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The use of substituted cyclodextrins in capillary electrophoresis for the separation and identification of methorphan enantiomers

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The Use of Substituted Cyclodextrins in Capillary Electrophoresis for the Separation and Identification of Methorphan Enantiomers

By

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Abstract

The goal of this project was to separate the enantiomers levo and dextromethorphan using capillary electrophoresis (CE). Levomethorphan is a controlled substance, and dextromethorphan is an active ingredient in many cough syrups. Methylated-β-cyclodextrins (CDs) are used as additives because the CDs have chiral centers, allowing them to bind differently to the two enantiomers and thereby changing the enantiomer migration times in CE. Dimethyl-β-CD did not separate the levo and dextromethorphans; however the trimethyl-β-CD was successful at producing baseline separation with migration times of roughly 6 minutes. We are optimizing it by changing variables such as type of buffer, buffer concentration, buffer pH, CD concentration and applied voltage. In addition, we are comparing the use of other substituted β-CDs for separating these enantiomers with the results obtained with the trimethyl-β-CD.

Acknowlec'gments

I would like to thank Cerestar Inc. for providing cyclodextrins, the Union College Chemistry Department for all of their help and support. In addition, I want to thank Tania Magoon ('01) and Keiko Ota ('01) for the research that they did on propoxyphene. I would especially like to thank my advisor, Tom Werner, for guiding me through my research and for being an excellent mentor.

Sincerely,

Colleen Strait

Colleen Strait ('02)

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Introduction

Within the past ten to fifteen years, capillary electrophoresis (CE) has become a very important tool in analytical chemistry and in drug analysis. There are many advantages of using CE over other standard instruments, such as high performance liquid chromatography (HPLC). Electrophoresis is preformed in a very narrow capillary that can range from 25-150 micrometers in diameter and can have a length of 50 to 200 cm. Because the capillary is so narrow, only nanoliters of the sample are needed. In performing CE, the two ends of the capillary are placed into an inlet and outlet vial. The sample is run under high voltages (10-30 kV) and high electric fields (100-1000 V/cm) (Figure 1).

CE separates ions and molecules on the basis of their charge to size ratio. The detector is usually at the cathode, which is negatively charged. The detector measures analyte absorption in the UV/Visible range, and therefore the capillary walls are made of fused silica, which does not absorb in the UV. An external coating covers the fused silica because it is very brittle, so this gives the capillary strength. Since detection is usually performed on the capillary, a small portion of the protective coating is removed at the detector site [2].

The fused silica contains silanol groups, which ionize under moderately high pH. This ionization causes the wall to have a negative charge; positive ions associate with the wall to keep the overall charge neutral. This is known as the electrical ion double layer. The surface charge is known as the zeta potential (Equation 1):

$$\zeta = 4\pi\delta e/ \qquad (1)$$

where is the buffer's dielectric constant, e is the total excess charge in solution per unit area, and δ is the double layer thickness (Equation 2):

$$\delta = 3x10^{7}(Z)/(C^{1/2}) \qquad (2)$$

Z is the number of valence electrons and C is the buffer concentration. When a voltage is applied, the cations in the buffer migrate toward the cathode. This produces a net flow that pulls all ions and neutral species toward the cathode. This net flow is called the electroosmotic flow (EOF) (Figure 2). The electroosmotic mobility (μ_{EOF}) is related to the charge on the capillary, the buffer viscosity and the dielectric constant (Equation 3) [2]:

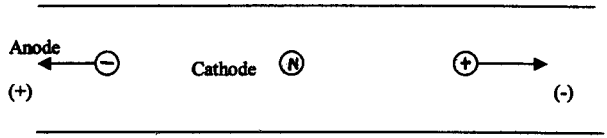
$$\mu_{EOF} = o \quad \zeta/4 \pi \eta \qquad (3)$$

where is the dielectric constant, η is the viscosity constant of the buffer, ζ is the zeta potential.

The pH has an effect on the μ_{EOF}; as pH increases the μ_{EOF} increases up to about a pH of nine. By increasing the pH the silanol groups on the capillary become ionized which increases the zeta potential, in turn increasing the μ_{EOF} (Figure 3). However, at a pH of nine or greater, the silanol groups are fully ionized, and the μ_{EOF} reaches a maximum and levels off. The μ_{EOF} has the least reproducibility in the pH range 4-6 because a slight change in pH causes a dramatic change in the μ_{EOF}.

There is a second mass flow mechanism that affects the mass transfer in the capillary, the electrophoretic mobility (μ_{EPF}). The μ_{EPF} is governed by the direction in which the species is attracted (Figure 4).

Figure 4: Electrophoretic Flow



Equation 4 shows the relationship between μ_{EPF} and charge to size ratio:

$$\mu_{EPF} = q / 6\pi r * \eta \qquad (4)$$

q is the charge, r is the radius and η is the viscosity. This mobility is often not as great as μ_{EOF}, but it does have a significant effect on the apparent mobility. Mobility is the proportionality constant that relates ion velocity (v) and electric field (E) (Equation 5):

$$v = \mu E \qquad (5).$$

The apparent mobility depends upon both the μ_{EOF} and the μ_{EPF} (Figure 5). A positive ion will have an μ_{EPF} that is in the same direction of the μ_{EOF}, since the flows are additive, the cations will reach the cathode faster than other species. An anion is attracted to the anode and does not want to go to the cathode. The anion tries to go to the anode, but is swept along with the EOF. It is similar to a fish swimming up stream; it will eventually go downstream like all the other fish, however, it will take longer. Equation 6 shows how the apparent mobility is calculated [2]:

$$\mu_{APP} = (L_d/t_m)/(V/L_t) = \mu_{EOF} + \mu_{EPF} \qquad (6)$$

L_d is the length of the capillary to the detector (0.560 m), L_t is the total capillary length (0.645 m), t_m is the migration time (seconds) and V is the applied voltage (20×10^3 V). Neutral species will have no electrophoretic flow, so the apparent mobility is equal to the μ_{EOF}. This feature makes neutral species good markers of the μ_{EOF}.

