Chapter 6

Tuning of the Selectivity in Capillary Electrophoresis by Neutral Cyclodextrins Illustrated by the (Non-) Chiral Separation of some Structurally Related Phenothiazines

---

Abstract

Cyclodextrins were used for affecting the selectivity of the capillary electrophoresis system in the separation of ten widely used phenothiazines. It was shown that the addition of cyclodextrins substantially improved the selectivity. The effect of temperature and cyclodextrin concentration on the resolution between the screened phenothiazines was studied. The best results were obtained with 8 mM hydroxypropyl-β-cyclodextrin at 15.5 °C. Under these conditions a resolution of at least 1.5 between all phenothiazines could be obtained. In addition the chiral separation of the enantiomers of trimeprazine could be accomplished. Structure separatability relations between the phenothiazines showed that a change in the side chain at the R10 position had the largest effect on the migration.

6.1 Introduction

In capillary zone electrophoresis (CZE) the selectivity in non-chiral separations can be influenced by various factors, e.g. by changing the pH or by adding organic modifiers to the run-buffer [1-5]. When separating closely related substances, the effect of these parameters on the selectivity is restricted, because the pKa-values of these analytes will all be in the same range and therefore changing the pH may have a unilateral effect for all analytes involved. This is especially true when a pH is used that warrants full protonation. The addition of organic modifiers may also lead to a general increase in migration time because the separation window will only be enlarged: all analytes will have a similar extension of their residence-time. Micellar electrokinetic chromatography (MEKC) is another way to affect selectivity, being dependent on the interactions of the analytes with micelles (as the pseudo-stationary phase) and the mobile phase. Even slight differences in these interactions may result in successful separations. In these cases, selectivity is dependent on and restricted by the type and solubility of the micelles used [6-9]. In our studies on the separability of some closely related phenothiazines, we found that straight CZE was unable to separate the ten drugs under study. This Chapter describes the use of hydroxypropyl-
\(\beta\)-cyclodextrins (HP-\(\beta\)-CD) to provide the necessary selectivity to obtain baseline separation between the analytes.

Furthermore, we tried to investigate the possibility of a structure-relationship between the mentioned phenothiazines and their migration time, due to complexation with the HP-\(\beta\)-CD, under the prescribed conditions. A possible advantage of CDs compared to MEKC is that the CDs, instead of micelles, do not migrate towards the electrode under these conditions; thus a higher selectivity can be expected. Also, the nature of the selectivity is not based on chromatography but on the complexation of the specific analyte with the CDs.

The choice of CDs as a non-chiral selector in CE was inspired by recent observations on the enantiomeric separation of promethazine \[10,11\] and structure-related \(\beta\)-adrenergic sympathicomimetics (see Chapters 4 and 5). Similar applications have been described for anthracenes \[12\], monosialogangliosides \[13\], estrogens \[14\] and tetracyclines \[15\]. CDs are oligosaccharides consisting of 6, 7 or 8 D-\((+)-\)glucopyranose units and are designated as \(\alpha\), \(\beta\) and \(\gamma\)-CD. Derivatization of the plain cyclodextrins leads to a large variety of cyclodextrins, all with their own selectivity in their interaction with chiral or non-chiral compounds.

Derivatization of CD improves the solubility in water/methanol solutions \[16\].

Phenothiazines are mainly used in the therapy of schizophrenia, organic psychoses, the manic phase of manic-depressive illness and other acute idiopathic psychotic illnesses. They may also be used as antiemetics, antinausea and antihistaminics. The molecular structures of the phenothiazines under study, along with their pKa-values, are given in Table 6.1. The nature of the substituent at position 10 influences the pharmacological activity. According to their substituent, we can divide the phenothiazines into three groups, phenothiazines with an aliphatic side chain (A), phenothiazines with a piperidine moiety (B) and phenothiazines with a piperazine group (C). The latter is found to be the most potent group of phenothiazines. Substituents on position 2 with an electron-withdrawal group increase the efficacy of the phenothiazines (CF\(_3\) > Cl > H) \[17,18\].
Table 6.1. Molecular structure of the ten most frequently used phenothiazines and their pKa-values. Possible chiral centers are depicted with an "*".

