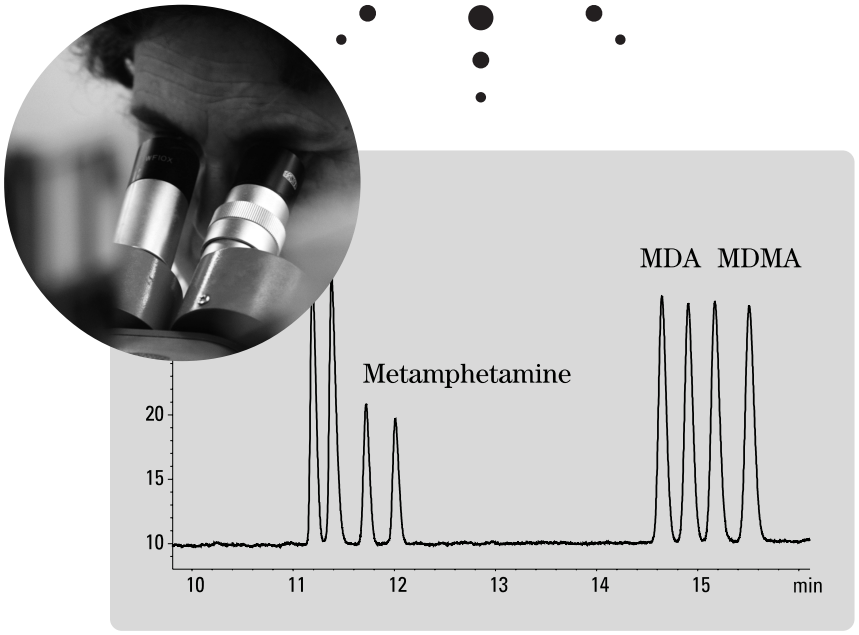
# Chiral Analysis
with the Agilent Capillary Electrophoresis System




Agilent Technologies

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## Chiral Separations by Capillary Electrophoresis with Cyclodextrins
## Development and Optimization of Methods for Determining Enantiomeric Purity

This note is meant to facilitate development of chiral separations by CE.

The development and optimization of methods for the separation and determination of chiral compounds are described in detail. The impact of cyclodextrin type and concentration, buffer ionic strength, pH and capillary temperature are illustrated to help understand the process of developing robust methods for quantitative analysis of chiral species.

## 1. Introduction

The challenges of chiral recognition spurred the development of analytical methods for the separation of enantiomers. In order to accomplish enantio-separation the optical isomers have to come into contact with a chiral enviroment to form two diastereomeric complexes. According to the "three-point-interaction" rule of Dalgliesh, chiral recognition depends on a minimum of three simultaneous interactions between selector and selectands and at least one of these interactions has to be stereo-selective in order to discriminate between the enantiomers.
In capillary zone electrophoresis direct separation is usually performed by adding an optically pure additive which is dissolved in the buffer electrolyte. Of the hundreds of reports in literature on chiral seperations by CE, about 80% use cyclodextrins as the optically active reagent.

## 2. Theory

The basis for chiral recognition and CE-separation is obtained simply by addition of mobility-modifying buffer additives which form transient diastereomeric molecular complexes with the analytes. Most commonly, cyclodextrins are used as buffer additives. When a charged solute complexes with a neutral cyclodextrin its charge-to-mass ratio and thus its mobility decreases compared to an uncomplexed solute. Differences in the equilibrium constants determine the ratio of free to complexed material. If the equilibrium constants are sufficiently different, enantiomer separation will occur.
The primary mechanism of cyclodextrin function involves inclusion complexation of the analytes. The inclusion complexation mechanism is based on dynamic equilibrium in which water, or other compounds (the analytes), replace each other as guests in the cavity of the cyclodextrin molecule. The stability of the inclusion complex is influenced by several parameters, including chemical structure and hydrophobicity of the guest molecule, structure and concentration of the cyclodextrin, buffer counterion, pH, organic solvent, and capillary temperature. Cyclodextrins are torus shaped molecules which have a relatively hydrophobic internal cavity which influences the formation of inclusion complexes with analytes conforming to the interior size dimensions of the cyclodextrin. If the analyte is too large, it cannot enter the cavity and no complex is formed. If it is too small, the molecular contact with the cyclodextrin may not be strong enough to effect the separation. Figure 1a shows the structure of cyclodextrins and figure 1b the proposed interaction of epinephrine with dimethyl-β-cyclodextrin.

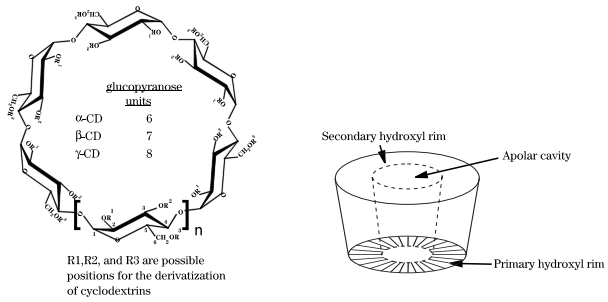



**Figure 1a: Structure of Cyclodextrins**

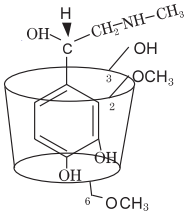



**Figure 1b: Interaction of epinephrine enantiomer with dimethyl-β-cyclodextrin**

Chiral recognition is also dependent on derivatization of the hydroxyl group(s) on the cyclodextrin ring. Through derivatization, hydrophobicity and charge can be altered to modify either the electrophoretic mobility or the complex-forming character of the cyclodextrins.

Enantiomeric separation by CE requires that either the analyte or the cyclodextrin be charged. For the separation of charged analytes neutral cyclodextrins are usually used. Method development strategies for the analysis of charged analytes (using neutral cyclodextrins) are described in this note.

For neutral analytes, either charged cyclodextrins or neutral cyclodextrins in an MEKC system are used.

## 3. Ordering Information

The following items should be supplied by the user:

analytical balance and weighing paper/dishes

spatula

syringe (plastic or glass) , 1–5 ml

pipette with micro pipette tips, 1-10 µl, 50-200 µl

graduated cylinder, 10-100 ml

beaker or flask, 10-100 ml

magnetic stir plate and stir bar

ultrasonic bath

Pasteur pipettes and bulb, 1-2 ml

The following chemicals must be ordered from 3rd party vendors. For ordering please refer to **http://www.sigald.sial.com/fluka** for Sigma and to **www.cyclolab.hu** for Cyclolab.

**Vendor and Product Information**

| Component | Vendor | Order Number |
|---|---|---|
| (±)-Epinephrine Free Base | Sigma | E1635 |
| β-cyclodextrin | Cyclolab | CY-2001 |
| (2-hydroxy)propylated-β-cyclodextrin | Cyclolab | CY-2005.2 |
| Heptakis(2,6-di-0-methyl)-β-cyclodextrin | Cyclolab | CY-2004.0 |
| Heptakis(2,3,6-tri-0-methyl)-β-cyclodextrin | Cyclolab | CY-2001 |
| Gamma-cyclodextrin | Cyclolab | CY-3001 |

The following parts can be ordered from Agilent:

| Component | Quantity | Part No. |
|---|---|---|
| 50 mM phosphate, pH 2.5 | 250 ml | 5062-8571 |
| 50 mM phosphate, pH 7 | 250 ml | 5062-8572 |
| 89 mM Tris, 89 mM boric acid, pH 8.3 | 250 ml | 8500-6783 |
| 50 mM sodium tetraborate, pH 9.3 | 250 ml | 5062-8573 |
| 0.1 N NaOH (for conditioning and pH adjustment) | 250 ml | 5062-8575 |
| 0.1 N Phosphoric acid (for conditioning and pH adjustment) | 250 ml | 5062-8577 |
| CE water | 500 ml | 5062-8578 |
| CE buffer vials, 2 ml glass | 100/pk | 5181-3375 |
| CE sample vials, 100 µl | 1000/pk | 9301-0978 |
| CE caps, polyurethane | 100/pk | 5181-1512 |
| sample filters, 0.2 µm, 30 mm | 100/pk | 5061-3354 |
| or 0.2 µm, 3 mm | 100/pk | 5061-3368 |
| Capillary, 50 µm i.d., l=72 cm | 2/pk | G1600-62211 |
| Alignment Interface for 50 µm i.d. capillary (color code: green) | | G1600-60210 |
| CE Column cutter* | 1 | 5183-4620 |

*can be used if the capillary length needs to be reduced.