Both migration time and resolution are important in CE. To enhance resolution and reduce migration times, the μ_{EOF} can be changed, the μ_{EPF} can be changed or the voltage increased. Parameters of the method can be altered such as the buffer concentration and pH that can effect both mass flow mechanisms. By increasing the buffer concentration (increasing ionic strength) the μ_{EOF} decreases. Therefore, at higher buffer concentrations the migration time should be longer [2]. The reason for this lies in the equation for the zeta potential (Equation 1). As the buffer concentration increases, the zeta potential and therefore the μ_{EOF} decreases in proportion to the square root of the buffer concentration.

The pH not only alters the μ_{EOF}, as mentioned earlier, it can change the μ_{EPF}. The charge on a species will change according to the pH and this alters the rate at which it will flow. For example, if a species becomes more positive, the apparent mobility will increase and the migration time will decrease. In most cases this is the most important factor in getting good resolution. As the difference in the charge and size of the species increases, separation is easier, thereby gaining better resolution.

Increasing the voltage can also lead to better resolution. Unfortunately, by doing this there is a production of heat. This subsequent heating (joule heating) can cause substantial band broadening [2].

It is possible to separate many different molecules with CE such as neutral and charged species, and biomolecules. Separating enantiomers becomes increasingly difficult because they are chemically equivalent. In order to make the separation occur, an additional species needs to be added to interact with the enantiomers. The additive, which is added to the background electrolyte, must be optically pure. The separation will

occur if there are different interactions between the enantiomers and the additive. These types of interactions may be dipole-dipole interactions, hydrogen bonding, and hydrophobic interactions.

Cyclodextrins (CD) have become some of the most widely used additives to separate chiral molecules. CDs are versatile, easy to use and fairly inexpensive. They have been made functional; anionic, cationic, hydroxylated and methylated CDs are available [1].

CD's are compounds formed with 6, 7, 8 glucopyranose units (Figure 6). These different CDs are called α-, β-, γ-, respectively. They have a torus shape and have a hydrophobic cavity. This cavity allows the CD to have hydrophobic interactions and form complexes (Figure 7). Because there is a specific size to the cavity, the CD can only have good interactions with certain size molecules. If the analyte is too small or too large, with respect to the cavity size, there may not be strong enough interaction to have any effect on separation [1]. However, by having three different CD sizes, a variety of molecular sizes can be accommodated.

When a charged analyte interacts with the neutral CD, the effective charge/mass of the analyte decreases. This reduces its electrophoretic mobility. Not all of the analyte will be in a complex state with the CD, so there is a ratio between free analyte and complexed analyte. Differences in the equilibrium constants determine the ratio of free/complexed analyte. If the differences in the equilibrium constants are great enough, separation will occur (Equation 7)[1].

$$\Delta\mu = [C](\mu_1-\mu_2)(K_1-K_2)/1+[C](K_1+K_2)+K_1K_2[C]^2 \qquad (7)$$

$\Delta\mu$= difference in the apparent electrophoretic mobility of the two enantiomers
μ_1= mobility of uncomplexed solute
μ_2= mobility of complexed solute
C= concentration of chiral selector
K_1= equilibrium constant of enantiomer #1
K_2= equilibrium constant of enantiomer #2

Being able to separate enantiomers with CE using CDs has impacted drug analysis. Since the CE does not need large amounts of sample, this makes it a better method for analyzing than HPLC in many cases.

The two enantiomers that we wanted to separate are levo and dextromethorphan (Figures 8 and 9). Levomethorphan is a schedule II controlled substance and it is a narcotic analgesic. It relieves pain but is highly addictive. Dextromethorphan (DXM), however, can be purchased in a variety of over-the-counter cough syrups. DXM has replaced codeine as a cough suppressant, it has no opiate-like activity, but codeine is an analgesic, and DXM is not [3]. High doses of DXM can cause nausea, visual and audial hallucinations, loss of motor control and itchy skin. Symptoms of overdose include stupor, hyperexcitability, respiratory depression, tachycardia, nausea and vomiting occur. No one has been reported as dying from a DXM overdose [4].

The goal of this work is to develop a method of separating levo and dextromethorphan using CE and neutral CDs as additives. By changing the type and concentration of CD, as well as buffer concentration, we hope to develop an optimized method of separation. The pH range used is such that the methorphans will be protonated and have an electrophoretic flow in the same direction as the electroosmotic flow. The CDs that were used in this experiment were dimethyl-β-CD, trimethyl-β-CD, α-CD and γ- CD, α-CDP, γ- CDP and β-CDP.