<table>
<thead>
<tr>
<th>Group</th>
<th>Name</th>
<th>pKa</th>
<th>R2</th>
<th>R10</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Acepromazine</td>
<td>9.30</td>
<td>-C(O)CH₃</td>
<td>CH₂-C₆H₄-N(CH₃)₂</td>
</tr>
<tr>
<td>A</td>
<td>Chlorpromazine</td>
<td>9.30</td>
<td>-Cl</td>
<td>CH₂-C₆H₄-N(CH₃)₂</td>
</tr>
<tr>
<td>A</td>
<td>Promazine</td>
<td>9.40</td>
<td>-H</td>
<td>CH₂-C₆H₄-N(CH₃)₂</td>
</tr>
<tr>
<td>A</td>
<td>Promethazine</td>
<td>9.10</td>
<td>-H</td>
<td>CH₂-C₆H₄-N(CH₃)₂</td>
</tr>
<tr>
<td>A</td>
<td>Trifluopromazine</td>
<td>9.41</td>
<td>-CF₃</td>
<td>CH₂-C₆H₄-N(CH₃)₂</td>
</tr>
<tr>
<td>A</td>
<td>Trimeprazine</td>
<td>9.00</td>
<td>-H</td>
<td>CH₂-C₆H₄-N(CH₃)₂</td>
</tr>
<tr>
<td>B</td>
<td>Thioridazine</td>
<td>9.50</td>
<td>-SCH₃</td>
<td>CH₂-C₆H₄-N(CH₃)₂</td>
</tr>
<tr>
<td>C</td>
<td>Perphenazine</td>
<td>7.80</td>
<td>-Cl</td>
<td>CH₂-C₆H₄-N(C₂H₅)OH</td>
</tr>
<tr>
<td>C</td>
<td>Prochlorperazine</td>
<td>8.10</td>
<td>-Cl</td>
<td>CH₂-C₆H₄-N(C₂H₅)N-CH₃</td>
</tr>
<tr>
<td>C</td>
<td>Trifluoperazine</td>
<td>8.10</td>
<td>-CF₃</td>
<td>CH₂-C₆H₄-N(C₂H₅)N-CH₃</td>
</tr>
</tbody>
</table>
6.2 Experimental

6.2.1 Apparatus

The CE system was a Model PRINCE with a four-position sample tray and a programmable injector system from Lauerlabs (Emmen, The Netherlands). Detection at 210 nm was carried out with a LAMBDA 1000 UV/VIS VWL detector (Bischoff, Leonberg, Germany). The fused-silica capillary (50 µm i.d., 375 µm, o.d.) with an outer polyimide coating was from Polymicro Technologies (Phoenix, AZ, USA). Data acquisition of CE/UV was performed by the Maclab system (ADinstruments, Castle Hill, Australia) using the Chart program (version 3.3) for recording of the electropherograms. For interpretation of the electropherograms, the Peaks program (ADinstruments) was used. The vials used were 4 ml glass vials and were obtained from PhaseSep (Waddinxveen, The Netherlands).

6.2.2 Chemicals and solutions

Acepromazine maleate, chlorpromazine hydrochloride, perphenazine base, prochlorperazine base, promazine hydrochloride, promethazine hydrochloride, thioridazine base, trifluoperazine hydrochloride, trifluromazine hydrochloride and trimeprazine tartrate, were all of pharmacopoeia quality and were dissolved in a solution containing 1 part run-buffer and 9 parts of water to a final concentration of 20 µg/ml. Thioridazine, trimeprazine and promethazine were used as racemates. The CE run-buffer was prepared by dissolving sodium dihydrogen phosphate monohydrate, p.a., Merck, Darmstadt, Germany to a concentration of 100 mM and adjusting the pH with concentrated ortho-phosphoric acid (p.a. 85%, Merck) to a pH of 2.5, giving a conductivity of 7.0 mS/cm. Hydroxypropyl-β-cyclodextrin (HP-β-CD), analytical grade was obtained from Wacker-Chemie, Munich, Germany. Water was purified with a Milli-Q system (Millipore, Bedford, MA, USA). The conductivity of the purified water was always less than 2 µS/cm. All solutions were filtered through a membrane filter (0.45 µm) and degassed for five minutes in an ultra-sonic
bath (50 kHz, Branson Europa, Soest, The Netherlands), immediately prior to use.