## 4. Method Development Strategies

Practical methodology for chiral separations is described in this section. Important experimental parameters are outlined and a general separation scheme is given. Listed below are primary and secondary experimental parameters. The former require the most attention and their optimization is usually critical for successful operation. The secondary parameters may be critical, but are most often important only in fine-tuning a separation.

Primary experimental parameters
- Buffer pH
- Cyclodextrin type
- Cyclodextrin concentration

Secondary experimental parameters
- Buffer type
- Buffer concentration
- Electric field
- Injection amount
- Capillary temperature
- Capillary dimensions

General remark:
It is still difficult to predict which type of cyclodextrin will exhibit the best chiral recognition ability for a defined racemate (see the Dns-Phe example), and screening is therefore absolutely necessary.

A flow chart defining recommended experiments is shown in figure 2.

### Step 1: Buffer pH

The first step in method development is identification of analyte charge and appropriate buffer pH. If the analyte can be charged it is important that the buffer pH be 1-2 units above or below the pI. Generally pH 2-3 is first selected for basic analytes and pH 7-10 for acidic analytes. If the analyte is not charged either an MEKC system or charged cyclodextrins will be necessary (see below).

### Step 2: Cyclodextrin screening

In this step all cyclodextrins will be used at a nominal concentration of 20 mM. This process can be automated so screening requires minimal user attention. The cyclodextrin that best resolves the enantiomers should be selected for further optimization. When more than one is successful the least expensive cyclodextrin may be the best choice. If absolutely no resolution is obtained refer to the flow chart in Figure 2 for suggestions.

### Step 3: Cyclodextrin concentration screening

Optimization of cyclodextrin concentration is extremely important. As will be shown in the examples below, there is often an optimum cyclodextrin concentration, above and below which resolution may be decreased. It may be necessary to fine tune the concentration beyond what is described in the flow chart (from 1 to 100 mM, for example). At this point the separation should be nearly complete.

### Step 4: Optimization of secondary parameters

Numerous experimental parameters, many of which are instrumental, can be adjusted to complete the optimization of the separation. Many of these parameters are important to obtain complete resolution of the enantiomers, but are secondary to the selection of buffer, cyclodextrin, and cyclodextrin concentration. These parameters, given in the flow chart of figure 2, are described in the examples below. For more information on the fundamentals of CE please refer to Agilent Publication No. 5968-9963E.

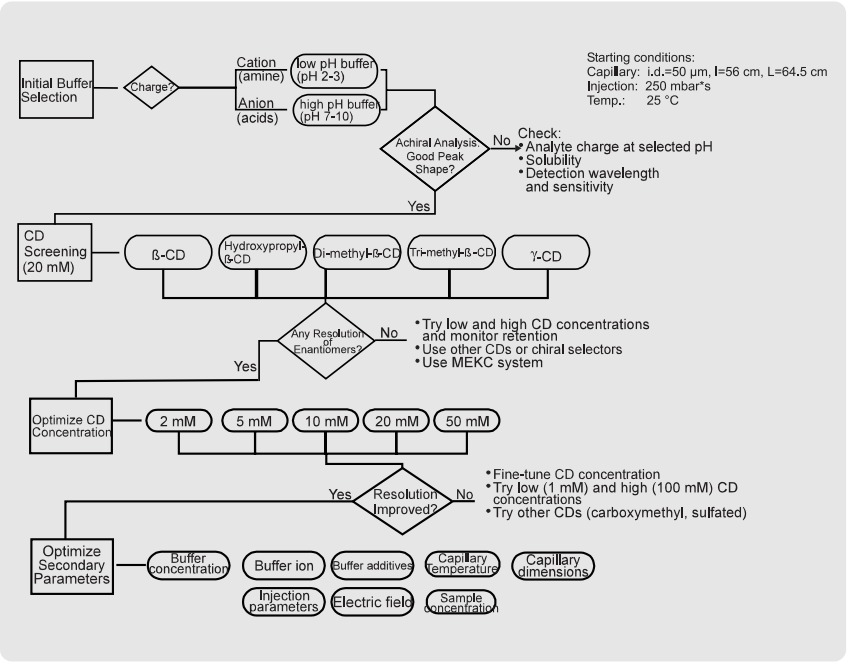



**Figure 2: Method development flow chart**

## 5. Experimental

The preparation of cyclodextrin-containing buffers, capillary conditioning and instrumental set-up are described in this section.

### 5.1 Buffer preparation

The following procedure describes the preparation of 2 ml of each cyclodextrin at a concentration of 40 mM for initial method development. The cyclodextrin-solutions are prepared in small volumes at relatively high concentration for the following reasons:

1) Cyclodextrins are relatively expensive
2) Only one or two runs are necessary to determine if the cyclodextrin shows appropriate interaction with the analyte
3) If cyclodextrin selection starts at a relatively high concentration the buffer can be used for further development at lower concentrations by dilution with buffer.

The cyclodextrins should be prepared in one of the buffers listed under 3. (Ordering Information). For acidic (anionic) analytes use 50 mM phosphate pH 7, 89 mM Tris/89 mM boric acid pH 8.3, and/or 50 mM sodium tetraborate pH 9.3 buffers. For basic (cationic) analytes use 50 mM phosphate, pH 2.5.

The cyclodextrin-solutions can be prepared in 2–5 ml vials by dissolving the appropriate amount of cyclodextrin in 2 ml buffer. The following table gives the mass needed for each of the cyclodextrins (concentration 40 mM):

| | |
|---|---|
| β-cyclodextrin | 91 mg |
| γ-cyclodextrin | 104 mg |
| hydroxypropyl-β-cyclodextrin | 111 mg |
| di-methyl-β-cyclodextrin | 106 mg |
| tri-methyl-β-cyclodextrin | 114 mg |

Molecular weight (MW) of cyclodextrins:

| | |
|---|---|
| β-cyclodextrin | 1135 |
| γ-cyclodextrin | 1297 |
| hydroxypropyl-β-cyclodextrin (Ds~4.6) | 1384 |
| di-methyl-β-cyclodextrin | 1331 |
| tri-methyl-β-cyclodextrin | 1430 |

The solution should be drawn or poured into a 1-5 ml syringe and filtered through a 0.2 µm filter. The final volume of cyclodextrin solution should then be divided equally into the inlet and outlet vials.

If a separation of the enantiomers can be observed the fine-tuning of the method can be started. If no separation occurs the buffer should be diluted 1:1 with the appropriate cyclodextrin-free buffer and the analysis repeated. Further dilution of the buffer should be done until separation is observed. If no separation is obtained, higher cyclodextrin concentrations (starting at 75 to 100 mM) should be tried.

### 5.2 Capillary Conditioning

Prior to First Use Conditioning

Preconditioning of capillaries prior to their first use can dramatically improve migration time reproducibility by cleaning and stabilizing the internal capillary wall. It is recommended that all capillaries are carefully washed before their first use, and further, that a capillary is used only for one analytical method for highest reproducibility (not including method development). A variety of wash methods have been described. Here we recommend two procedures:

I. Temperature 20–25 °C
Step 1 flush 5 minutes with methanol
Step 2 flush 5 minutes with distilled deionized $H_2O$
Step 3 flush 5 minutes with 1N NaOH
Step 4 flush 5 minutes with distilled deionized $H_2O$
Step 5 flush 10 minutes with run buffer.

II. Temperature 20–25 °C
Step 1 flush for 10 minutes with 1N NaOH
Step 2 wait for 5 minutes
Step 3 flush for 20 minutes with run buffer.

Wash I is recommended for general operation or method development. Wash II is recommended if the method used is intended to operate at high pH (>7). In both cases these have been developed for 50 µm capillaries with a total length of 64 cm. If longer capillaries are used the wash time can be increased slightly. If larger bore capillaries are used then these times should not be changed. When using the Agilent CE system, monitor the ONLINE signal at 200-214 nm to visualize flow through the detector.

