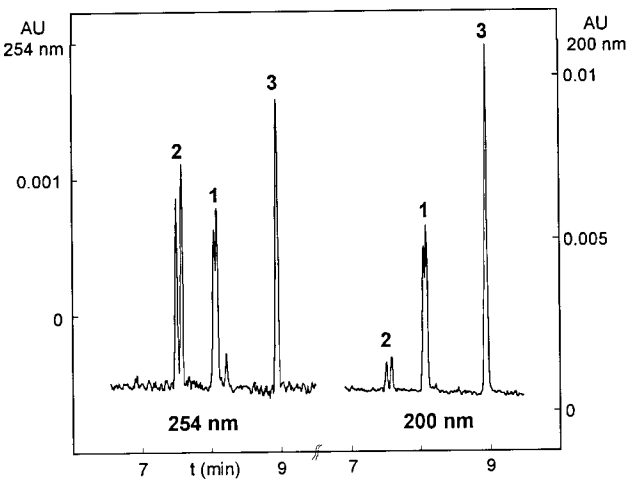


Figure 4. Separation of a standard mixture in buffer E using detection at different wavelengths.

Table 2. Complex Stability Constants for Cyclobarbital with α- and β-Cyclodextrin

Cyclobarbital Enantiomer	Complex Stability Constant (L/mol)	
	α-Cyclodextrin	β-Cyclodextrin
R	208	151
S	220	131

Determination of Migration Order of Enantiomers

The stability constants for the inclusion complexes of the *R*- and *S*-forms of the enantiomeric pair are the basis for determining the migration order. They can be determined by spectroscopic, polarographic, or potentiometric means. Once the constants as well as the specific separation conditions are known, the identity of the peaks can be determined by logical reasoning.

The constants for cyclobarbital are presented in Table 2 (*7*). In a capillary electrophoretic separation with either α- and β-cyclodextrin containing buffers the reversal of the migration order of the enantiomers must be anticipated. This is due to the fact, that the complex of α-CD with the *S*-form and the complex of β-CD with the R-form have the higher stabilities.

During complex formation the mass of the analyte increases substantially while its charge remains constant. The migration time is proportional to the ratio of mass to charge. An increase in mass leads therefore to an increase in migration time. The formation of complexes between uncharged chiral selectors and charged analytes described here equilibrates very rapidly. This makes the separation of the free and complexed form of enantiomers impossible. They occur in a single peak with an average migration time that is determined by the ratio of the two compounds. For complexes of different stabilities the more stable complex yields more of the complexed and slower species in the equilibrium than the less stable one. Therefore the more stable complex migrates more slowly and separation is achieved.

Under the conditions used in this study (pH 9.60) the analytes are negatively charged (pK_a about 8) and migrate towards the anode (i.e., the inlet). In bare fused-silica capillaries the electroosmotic flow can be substantial at this high pH. Since electroosmosis causes transport toward the cathode (i.e. the outlet), analytes move with an effective velocity that is the sum of the two opposite velocity vectors. In our case the vector of osmosis is higher in magnitude than the vector of the electrophoretic mobility of the analytes. This results in a net mobility toward the cathode. For a labile complex the resulting velocity will be smaller because its electrophoretic velocity is larger. The labile complex moves toward the detector slower than the analyte forming the more stable complex.

For cyclobarbital in an α-CD-containing buffer the *S*-form moves faster than the *R*-form; in a β-CD-containing buffer the *R*-form reaches the detector first, followed by the *S*-enantiomer.

The experiment described offers students an introduction to chiral separations by CE. In addition, the use of racemic mixtures of barbiturates may be a springboard for discussing the necessity of enantiomeric purity of drugs and pharmaceuticals (e.g., Methadone, Contagan). The simple and fast method development along with the potential to solve difficult analytical problems such as chiral separations makes capillary electrophoresis very attractive. Since this technique has many advantages it is increasingly applied in industry for quality control of a variety of products. At the same time, CE is so easy to handle that it could and should be taught and practiced in undergraduate and graduate education.

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