Experimental

Stock Solutions. The levomethorphan was purchased as a solid from Cerilliant; 9.2 mg were transferred into a vial and 9.00 mL of methanol was added to make the concentration 1.00 mg/mL. The dextromethorphan was purchased as a solution from Cerilliant that was already at a concentration of 1mg/mL in methanol. The dextromethorphan was transferred into a vial that would decrease loss due to evaporation.

Sample Solution. The sample solution was prepared with 0.10 mL of 1mg/mL of both dextro and levomethorphans and 0.10 mL of methanol and 0.70 mL of water. This made the final concentration of both enantiomers to be 0.10 mg/mL

Buffer Solutions. Three borate buffer solutions with concentrations of 25, 50 and 75 mM, were prepared using boric acid and were adjusted to the desired pH by 1 M NaOH. Each buffer was prepared in a 250 mL beaker. Buffers were made at pH's of 8.14, and 8.21, 8.27, 8.38, 8.64, 9.02.

Three phosphate buffer solutions with concentrations of 25, 50, and 75 mM were prepared using phosphoric acid with a concentration of 14.8 M. The stock solution was diluted and adjusted to the desired pH with 1 M NaOH. The buffers were made at a pH of 8.31.

Cyclodextrins. The first cyclodextrin (CD) that was used was heptakakis di-(o-methyl)-β-CD, (DM-β-CD, average degree of substitution = 13). The DM-β-CD was a gift from Cerestar USA, Inc.. The second cyclodextrin used was heptakis (2,3,6-O-methyl)-β-CD (TM-β-CD) which was purchased from Aldrich Chemical company. Other CDs that were tested were α and γ CD as well as α, β, and γ polymer CDs.

Varying concentrations of CDs were used in the buffer solutions. Three CD solutions were prepared with concentrations of 20, 30, 40 mM. The appropriate amount of CD was weighed directly into a vial and 3mL of buffer were added. To ensure that the solution was mixed, it was stirred for 3 minutes.

CE. All experiments were performed on a Hewlett Packard (HP) 3D CE instrument. The detector was at the cathode end and the other specifications were as follows:

Capillary Length (to detector)	64.5 cm (56 cm)
ID	50 μm
Temperature	25 C
Applied Voltage (time)	20 kV
Injection Pressure	50 mBar (4 seconds)
Absorption Wavelength	210 nm
Run Time	10 min
Flush Time	5 min
All runs were done in triplicate	

Three vials of the same buffer are needed to do one run. There is an inlet buffer, and outlet buffer and a wash buffer. All of the buffer solutions contain the desired concentration and type of CD. Since running a sequence is possible, multiple buffer concentrations and or CD concentrations can be run at the same time. A sample setup is shown in the following table:

Vial #	
10	25 mM Borate Buffer, pH 8.2, 40 mM TM-β-CD (inlet)
11	25 mM Borate Buffer, pH 8.2, 40 mM TM-β-CD (outlet)
12	25 mM Borate Buffer, pH 8.2, 40 mM TM-β-CD (wash)
13	50 mM Borate Buffer, pH 8.2, 40 mM TM-β-CD (inlet)
14	50 mM Borate Buffer, pH 8.2, 40 mM TM-β-CD (outlet)
15	50 mM Borate Buffer, pH 8.2, 40 mM TM-β-CD (wash)
16	75 mM Borate Buffer, pH 8.2, 40 mM TM-β-CD (inlet)
17	75 mM Borate Buffer, pH 8.2, 40 mM TM-β-CD (outlet)
18	75 mM Borate Buffer, pH 8.2, 40 mM TM-β-CD (wash)
19	Sample containing 0.10 mg/mL of Levo and Dextromethorphan

Each buffer run is given a different method name. For example vials 10-12 would be method LD25.m, 13-15 LD50.m and 16-18 LD75.m. The sequence parameters table would be setup the following way:

Sample Vial	Method	Injections/Vial
19	LD25.m	3
19	LD50.m	3
19	LD75.m	3

This proves to be a quick and easy method and it enables a lot of data to be collected in a relatively short period of time.

Results

A. Dimethyl-β-CD

There was no separation of the two enantiomers with the dimethyl-β-CD (DM-β-CD); CD concentrations of 20, 30 and 40 mM in buffer pHs of 9.02 and 8.14, were tried and all failed to produce separation. Figure 10 is an example of an electropherogram that was obtained by these conditions.

B. Trimethyl-β-CD

1. Varying TM-β-CD Concentrations

The concentrations of trimethyl-β-CD (TM-β-CD) were varied while maintaining the same concentration of borate buffer (50 mM) at a pH of 8.14. Table 1 contains the average migration times, resolution and standard deviations, for triplicate runs, for 20, 30 and 40 mM TM-β-CD. Resolution is based on the equation:

$$R=\Delta t/W_{ave} \qquad (1)$$

Δt is equal difference in migration time between the two peaks, and W_{ave} is equal to the average width of the two peaks.

Table 1: Migration Times and Resolutions using 20, 30, 40 mM TM-β-CD, 50 mM Borate Buffer, pH 8.14

20 mM TM-B-CD	Migration Time Peak 1 (min)	Migration Time Peak 2 (min)	Resolution
Average	4.805	4.987	1.5
Std. Dev.	0.05	0.05	0.0
% Std. Dev.	1%	1%	0.0%
30 mM TM-B-CD			
Average	5.050	5.152	0.713
Std. Dev.	0.02	0.02	0.03
% Std. Dev.	0.4%	0.3%	5%
40 mM TM-B-CD			
Average	5.597	5.895	2.7
Std. Dev.	0.06	0.06	1.4
% Std. Dev	1%	1%	51%

2. Varying Borate Buffer Concentration

The concentration of the TM-β-CD was held constant at 40 mM, while the buffer concentration was changed from 25 to 50 to 75 mM. The same calculations were completed for these data as for section A1. The pH (8.14) was the same for the 50 and 75 mM buffer solutions, but it was 8.21 for the 25 mM buffer solution, which may have skewed the results. In addition, the buffer solutions were not all made at the same time so this could change the results as well. The sample remained the same for all buffer solutions.