Between Run Conditioning

Washing capillaries between analyses need not use such high concentrations of wash solution or such extreme pH values. In particular large changes in pH over a short wash cycle should be avoided since the EOF is pH dependent and the equilibration time for the capillary wall can be very long if pH is drastically altered.

Inter-analysis wash regimens should be suited to the analysis in

question. At neutral or high pH (>7) 0.1 N NaOH flushing followed by buffer can be used and can be particularly effective. This type of wash, or even using a higher concentration of NaOH, may be mandatory if the sample or matrix is prone to sticking onto the capillary wall (e.g. strong bases). However if the sample or matrix shows no wall-interaction then simply flushing between runs with the run buffer is sufficient to maintain EOF stability and prevent carry-over from sample to sample.

With low pH buffers (<4) washing with strong alkali should be avoided if possible. In this case washing with 0.1 M HCl or 0.1 M $H_3PO_4$, may be more effective in stabilizing the EOF.
Within the pH range 4 to 7 the capillary wall and EOF are extremely sensitive to pH changes. In this case avoid any pH changes by washing only with the running buffer or ensure that the timing of washes with either acid or alkali is very reproducible between analysis and that the wash time of the running buffer is extended (5–15 minutes). It may also be beneficial to perform two or three blank runs before starting the analysis, or to subject the conditioned capillary to an applied voltage for 5 to 20 minutes prior to performing analyses.

For method development purposes, between runs the capillary should be rinsed with the buffer containing the cyclodextrin for 5 minutes. When the cyclodextrin-solution is diluted to different concentrations the capillary should be reconditioned by performing a 10 minute rinse with the new concentration. When the cyclodextrin-type is changed the capillary should be rinsed with water for 5 minutes and then conditioned with the new cyclodextrin-solution for a further 10 minutes prior to performing a run.

### 5.3 Capillary storage

When the capillary is to be removed from the instrument it should be washed for 15 to 30 minutes with distilled deionized water and then flushed with air (e.g. using an empty vial). When the capillary is to be reinstalled, it may be sufficient to use a "between wash" regimen before analyses, however if the migration time or peak shapes have changed it may be necessary to perform prior the "first use" routine.

### 5.4 Procedures for Analysis of Epinephrine

This section describes the procedures and method for anlaysis of epinephrine (dissolved to 0.1 mg/ml in 0.1N HCl). This analysis can be used to verify that the CE system is functioning properly. In addition, it provides the opportunity to become familiar with chiral separations by CE. Below are the analysis conditions followed by buffer preparation and a copy of the method which can be programmed into the Agilent ChemStation. Comments are given in italics.

#### Procedures

Buffer: 25 mM phosphate, pH 2.5
20 mM dimethyl-β-cyclodextrin

Capillary: 50 µm i.d. , l=72 cm, L=80.5 cm
*Capillary length may be reduced if resolution of samples exceeds requirements*

Flush new capillary for 10 minutes with 1 N NaOH

Flush capillary for 1–3 minutes with 0.1 N NaOH

Rinse 0.1 N NaOH off capillary prior to flushing with buffer

Flush capillary for 2-10 minutes between analyses with buffer

Injection: Pressure, 50 mbar, 2 seconds
Adjust as needed

Power supply polarity:
Positive

Applied voltage:
30 kV with a voltage ramp time 0.3 minutes

Temperature: 25 °C

Stop Time: 20 minutes

Detection wavelength:
Signal = 200/10 nm
Reference = 380/50 nm
*Use detection wavelength optimized for the analyte*

#### Buffer Preparation

1. Prepare 25 mM phosphate, pH 2.5: Dilute 1 ml of 50 mM phosphate, pH 2.5 to 2 ml using CE water.
2. Prepare 20 mM dimethyl-β-cyclodextrin in 25 mM phosphate, pH 2.5: Weigh and dissolve 53 mg of dimethyl-β-cyclodextrin in the buffer.
3. Filter and transfer to CE buffer vials

#### Instrument Set-up

| Vial Contents | Carousel Location |
|---|---|
| Conditioning Vial containing 1 N NaOH | 1 |
| Conditioning Vial containing 0.1 N NaOH | 2 |
| Wash Vial containing water | 3 |
| Waste vial 1/4 full with water | 4 |
| INHOME<br>Vial containing cyclodextrin buffer | 5 |
| OUTHOME<br>Vial containing cyclodextrin buffer | 6 |

## **Method** (comment in italic)

**Capillary Electrophoresis**

**Home values:**

| | | |
|---|---|---|
| Lift offset | 4 | |
| Cassette Temperature | 25.0 °C | |
| Inlet Home Vial | 5 | |
| Outlet Home Vial | 6 | *Vial locations are exemplary only* |

**Replenishment and Preconditioning:**
Serial Processing

**Replenishment Entries:**

| | | |
|---|---|---|
| No Replenishment used | | *Buffer replenishment can be implemented for repetitive analyses* |

**Preconditioning Entries:**

| Function | Parameter | |
|---|---|---|
| 1 Flush | 10.00 min, I:1, O:4 | *1.0 NaOH flush for new capillaries* |
| 2 Flush | 3 .00 min, I:2, O:4 | *0.1 NaOH flush between analyses* |
| 3 Inlet | I:3 | *Raises buffer or water vial to remove residual NaOH* |
| 4 Flush | 5 .00 min, I:5, O:4 | *Buffer flush between analyses. Do not flush into OutHome vial. Avoid over-use of buffer* |
| | | *Use of 0.1 N NaOH is optional. Migration time stability may be sufficient without it. In addition, flush times may be varied as necessary.* |

**Postcondition Entries:**
No Postconditioning used

**Electric:**

| | | |
|---|---|---|
| Electric | On | |
| Polarity | Positive | |
| Voltage | 0.00 kV | |
| Current | 100.0 µA | *Current limit is not necessary but may be used to prevent excessive current generation for highly conductive buffers* |
| Power | System Limit | |
| Low Current Limit | 0.00 µA | |

**Injection:**

| | |
|---|---|
| Inject by PRESSURE 50 mbar s, 2.00 sec | *May be increased or decreased depending on result with sample. Alternatively electrokinetic injection can be used for dilute samples.* |

**Store Data:**

| | | |
|---|---|---|
| Collect voltage | Yes | |
| Collect current | Yes | *It is recommended to store current. It can be used to identify the source of problems if they occur* |
| Collect power | Yes | |
| Collect pressure | No | |
| Collect temperature | Yes | |

**Time entries:**

| | | |
|---|---|---|
| Stop time | 20.00 min | *Adjust as needed.* |
| Post time | Off | |

**Time Table:**

| Time[min] | Function | Parameter | |
|---|---|---|---|
| 0.30 | VOLTAGE | 30 kV | *Decrease if capillary length is greatly reduced or current generation is too high.* |

**Diode Array Detector**

**Settings**

| | | |
|---|---|---|
| Stop time | as HPCE: 20.00 min | |
| Post time | Off | |
| Response time | 0.3 sec | |
| Peak width | 0.03 min | |
| | *Adjust as necessary* | |
| Prerun autobalance | Off | |
| Postrun autobalance | Off | |

**Spectrum:**

| | | |
|---|---|---|
| Store | All | *Spectra are useful for peak identification, purity analysis, spectral library searching* |

**Signals:**

| Store | Signal,Bw | Reference,Bw [nm] | |
|---|---|---|---|
| A: Yes | 200/10 | 380/50 | *Optimize for analyte* |

### Separation of Epinephrine

The separation of epinephrine should be similar to that shown inFigure 3.
If results are not satisfactory, please refer the the Trouble-shooting section.

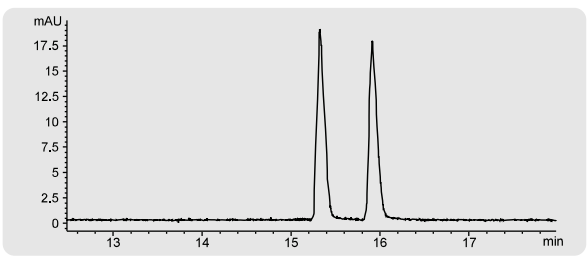



**Figure 3: Separation of epinephrine**

| | |
|---|---|
| Sample: | Epinephrine, 0.1 mg/ml in 0.1 N HCl |
| Buffer: | 25 mM Phosphate pH 2.5<br>20 mM dimethyl-β-cyclodextrin |
| Injection: | 100 mbar*s |
| Voltage: | 30 kV |
| Capillary: | l=72 cm, 50 µm i.d. |
| Temperature: | 25 °C |
| Detection: | 200 nm, 10 nm Bw |

## 6. Method Development Examples

The following sections describe the method development results for a variety of analytes. Each section contains chemical structures, experimental conditions, and representative electropherograms. These examples may be useful in determining initial conditions for similar analytes.

### 6.1 Method development for the separation of basic enantiomeric drugs

The following chapter describes the influence of
- Type of cyclodextrin
- Cyclodextrin concentration
- Buffer concentration and pH
- Capillary temperature

on the resolution on epinephrine enantiomers.

#### 6.1.1 Type of cyclodextrin

One of the first steps in method development is selection of the appropriate cyclodextrin. The size of the cyclodextrin cavity in conjunction with its chemical modifications determines the degree of solute interaction. Figure 4 demonstrates the impact of cyclodextrin type on the resolution of epinephrine enantiomers. With α-cyclodextrin no resolution is obtained since the cyclodextrin presumably has too small a molecular cavity to complex the solute. Using β-cyclodextrin, the results are improved but without baseline separation. Increasing the β-cyclodextrin concentration further would help, but is limited by the cyclodextrin solubility. Of the two methylated cyclodextrins studied, dimethyl-β-cyclodextrin is clearly superior.

#### 6.1.2 Cyclodextrin concentration

Figure 5 shows the impact of cyclodextrin concentration on the average migration time and resolution of the epinephrine enantiomers.
As the cyclodextrin concentration is increased, the migration time increases in an almost linear fashion. The higher cyclodextrin concentration naturally favors complex formation so the drug spends more time in complexed form. The charge to mass ratio is reduced and likewise its mobility. If the cyclodextrin concentration is too high, valuable separation time may be wasted without any meaningful gain in resolution. In some cases there can be an optimum cyclodextrin concentration, below and above which resolution decreases.

#### 6.1.3 Buffer concentration and pH

Figure 6 shows the impact of buffer ionic strength. As expected, increasing the ionic strength decreases the mobility by shielding the effective charge. The decrease in mobility may also be due in part to raising the equilibrium constant. This may be a "salting-in" effect where the ionic buffer environment is less preferred to the hydrophobic core

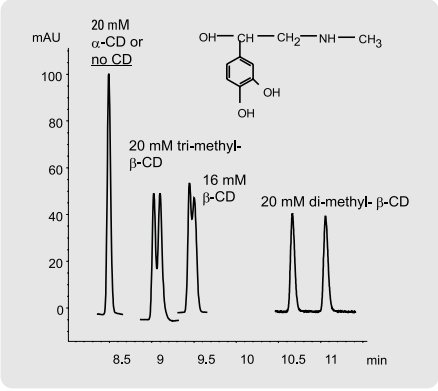



**Figure 4: Effect of cyclodextrin-type on the separation of epinephrine enantiomers.**

| | |
|---|---|
| Buffer: | 50 mM Tris-Phosphate pH 2.4, different cyclodextrins |
| Injection: | 100 mbar*s |
| Voltage: | 30 kV |
| Capillary: | l=56 cm, 50 µm i.d. |
| Temperature: | 25 °C |
| Detection: | 200 nm, 10 nm Bw |

**note: Phosphate buffer can be substituted for Tris-phosphate*

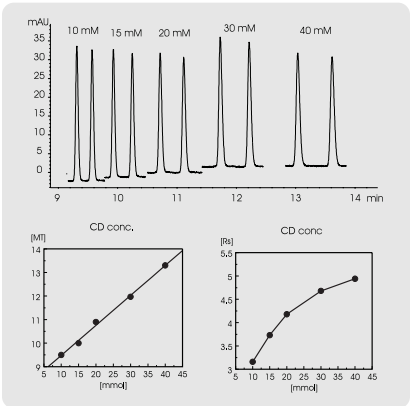



**Figure 5: Effect of cyclodextrin-concentration on the separation of epinephrine enantiomers.**

| | |
|---|---|
| Buffer: | 50 mM Tris-Phosphate pH 2.4, different concentrations of dimethyl-β-cyclodextrin |
| Injection: | 100 mbar*s |
| Voltage: | 30 kV |
| Capillary: | l=56 cm, 50 µm i.d. |
| Temperature: | 25 °C |
| Detection: | 200 nm, 10 nm Bw |

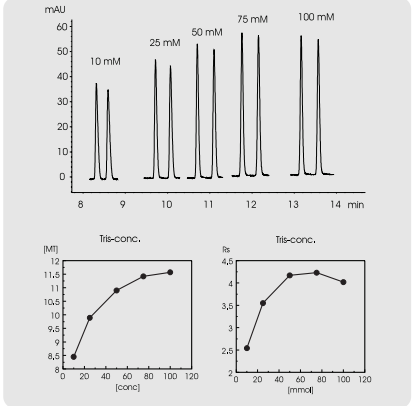



**Figure 6: Effect of buffer concentration on the separation of epinephrine enantiomers.**

| | |
|---|---|
| Buffer: | Tris-Phosphate pH 2.4, 20 mM dimethyl-β-cyclodextrin |
| Injection: | 100 mbar*s |
| Voltage: | 30 kV |
| Capillary: | l=56 cm, 50 µm i.d. |
| Temperature: | 25 °C |
| Detection: | 200 nm, 10 nm Bw |

of the cyclodextrin. Resolution only shows modest improvement as the buffer concentration is raised from 10 to 75 mM. At higher concentrations resolution drops, presumably as a result of increased current generation and Joule heating.

The effect of buffer pH is shown in figure 7. As the pH is increased, the migration time is reduced since the EOF becomes more significant and speeds elution. This effect will be more predominant at pH values between 6 and 9 where there is a high EOF dependence on pH.

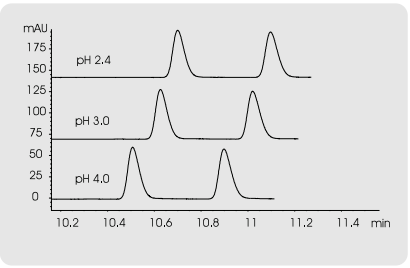



**Figure 7: Effect of buffer pH on the separation of epinephrine enantiomers.**

| | |
|---|---|
| Buffer: | Tris-Phosphate at different pH, 20 mM dimethyl-β-cyclodextrin |
| Injection: | 100 mbar*s |
| Voltage: | 30 kV |
| Capillary: | l=56 cm, 50 µm i.d. |
| Temperature: | 25 °C |
| Detection: | 200 nm, 10 nm Bw |

### 6.1.4 Capillary temperature

As illustrated in figure 8, lowering the capillary temperature increases the migration times since mobility always decreases as viscosity increases. A secondary effect might be an increase in the equilibrium constant since complex stability may be enhanced at low temperature. The longer the solutes remain in the capillary, the better the differentiation based on the difference of the mobility of the enantiomers. In some cases, separation can only be obtained at subambient temperatures.

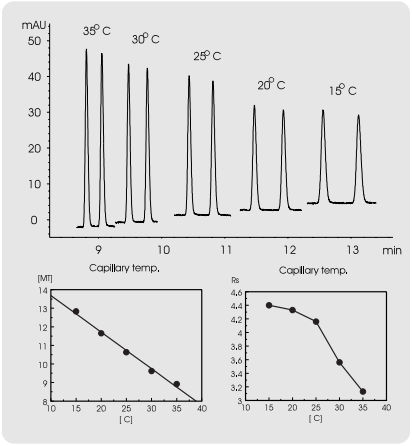



**Figure 8: Effect of capillary temperature on the separation of epinephrine enantiomers.**

| | |
|---|---|
| Buffer: | Tris-Phosphate pH 2.4, 20 mM dimethyl-β-cyclodextrin |
| Injection: | 100 mbar*s |
| Voltage: | 30 kV |
| Capillary: | l=56 cm, 50 µm i.d. |
| Temperature: | range from 15 to 35 °C |
| Detection: | 200 nm, 10 nm Bw |

### 6.1.5 Application of the method to different basic drugs

The method developed in the former section is now applied to different drugs as shown in figure 9. Shown here are the optimized separation conditions. All enantiomer pairs could be resolved from each other.