Table 2 shows the average migration times, resolution and standard deviations, for triplicate runs, for 25 mM borate buffer with 40 mM TM-β-CD at a pH of 8.21 and 50, 75 mM borate buffer with 40 mM TM-β-CD at a pH of 8.14.

Table 2: Migration Times and Resolutions using 25 mM Borate Buffer at pH 8.21 and 50, 75 mM Borate Buffer at pH 8.14 with 40 mM TM-β-CD

25 mM Borate Buffer	Migration Time Peak 1 (min)	Migration Time Peak 2 (min)	Resolution
Average	5.943	6.327	3.063
Std. Dev	0.04	0.05	0.085
%Std. Dev	0.7%	0.8%	3%
50 mM Borate Buffer			
Average	6.006	6.339	1.77
Std. Dev	0.01	0.01	0.02
%Std. Dev	0.2%	0.2%	1%
75 mM Borate Buffer			
Average	5.784	6.200	2.22
Std. Dev	0.003	0.004	0.03
%Std. Dev	0.05%	0.06%	1%

These data were redone using buffers made at the same time and at the same pH, because of this the results should be more reliable. The same buffer concentrations were

used; 25, 50 and 75 mM all at a pH of 8.38. Table 3 shows the average migration times, resolutions and standard deviations, for triplicate runs, for 25, 50 and 75 mM borate buffer.

Table 3: Migration Times and Resolutions using 25, 50 and 75 mM Borate Buffer, 40 mM TM-β-CD, pH 8.38

25 mM Borate Buffer	Migration Time Peak 1 (min)	Migration Time Peak 2 (min)	Resolution
Average	6.152	6.563	3.1
Std Dev	0.02	0.03	0.2
%Std. Dev	0.3%	0.5%	5%
50 mM Borate Buffer			
Average	5.376	5.693	3.7
Std. Dev	0.03	0.03	0.1
%Std. Dev	0.6%	0.5%	3%
75 mM Borate Buffer			
Average	6.460	6.890	4.5
St. Dev	0.04	0.05	0.3
%St. Dev	0.6%	0.7%	6%

These data were done over again because an expected trend of increased migration time with increase buffer concentration did not occur. As the buffer concentration increases, the electroosmotic mobility is expected to decrease, and this would slow the migration times. An unexpected trend occurred instead, migration times increase from 25 to 50 mM and then decreases from 50 to 75 mM. The same buffer concentrations were used, at a pH of 8.27. Table 4 shows the average migration times, resolutions and standard deviations for borate buffer concentrations of 25, 50 and 75, all have a 40 mM TM-β-CD concentration at a pH of 8.27.

Table 4: Migration Times and Resolutions of 25, 50, and 75 mM Borate Buffer, 40 mM TM-β-CD, pH 8.27

25 mM Borate Buffer	Migration Time Peak 1 (min)	Migration Time Peak 2 (min)	Resolution
Average	5.23	5.55	2.6
Std Dev	0.03	0.02	0.2
%Std Dev	0.6%	0.3%	7%
50 mM Borate Buffer			
Average	5.07	5.36	3.3
St. Dev	0.03	0.04	0.1
%St. Dev	0.7%	0.7%	3%
75 mM Borate Buffer			
Average	5.558	5.906	4.0
St. Dev	0.05	0.06	0.6
%St. Dev	1.0%	1.0%	15%

3. Varying the pH of the Borate Buffer

Since the same unexpected trend did occur when the three borate buffer concentrations were tested again at only a slightly lower pH, we decided to increase the pH by half a pH unit to see if the trend still occurred. Table 5 shows the average migration times, resolutions and standard deviations of 25, 50, 75 mM borate buffer, 40 mM TM-β-CD at a pH of 8.64.

Table 5: Migration Times and Resolutions using 25, 50 and 75 mM borate buffer, 40 mM TM-β-CD, pH 8.64

25 mM Borate Buffer	Migration Time Peak 1 (min)	Migration Time Peak 2 (min)	Resolution
Average	5.18	5.48	2.7
Std Dev	0.02	0.03	0.1
%Std. Dev	0.4%	0.5%	4%
50 mM Borate Buffer			
Average	5.26	5.57	2.6
Std. Dev	0.01	0.01	0.9
%Std. Dev	0.2%	0.2%	30%
75 mM Borate Buffer			
Average	5.76	6.12	3.5
Std. Dev	0.04	0.04	0.8
%Std. Dev	0.6%	0.6%	20%

The expected trend was seen at the higher pH and this can be clearly pointed out by how the electroosmotic and electrophoretic mobility differed from each other. Table 6 is a comparison of the average electroosmotic and electrophoretic mobility at all buffer concentrations and at each pH (8.27, 8.64). Only electrophoretic values are given for the first peak. The same trend was seen with the second peak as well.