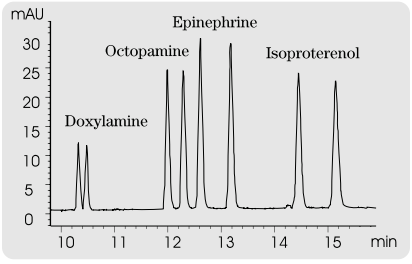



**Figure 9: Buffer: 50 mM Tris-Phosphate, pH 2.4, 20 mM dimethyl-b-cyclodextrin**

| | |
|---|---|
| Capillary: | l=56 cm, 50 µm i.d. |
| Injection: | 100 mbar*s |
| Voltage: | 30 kV |
| Temperature: | 15 °C |
| Detection: | 200 nm, 10 nm Bw |

The structures of the drugs separated in the previous example are shown below in figure 10.

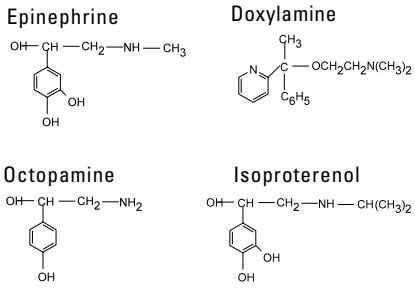



**Figure 10: Structures of the drugs separated in figure 9**

Figure 11 shows the determination of 1.1% (+)-Epinephrine impurity. Quantitative analysis of enantiomeric impurities will be highly dependent on resolution from the main component since overloading is often necessary to obtain low-level impurity detection.

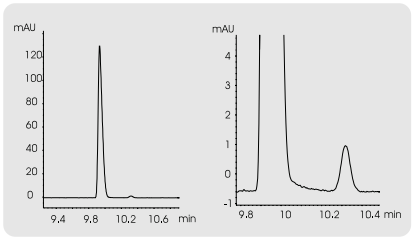



**Figure 11: Determination of 1.1% (+)-Epinephrine trace impurity**

| | |
|---|---|
| Buffer: | 50 mM Tris-Phosphate, pH 2.4, 20 mM dimethyl-β-cyclodextrin |
| Capillary: | l=56 cm, 50 µm i.d. |
| Injection: | 100 mbar*s |
| Voltage: | 30 kV |
| Temperature: | 15 °C |
| Detection: | 200 nm, 10 nm Bw |

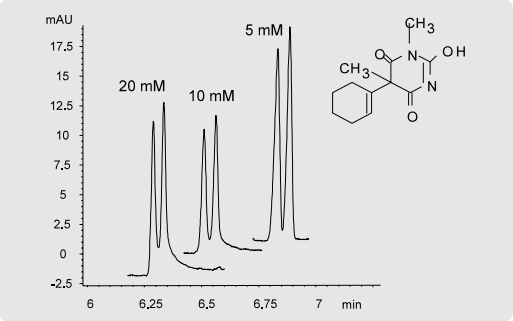
### 6.2 Method development for the separation of hexobarbital enantiomers

The results in figures 12-14 show the method optimization for separation of hexobarbital enantiomers using Tris-Borate buffer at pH 9 and hydroxypropyl-β-cyclodextrin as chiral selector.

The influence of the cyclodextrin concentration on migration time and selectivity is shown in figure 12. Selectivity increases and migration time decreases as the cyclodextrin concentration increases. Above 10 mM hydroxypropyl-β-cyclodextrin the migration time continues to decrease but there is no improvement in selectivity.

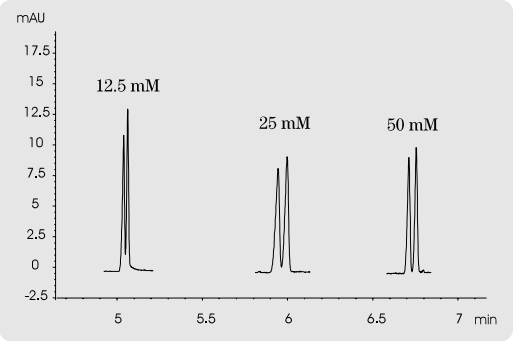
With increasing buffer concentration the resolution and migration time increases probably due to the decrease in EOF and longer time spent in the capillary. These results are shown in figure 13.

Numerous parameters such as the viscosity of the buffer solution, the $pk_a$ of the solute and the complexation constant with cyclodextrin are directly affected by variations of the temperature. Therefore it is not surprising that the temperature has a great influence on the separation as illustrated in figure 14 which shows the electropherogram of hexobarbital enantiomers at 10, 20, and 25°C.



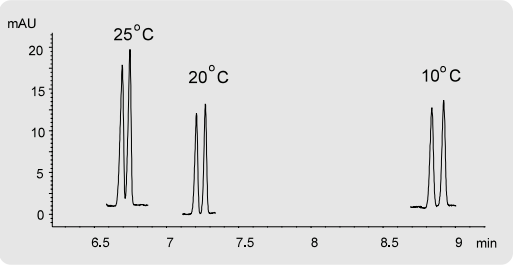

Buffer: 50 mM Tris-Borate, pH 9.0, different concentrations of hydroxypropyl-β-cyclodextrin
Capillary: l=72 cm, 50 mm i.d.
Injection: 100 mbar*s
Voltage: 30 kV
Temperature: 10 °C
Detection: 205 nm, 10 nm Bw

**Figure 12: Influence of cyclodextrin-concentration on the separation of hexobarbital enantiomers**




Buffer: Different Tris-borate concentrations at pH 2.4, 5 mM hydroxypropyl-β-cyclodextrin
Capillary: l=72 cm, 50 µm i.d.
Injection: 100 mbar*s
Voltage: 30 kV
Temperature: 10 °C
Detection: 205 nm, 10 nm Bw

**Figure 13: Influence of Tris-borate concentration on the separation of hexobarbital enantiomers**

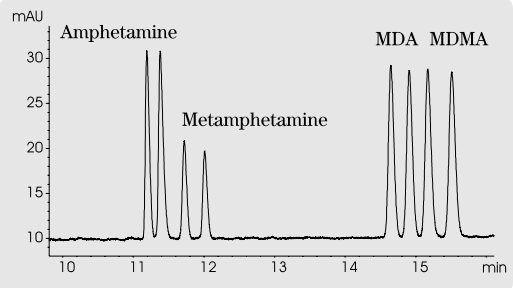



Buffer: 50 mM Tris-borate, pH 9.0, 5 mM hydroxypropyl-β-cyclodextrin
Capillary: l=72 cm, 50 µm i.d.
Injection: 100 mbar*s
Voltage: 30 kV
Temperature: range from 10 to 25 °C
Detection: 205 nm, 10 nm Bw

**Figure 14: Influence of the capillary temperature on the separation of hexobarbital enantiomers**

### 6.3 Method for the separation of amphetamines enantiomers

Another method using hydroxypropyl-β-cyclodextrin is shown in figure 15 in which four different amphetamines are separated. Excellent peak shape and baseline resolution of all amphetamines enantiomers were obtained in about 15 minutes using the optimal separation conditions.




Buffer: 50 mM Tris-Phosphate, pH 2.4, 20 mM hydroxy-propyl-β-cyclodextrin
Capillary: l=72 cm, 50 µm i.d.
Injection: 100 mbar*s
Voltage: 30 kV
Temperature: 15 °C
Detection: 205 nm, 10 nm Bw

**Figure 15: Separation of 4 different amphetamine enantiomers.**

The structures of the amphetamines separated in the previous figure are shown in figure 16.

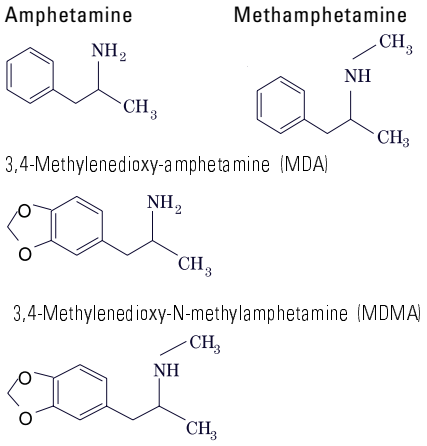



**Figure 16: Structures of amphetamines separated in figure 15**

### 6.4 Method development for the separation of dansylated phenylalanine enantiomers

The utility of different cyclodextrins on the resolution of 5-dimethyl-amino-1-naphthalene sulfonyl phenylalanine (Dns-Phe) enantiomers is investigated. As can be seen in the following examples, resolution is strongly influenced by the type and concentration of cyclodextrin employed. Figure 17 shows the structure of Dns-Phe and the experimental conditions employed. The cyclodextrins used are: α-cyclodextrin, β-cyclodextrin, γ-cyclodextrin, methyl-β-cyclodextrin, hydroxypropyl-β-cyclodextrin, di-methyl-β-cyclodextrin, tri-methyl-β-cyclodextrin.
The results of the experiments are summarized in figure 18.
The buffer with α-cyclodextrin shows no interaction with the analyte while the maximum resolution using β-cyclodextrin and di-methyl-β-cyclodextrin was 0.6 and 0.96 mmol respectively. The other cyclodextrins were applied successfully for the separation of Dns-Phe.

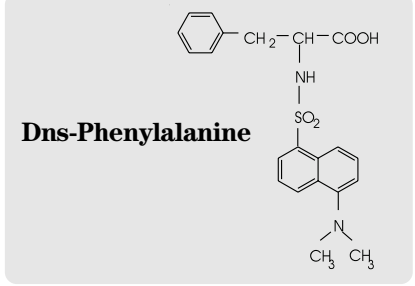



**Figure 17: Dns-Phe and experimental conditions for the separation**

Buffer: 50 mM borate, pH 9.3, different cyclodextrins
Voltage: 30 kV
Temperature: 15°C
Injection: 100 mbar*s
Capillary: l=72 cm, 50 µm i.d.
Detection: 200 nm, 10 nm Bw

Suitable cyclodextrins for enantioseparation of Dns-Phe are β-methyl-CD, HP-β-CD, tri-methyl-β-CD and γ-CD. An overview is given in table 1.

| Cyclodextrin | resolution | optimum CD-conc. |
|---|---|---|
| β-methyl-CD | 1.1 - 1.6 | 0.3 - 2.5 mM |
| HP-β-CD | 1.4 - 2.8 | 0.3 - 10 mM |
| tri-methyl-β-CD | 1.5 - 2.3 | 1.2 - 7.0 mM |
| γ-CD | 2.0 - 3.0 | 0.2 - 1.3 mM |

**Table 1: Overview of suitable cyclodextrins for enantioseparation of Dns-Phe**

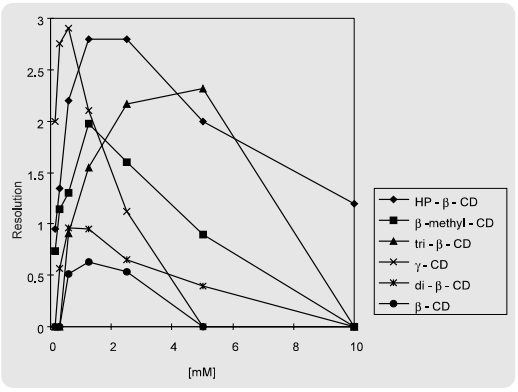
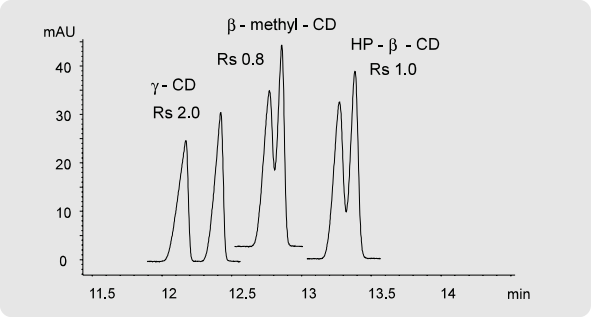
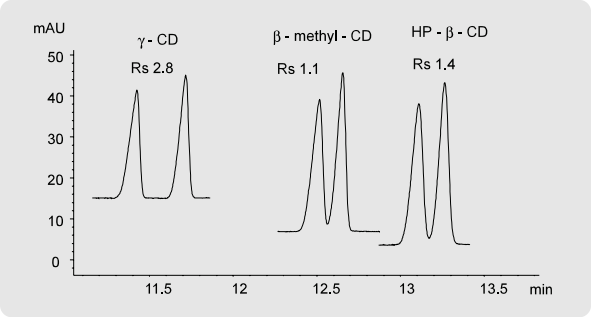



**Figure 18: Resolution of Dns-Phe using different cyclodextrins at varying concentrations**

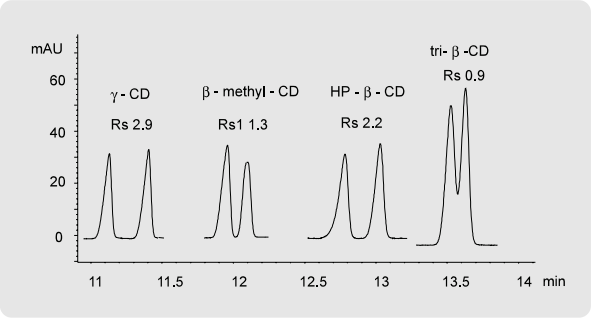
The electropherograms obtained by using different concentrations of cyclodextrins for the enantioseparation of Dns-Phe are shown in figures 19 (a-g).



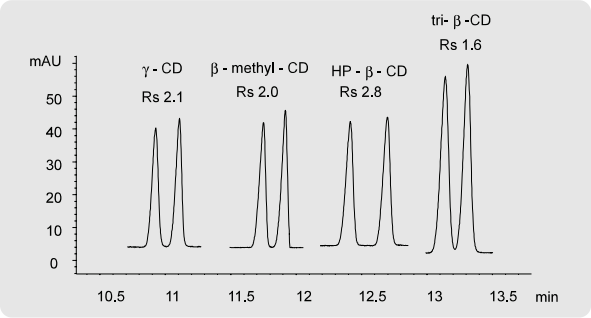

**a) 0.15 mM of different cyclodextrins**



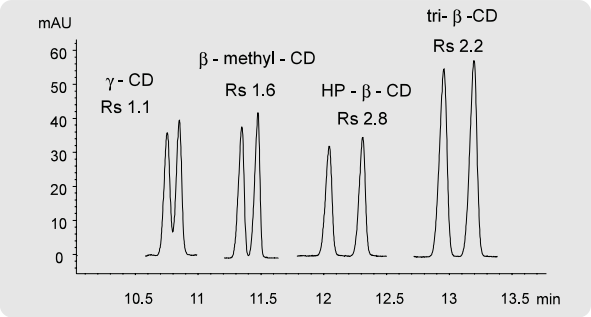

**b) 0.3 mM of different cyclodextrins**



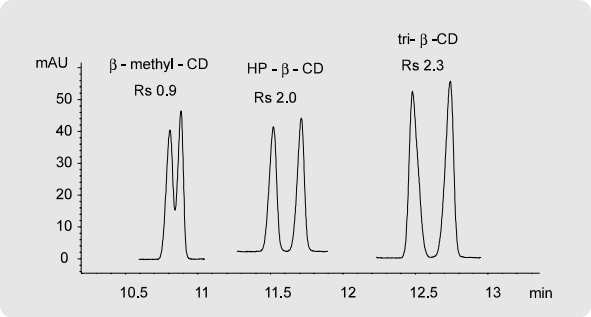

**c) 0.6 mM of different cyclodextrins**



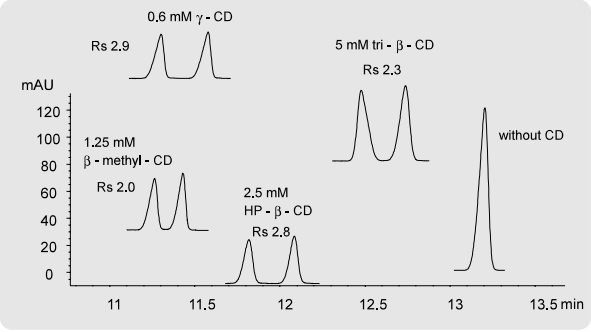

**d) 1.25 mM of different cyclodextrins**




**e) 2.5 mM of different cyclodextrins**




**f) 5 mM of different cyclodextrins**




**g) Optimal concentration of different cyclodextrins for the separation of Dns-Phe**

**Figure 19 (a-g): Separation of enantiomeric Dns-Phe using different cyclodextrins at varying concentrations**

## 6.5 Method development for the separation of a diastereomeric drug

Cyclodextrins can also be used to separate diastereomeric samples. The utility of cyclodextrins in the analysis of diastereomers is demonstrated in the development of the separation of (6R,S)-Leucovorin. Figure 20 shows the structure of Levoleucovorin Calcium.