Table 6: Comparison of electroosmotic mobility for all buffer concentrations at the pH of 8.27 and 8.64

Buffer Concentration	μ_{EOF} [1] pH 8.27	μ_{EPF} [1] pH 8.27 Peak 1
25 mM	4.8E-08 (0.3%)[2]	9.45E-09 (4.8%)
50 mM	4.9E-08 (0.8%)	1.09E-08 (0.2%)
75 mM	4.35E-08 (1.3%)	1.06E-08 (2.8%)
	μ_{EOF} [1] pH 8.68 Peak 1	μ_{EPF} [1] pH 8.68 Peak 1
25 mM	5.0E-08 (0.4%)	7.64E-09 (0.7%)
50 mM	4.9E-08 (0.5%)	8.19E-09 (3.9%)
75 mM	4.44E-08 (1.1%)	7.92E-09 (2.6%)

[1] $m^2/Vsec$
[2] +/- % Std. Dev

C. Varying Phosphate Buffer Concentrations

Since there was excellent resolution with the borate buffer, a phosphate buffer was tested to see if similar results would occur. The concentrations that were used with the borate buffer were kept the same (25, 50, 75 mM). The TM-β-CD was kept at 40 mM and the pH was 8.3. Table 7 shows the results of the 25, 50 and 75 mM phosphate buffer with the TM-β-CD.

Table 7: Migration Times and Resolution using 25, 50 and 75 mM phosphate buffer, 40 mM TM-β-CD, pH 8.3

25 mM Phosphate Buffer	Migration Time Peak 1 (min)	Migration Time Peak 2 (min)	Resolution
Average	5.706	5.938	2.6
Std Dev	0.13	0.14	0.2
%Std Dev	2.3%	2.4%	8%
50 mM Phosphate buffer			
Average	6.537	6.821	3.5
St. Dev	0.05	0.06	0.1
%St. Dev	0.8%	0.8%	3%
75 mM Phosphate Buffer			
Average	7.14	7.43	4.1
St. Dev	0.07	0.07	0.4
%St. Dev	0.9%	0.2%	10%

D. Other CDs

Several other CDs were tested to see if separation would be possible- alpha, gamma, alpha polymer, beta polymer and gamma polymer CDs were all tested. The same concentrations were used as with the DM-β-CD and TM-β-CD: 20, 30, 40 mM. Two different pH values were used similar to those in other methods, 8.23 and 8.64. There was no separation at any buffer concentration or pH with any CD.

E. Optimization

The separation can occur at a variety of buffer concentrations (25-75 mM) with at least two different buffers, borate and phosphate. Fluctuations in pH up to at least half a pH unit will not change the separation to any great degree (pH 8.2-8.64). However, only the TM-β-CD gave any separation of the methorphan enantiomers.

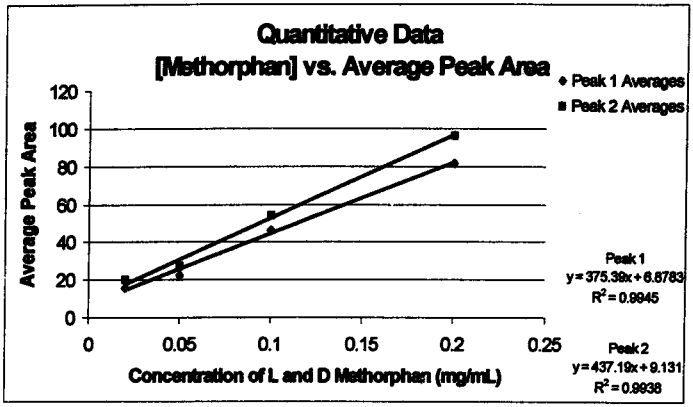
F. Quantitative Results of l, d Methorphan with TM-β-CD and Borate Buffer

Quantitative data were obtained by comparing the area under the curves in the electropherograms to methorphan concentration. A range from 0.02 to 0.2 mg/mL of the l and d methorphan was tested with 40 mM TM-β-CD, 50 mM borate buffer at a pH of 8.27. Table 8 shows the data at four concentrations of methorphan, 0.02, 0.05, 0.1, 0.2 mg/mL and the average peak area. Figure 11 shows the plot of these data.

Table 8: Quantitative data of Average peak area and Concentration of Methorphan

Concentration mg/mL	Peak 1 Average Area	Peak 2 Average Area
0.02	15.854	20.112
0.05	22.662	27.202
0.10	46.254	54.505
0.20	81.638	96.464

Figure 11: Concentration of Methorphan vs. Average Peak Area



Discussion

A. Varying dimethyl-β-CD concentration

The first combination of buffer concentration, p CD concentration that was used was a 50 mM borate buffer with a pH of 9.02 and DM-β-CD at concentrations of 20, 30 and 40 mM. There was no separation; however this was not a surprise because in a similar experiment done with propoxyphene the pH had to be dropped to around 8.2 to get some separation [5]. Figure 10 shows an electropherogram of the 30 mM DM-β-CD run. As can be seen, there is only one large peak at 6.048 min, which overlaps with the methanol peak at 6.231 min.

At a lower pH, there will be an increase in the charge on the methorphan enantiomers, and this will increase the electrophoretic mobility. When this occurs, the methorphan peak should be resolved from the methanol peak. Since there was some separation with the propoxyphene at a lower buffer pH, we tried the levo and dextromethorphan solution with DM-β-CD in a buffer with a pH of 8.14. Unfortunately, as shown in Figure 12, there was no separation of the enantiomers, but there was baseline resolution between the methanol peak and the methorphan peak. In addition, the migration times decreased, the methorphan peak is at 5.916 minutes.

B. Trimethyl-β-CD concentration

1. Varying TM-β-CD Concentrations

We next tried 20, 30, and 40 mM TM-β-CD at pH, 8.14, and a buffer concentration of 50 mM. Separation occurred for all three CD concentrations, and baseline separation occurred at 20 (Figure 13) and 40 mM (Figure 14). The runs were all performed in triplicate and the data are found in table 1. The precision of the CE results

were quite good with relative standard deviations of 1% or less in each case. In order for the resolution (R) to be considered base-line, R must equal 1.5 or greater. The 20 mM TM-β-CD produced an average R value of 1.5 and the 40 mM TM-β-CD produced an average R value of 2.7. The 30 mM TM-β-CD produced an average R value of less than 1. It is unclear as to why the 30 mM behaved this way since it shows no trend with the 20 and 40 mM TM-β-CD. Something may have gone wrong in the buffer preparations because an increasing trend in resolution is expected with increasing CD concentration.

2. Changing Buffer Concentration

Since 40 mM TM-β-CD gave the best resolution, this concentration of CD was used in varying buffer concentrations (25, 50, 75 mM). However, not all the buffers were at the same pH; the 25 mM buffer was at a pH of 8.21 and the 50, 75 mM buffers were at a pH of 8.14. The expected trend, and the trend that was seen in the propoxyphene experiment, was that t_m would increase with increasing borate concentration (see equations 1-3) [2]. With an increase in ionic strength, there will be a decrease in the electroosmotic mobility. This trend was not seen, as shown in table 2; the migration times do not follow any sort of pattern. The migration times increase from 25 to 50 mM and then decrease from 50 to 75 mM. This can be clearly seen in the migration times of Figures 15 and 16, which contain examples of electropherograms of 25 and 75 mM borate buffer at a pH of 8.21 and 8.14, respectively.

When doing these experiments it is very important to keep the pH exactly the same for all of the buffer concentrations. This unfortunately was not done in this trial. The pH of the 25 mM borate buffer was 8.21 and the other two were at the original pH of 8.14. Because the pH was not the same in all of the buffer concentrations, it is not

possible to draw conclusions about which buffer concentration gives the best resolution with the lowest migration time. Another problem that could skew the results was that all three buffers were not prepared at the same time. These data are suspected of being faulty because there was not very much change in migration time with the different buffer concentrations. There was a fairly drastic change in the propoxyphene data under these conditions [5].

The runs were precise with relative standard deviations of 1% or less for the migration times and there was still baseline resolution for all three buffer concentrations.

These data were repeated using freshly prepared buffers (25, 50, 75 mM) all at pH 8.38. Table 3 shows the average migration times and resolutions using these buffers. All of the buffers were made at the same time. Surprisingly, the results were very similar to those in table 2; there was not an increase in migration time as buffer concentration increased. We still did not understand why this was occurring and decided to check the data again.

The same buffer concentrations were used with 40 mM TM-β-CD, but the pH was dropped to 8.27. The data in table 4 confirm that the results in both tables 2 and 3. One theory to explain these seemingly odd results is that there may possibly be due to an ionic strength effect on the pKa reaction of the silico-OH groups. Because we are at a pH of 8.27, not all the silanol groups are deprotonated and an increase in ionic strength could lead to a shift in the pKa equilibrium to the right (see figure 18).

$$\text{-Si-OH} \leftrightarrow \text{-Si-O}^- + \text{H}^+ \qquad (18)$$

This would increase the electroosmotic mobility and decrease migration times. There is a competing effect going on at the same time, which causes the expected result, when there

is an increase in buffer concentration there is more shielding of the negative charges on the capillary wall and this would increase the migration times. At a higher pH, closer to a pH of 9, almost all the silanol groups are deprotonated so there would be no effect on the pKa equilibrium and the expected trend should be seen.

3. Varying the pH of the Borate Buffer

The pH of the borate buffers were increased to 8.64; table 5 shows the results. The expected trend of increasing migration time with increasing buffer concentration did occur. The reproducibility was excellent, with percent standard deviations all less than 1%, and there was baseline resolution with each buffer concentration. It can be seen a bit more clearly by looking at the electroosmotic mobilities at the two different pH's (8.27 and 8.64) in table 6. At a pH of 8.27 the electroosmotic mobility increases slightly from 25 to 50 mM borate buffer concentration and then decreases for 75 mM borate buffer concentrations. This also occurs with the electrophoretic mobility. However, at a pH of 8.64 the electroosmotic mobility decreases as the borate buffer concentrations decrease. The change in electroosmotic mobility between 25 and 50 mM is so small they can almost be looked at as the same; therefore a slight change could cause the solution of the 50 mM borate buffer to behave strangely and even cause faster migration times.

4. Peak Identification

A sample of l and d methorphan was run with twice as much l- as d- methorphan; the first peak was larger than the second in all cases which shows that the first peak that comes off the capillary is the l- methorphan.