Leucovorin is a reduced folate which is widely used clinically either as racemic (6R,S)- or pure (6S)-Leucovorin as an antidote to the toxic effects of methotrexate and as an enhancer of the antitumor activity of 5-fluoro-uracil in oncotherapy. Racemic Leucovorin, (6R)- and (6S)-Leucovorin were provided by Eprova AG (Schaffhausen, Switzerland). Samples were dissolved as a 0.4 mg/ml aqueous solution. All stock solutions were stored at -20°C.

Parameters affecting the separation, such as cavity size, type and concentration of cyclodextrin, buffer salt, SDS and capillary temperature were systematically investigated. The influence of the γ-cyclodextrin-concentration and the capillary temperature on the resolution of the sample is shown in figure 21. This cyclodextrin was chosen initially since the large cavitity seemed to best match the size of the analyte.

Interestingly, the resolution increased with increasing temperature but could not be improved by increasing the cyclodextrin concentration. The spikes in the electropherogram were caused by unknown reasons although the buffers were filtered before use. Urea was added to the buffer containing 250 mM γ-cyclodextrin to increase its solubility.

**Levoleucovorin Calcium**

(6S)-5-Formyl-5,6,7,8-tetrahydrofolic acid calcium salt

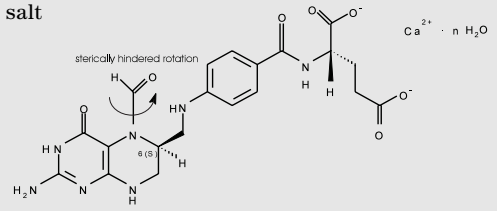
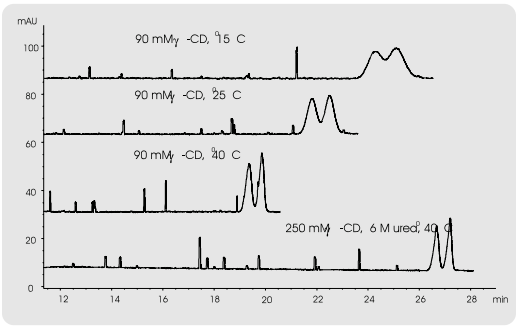
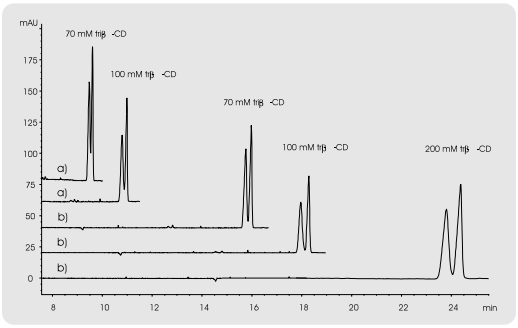



**Figure 20: Structure of Levoleucovorin Calcium**



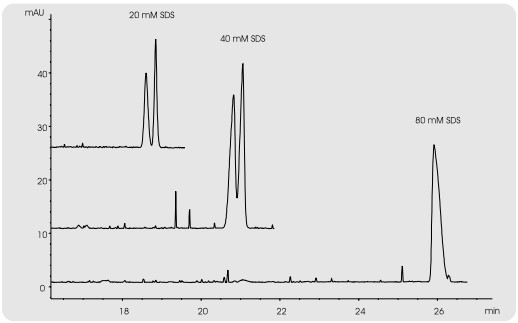

Capillary: l=56 cm, 50 µm i.d.
Buffer: 50 mM Phosphate pH 7.0, γ-cyclodextrin and urea cond. see electropherogram
Injection: 100 mbar*s
Temperature: see electropherogram
Voltage: 30 kV
Detection: 285 nm, 10 nm Bw

**Figure 21: Separation of Leucovorin using γ-cyclodextrin at different concentrations and different capillary temperatures.**




Capillary: a) l=56 cm, 50 µm i.d.
b) l=72 cm, 50 µm i.d.
Temperature: 15 °C
Buffer: 20 mM Borate pH 9.3, different concentrations of tri-methyl-β-cyclodextrin
Injection: 100 mbar*s
Voltage: 30 kV
Detection: 285 nm, 10 nm Bw

**Figure 22: Resolution of Leucovorin using different concentrations of tri-methyl-β-cyclodextrin**




Capillary: l=72 cm, 50 µm i.d.
Buffer: 20 mM Borate pH 9.3, 100 mM tri-methyl-β-cyclodextrin, SDS concentration, see electropherogram
Injection: 100 mbar*s
Temperature: 15 °C
Voltage: 30 kV
Detection: 285 nm, 10 nm Bw

**Figure 23: Separation of Leucovorin using tri-methyl-β-cyclodextrin and SDS.**

The influence of tri-methyl-β-cyclodextrin as chiral selector was examined in the next step. Because of its better solubility relative to γ-cyclodextrin addition of urea was not necessary. The result of the influence of the cyclodextrin concentration and capillary temperature on the separation of Leucovorin is shown in figure 22.

As expected, the migration time and selectivity increased as cyclodextrin concentration was increased. Longer capillaries also improved resolution. For further improvement of the resolution SDS was added at varying concentrations to the buffer and the influence examined. The results are shown in figure 23. The migration time increased with increasing SDS concentration because of the greater association of the solute with the micelles, but selectivity decreased.

The optimum capillary temperature was determined in the next experiment and the result is shown in figure 24.

Finally, the optimal conditions for the Leucovorin separation were used to determine the enatio-meric purity of (6R)- and (6S)-Leucovorin. The results are shown in figures 25 and 26.

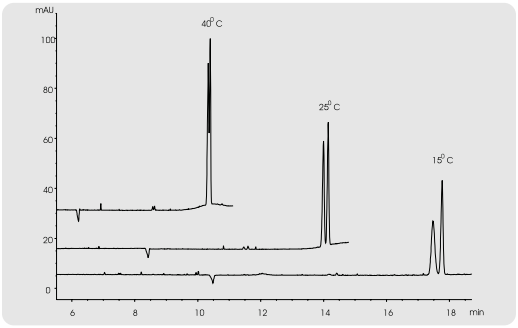



**Figure 24: Separation of Leucovorin at different temperatures.**

Capillary: l=72 cm, 50 µm i.d.
Buffer: 20 mM Borate pH 9.3, 100 mM tri-methyl-β-cyclodextrin
Injection: 100 mbar*s
Temperature: see electropherogram
Voltage: 30 kV
Detection: 285 nm, 10 nm Bw

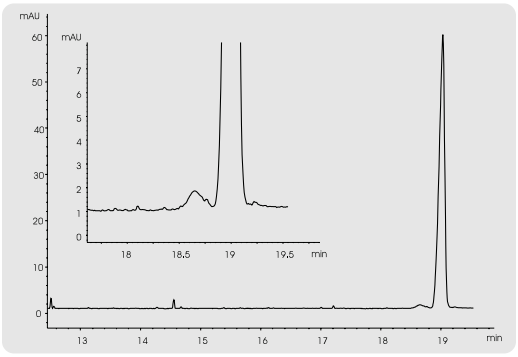



**Figure 25: Determination of the trace impuritiy of (6R)-Leucovorin.**

Capillary: l=72 cm, 50 mm id
Buffer: 20 mM Borate pH 9.3, 100 mM tri-methyl-β-cyclodextrin
Injection: 100 mbar*s
Temperature: 15 °C
Voltage: 30 kV
Detection: 285 nm, 10 nm Bw

The impurity of (6R)-Leucovorin was measured at 1.7%.