C. Varying Phosphate Buffer Concentration

Since the resolution of the separation with the borate buffer was excellent, we decided to see if a different buffer would give similar results. Three different concentrations (25, 50 and 75 mM) of phosphate buffer were used with 40 mM TM-β-CD at a pH of 8.3. Table 7 shows it was possible to achieve baseline separation with the phosphate buffer at all concentrations. The resolution was excellent but the migration times were a bit longer than with the borate buffer, especially with the 75 mM phosphate buffer concentration. Figure 17 shows an electropherogram using a phosphate buffer.

D. Other CDs

The study done on propoxyphene showed that a variety of CDs could be used to separate the enantiomers [5]. We tried five more CDs to see if we could get similar results as reported for propoxyphene. α, γ, α-polymer, β-polymer and γ-polymer CDs were tested at 20, 30 and 40 mM at 2 pH values, 8.23 and 8.64, with 50 mM borate buffer. There was not separation with any of these CDs. It seems as though methorphan enantiomers will only bind differently with the TM-β-CD.

E. Optimization

Baseline separation for the methorphan enantiomers can be achieved using TM-β-CD at a range of concentrations from 20-40 mM, pH's in the range of 8.14 to 8.64, and either phosphate or borate buffers. This is a very robust method that can withstand minor variations in conditions.

F. Quantitative Results of l, d Methorphan with TM-β-CD and Borate Buffer

Figure 11 shows how well the quantitative results can found with this method. The R^2 values are 0.9945 for peak 1 and 0.9938 for peak 2, for the line to be perfectly

linear the R^2 value must be equal to 1 and these are very close. This shows that the CE can obtain a linear relation between peak area and concentration within a tenfold increase in concentration from 0.02 to 0.2 mg/mL.

The goals of separation and optimization were both met and this method was given to the New York State Forensic Center so that it may be employed in their separation and identification of methorphan enantiomers. This method will replace a crystallization method, which takes much longer time and is subjective.

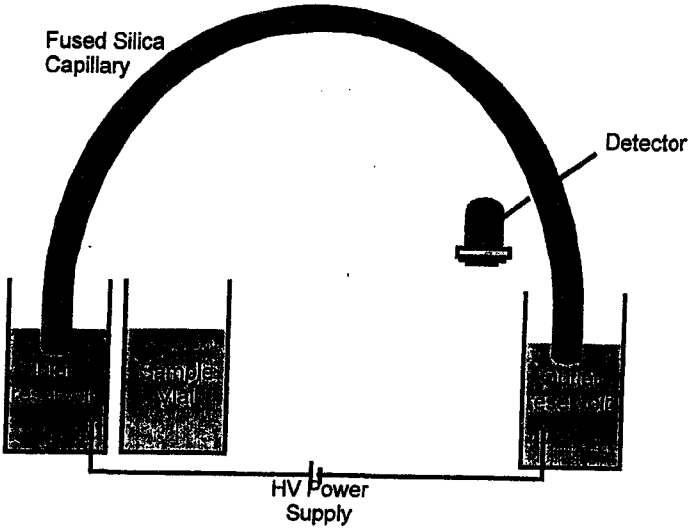


Figure 1

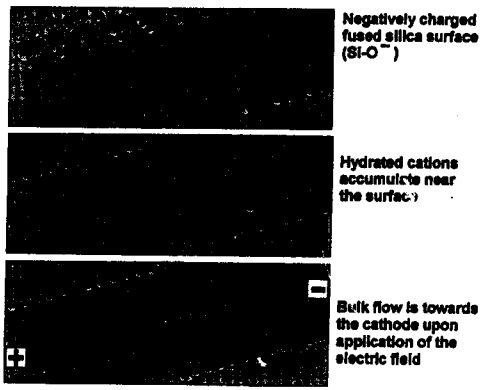


Figure 2

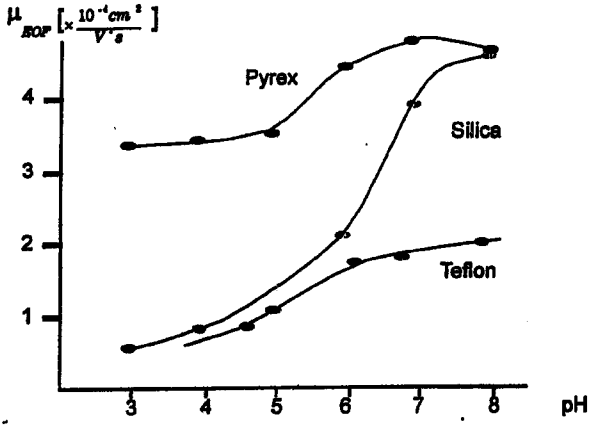


Figure 3

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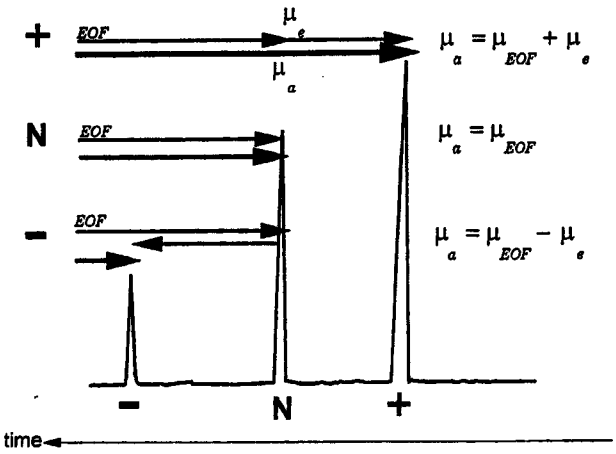