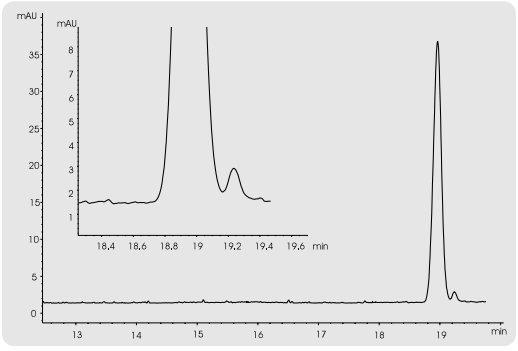



**Figure 26: Determination of the trace impuritiy of (6S)-Leucovorin.**

Capillary: l=72 cm, 50 mm id
Buffer: 20 mM Borate pH 9.3, 100 mM tri-methyl-β-cyclodextrin
Injection: 100 mbar*s
Temperature: 15 °C
Voltage: 30 kV
Detection: 285 nm, 10 nm Bw

The impurity of (6S)-Leucovorin was measured at 2.1%.

### 6.6 Separation of enantiomeric drugs using coated capillaries (PVA) and chargeable cyclodextrins

The utility of chargeable cyclodextrins and coated capillaries offer additional possibilities for the separation of enantiomers. Coated capillaries can reduce analyte-wall interactions, common for highly basic analytes. In addition, they can be used for both charged and neutral drugs.

The capillary used for the following experiments is permanently coated with poly(vinylalcohol) (PVA). The capillary shows virtually no electro-osmotic flow over a wide pH range (2.5-9.5). The carboxylic groups of chargeable (anionic, in this case) cyclodextrins are deprotonated at a pH above 5 resulting in self mobility of the chiral selector. This results in the separation of neutral compounds like hexobarbital at pH 7. Hexobarbital can also be separated with hydroxypropyl-β-cyclodextrin under alkaline conditions when the sample is deprotonated (pH >8), but hexobarbital is not stable under these conditions.
Figure 27 shows the separation of hexobarbital enantiomers using PVA capillary and carboxyethylated-β-cyclodextrin at pH 7.

The ion-pairing capability of charged cyclodextrins is demonstrated by separation of propranolol which is positively charged at the pH conditions used ($pk_a$ 9 +/-0.5). Hydrophobic interactions, affinity via hydrogen bonding and strong additional electrolyte interaction between the charged cyclodextrin and the analyte with opposite charges are responsible for the complex formation (figure 28).

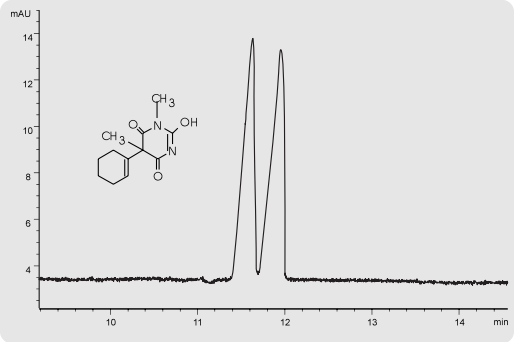



**Figure 27: Separation of Hexobarbital enantiomers**

| | |
|---|---|
| Buffer: | 50 mM Phosphate pH 7.0, 7 mmol carboxyethylated-β-cyclodextrin (not included in the kit) |
| Injection: | 100 mbar*s |
| Voltage: | -30kV |
| Temperature: | 40 °C |
| Capillary: | PVA, l=56 cm, 50 mm i.d. |
| Detection: | 220 nm, 10 nm Bw |

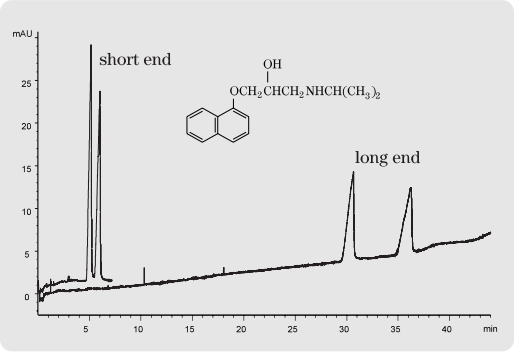



**Figure 28: Separation of propranolol enantiomers, injected at the short end (eff. length 8.5 cm) and at the long end (eff. length 56 cm) of a coated capillary**

| | |
|---|---|
| Buffer: | 50 mM Phosphate pH 7.0, 7 mmol carboxymethylated-β-cyclodextrin (not included in the kit) |
| Injection: | 100 mbar*s |
| Voltage: | 20 kV |
| Temperature: | 40 °C |
| Capillary: | PVA, l=56 cm, 50 mm i.d. |
| Detection: | 205 nm, 10 nm Bw |

## 7. Terminology used in Chiral Technology

**Chiral:** „Handed“, having the characteristic of „handedness“, which is having the potential to exist as two non-superimposable structures that are mirror images; not synonymous with the terms enantiomerically pure or optically active.

**D or L:** Absolute configuration of a molecule assigned according to experimental chemical correlation with that of D- or L-glyceraldehyde as the reference compound; often used for amino acids and sugars, but R and S are preferred.

**d or l:**Dextrorotatory or levorotatory according to experimentally determined rotation of the plane of monochromatic plane-polarized light to the right or left; since superseded by (+) or (-).

**Diastereoisomers:**
Stereoisomers with two or more centers of dissymmetry and whose molecules are not mirror images of one another; often contracted to „diastereomers“.

**Dissymmetric:**
Lacking an alternating axis of symmetry and so usually existing as enantiomers. Some prefer this to the term „asymmetric“.

**Enantiomers:**
Two stereoisomers whose molecules are nonsuperimposable mirrorimages of one another.

**Enantiomeric excess (ee):**
Percent by which one enantiomer, E1, is in excess in a mixture of the two;
ee = [(E1-E2)/(E1+E2)] x 100%.

**Enantioselectivity:**
Degree to which a chemical reaction produces one rather than the opposite enantiomer.

**Eutomer:**
The enantiomer that exhibits a particular drug related effect.

**Distomer:**
The enantiomer that lacks the particular drug effect or exhibits it to a lesser degree.

**Optical activity:**
Experimentally observed rotation of the plane of monochromatic plane-polarized light by a substance to the observer's right or left.

**Optical isomer:**
Synonym for enantiomer; now disfavored, because most enantiomers lack optical activity at some wavelengths of light.

**Optical purity:**
Percent of one enantiomer in a mixture of two according to experimentally measured angle of optical rotation; not valid to describe enantiomeric purities determined by other methods.

**R or S:**
Absolute configuration about a dissymmentric center, assigned by drawing the molecule according to specific conventions and finding wether a circle passes through certain atoms surrounding the center clockwise to the right (R, rectus) or counterclockwise to the left (S, sinister).

**Racemic:**
Existing as a racemate, or 50-50 mixture of the two enantiomers; also denoted dl (discouraged) or (+/-) (preferred). Racemates are also called racemic mixtures or racemic modifications.

**Racemization:**
Conversion of one enantiomer to a 50-50 mixture of the two.

**Scalemic:**
Existing as a mixture of two enantiomers in which one is in excess; coined in recognition that most syntheses or resolutions do not yield 100% of one enantiomer.

**Stereoisomers:**
Compounds whose molecules consist of the same number and types of atoms with the same connectedness but different configurations.

## 8. References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## 9. Troubleshooting

| Problem | Possible Cause | Solution |
|---|---|---|
| Unstable current | Capillary not filled with buffer | Increase flush time |
| | Air bubbles in buffer | Ultrasonicate buffer |
| | Capillary clogged | Flush with syringe or cut inlet end |
| | Capillary broken at window | Replace capillary |
| Poor resolution or broad peaks | Capillary not equilibrated | Flush and repeat analysis |
| | Sample overloaded | Reduce sample concentration or amount injected |
| No signal | Wrong setting of power supply polarity | Verify if injection and migration is toward anode |
| | Detection wavelength incorrect | Verify wavelength |
| | Sample not injected | Verify no air bubble trapped in bottom of sample vial |
| Noisy baseline | Buffer contains particles | Filter through 0.20 µm filter |
| | Alignment interface occluded | Examine under microscope and clean with MeOH |
| | Capillary window dirty | Examine and clean with lintfree paper/MeOH |
| | Lamp is old | Replace if more than 650-750 hours |
| Poor reproducibility | Capillary not equilibrated | Increase flush time with buffer |
| | Buffer overused | Replace buffer |


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