Agilent Technologies

Figure 5

Figure 6: Beta-Cylcodextrin

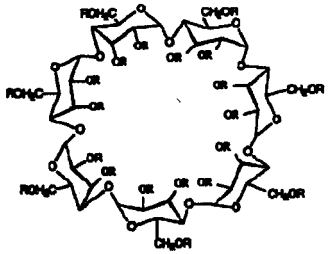


Figure 7: Cyclodextrin

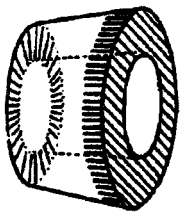


Figure 8: Levomethorphan

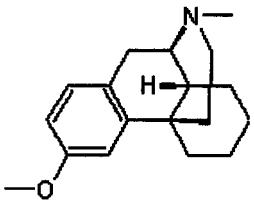
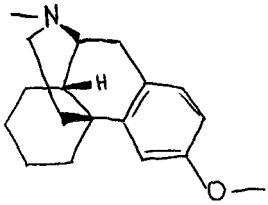


Figure 9: Dextromethorphan



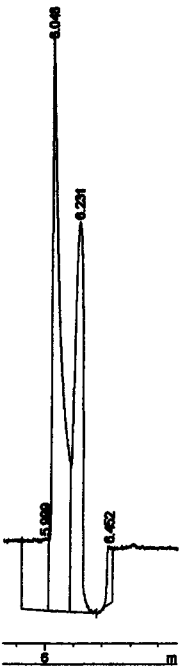


Figure 10

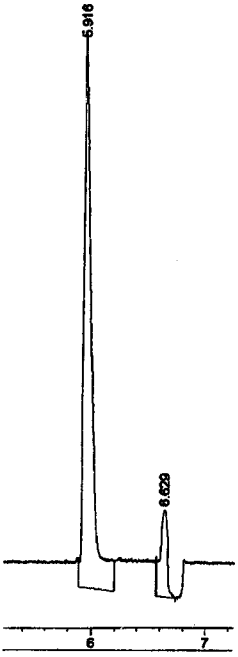


Figure 12

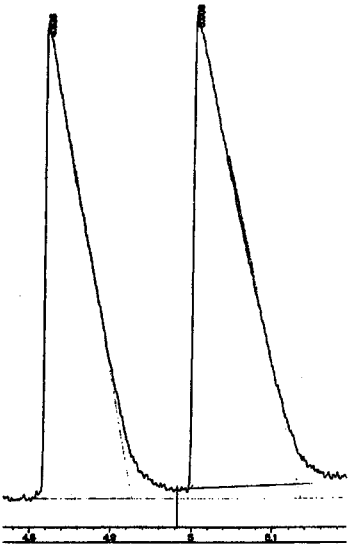


Figure 13

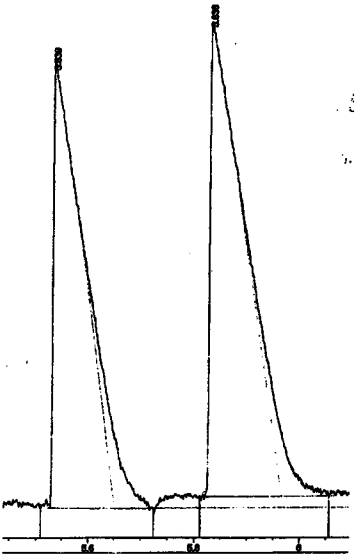


Figure 14

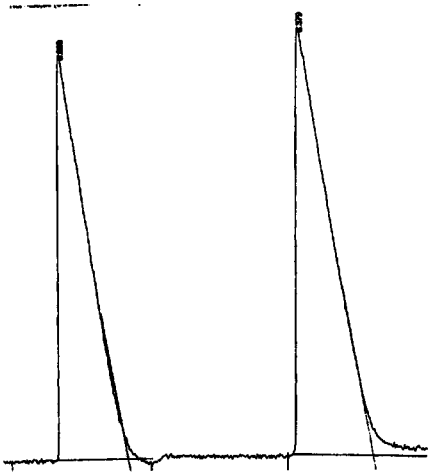


Figure 15

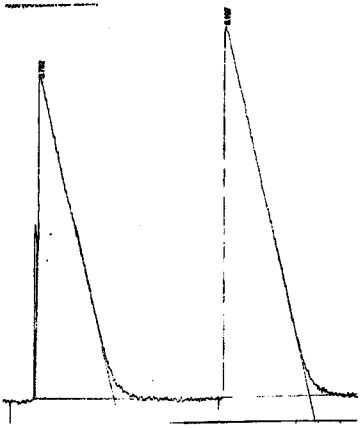


Figure 16

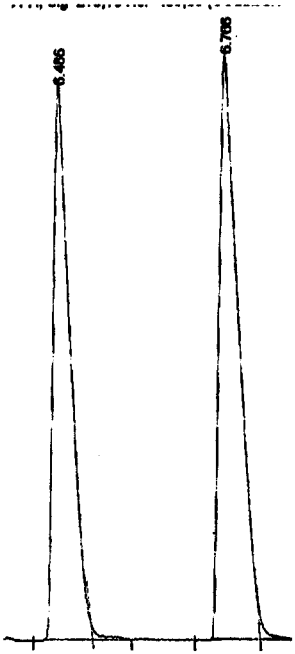


Figure 17

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