6.2.3 CE conditions

A capillary with a total length of 70 cm and an effective length of 55 cm was used. An optical viewing window with a length of 0.5 cm, obtained by burning off the polyimide coating, was aligned with the UV detection cell. The coating of the first 2 mm of the capillary was also stripped. New capillaries were rinsed with 1 M sodium hydroxide for 10 minutes at 1000 mBar, with water for 10 minutes at 1000 mBar and with the run buffer for 10 minutes at 1000 mBar, respectively. Cyclodextrins (8-22 mM) were dissolved in the run buffer and hydrodynamically injected until the capillary was fully filled. The latter was monitored by UV-detection. The injection (10 kV, 6s) and separation voltage (30 kV) were ramped at 6 kV/s and took place at a constant temperature of 15.5, 18 or 20.5 °C, respectively. After each run the capillary was refilled with fresh run medium. Because we inject the removable run medium directly into the capillary instead of adding it to the ground-electrolyte, the electro-osmotic flow (EOF) should be suppressed to avoid the former solution to be forced out of the capillary. The latter is accomplished by keeping the working pH of the ground-electrolyte below pH=3. This approach implies that the analyte(s) can only be introduced into the capillary by electrokinetic injection. Despite the fact that electrokinetic injection increases the sensitivity due to stacking, some precautions with respect to the amount of injected analyte(s) should be taken into account [19]. Especially possible vibrations that may occur during the injection process should be excluded and a constant volume in the sample vials should be warranted (see appendix).

After the injection, the electrode and the capillary-end were dipped in a vial containing water. The separation was started when the ground electrode and the capillary-end were placed into the vial containing the run buffer.
6.2.4 Statistical evaluations

One-way analysis of variance (ANOVA) and the paired two-sample t-test were performed with an Origin 2.9 (MicroCal Software, Northampton, MA, USA) program.

6.3 Results and discussion

In Figure 6.1 the resolutions of the ten phenothiazines are shown at 15.5 °C, 18.0 °C and 20.5 °C using a 8, 15 or 22 mM HP-β-CD solution as a (non-) chiral selector. These conditions were chosen based on recent experiences described in Chapters 4 and 5. Baseline separation is accomplished when the resolution is at least 1.5. The resolution is defined as the extent of separation between two compounds and is calculated by $R = 2 (t_2 - t_1) / (w_1 + w_2)$, where $t_1$ and $t_2$ are the retention times and $w_1$ and $w_2$ the peakwidths at the baseline of the more mobile (1) and the less mobile (2) analyte. As can be seen in Figure 6.1, only at 15.5 °C using an 8 or a 15 mM HP-β-CD solution all resolutions calculated between the measured phenothiazines including the enantiomers of trimeprazine, exceeded the 1.5 criterion for the resolution, resulting in 11 peaks in the electropherogram. Lower temperatures could technically not be achieved. The enantiomers of promethazine and thioridazine could not be separated under these conditions. ANOVA showed that there was not a significant difference between the resolutions of the phenothiazines at 15.5 °C, using a 8, 15 or 22 mM HP-β-CD solution at a 95% reliability level ($\alpha = 0.05$; $P = 0.961$), where $\alpha$ is the error in accepting the null-hypothesis and $P$ is the probability factor that indicates the chance that the given test-statistic is not correct. The latter was also found for the separation of the phenothiazines at 18 and 20.5 °C ($\alpha = 0.05$; $P = 0.965$ and $P = 0.973$, respectively). The effect of the temperature can be calculated by comparison of the resolutions obtained with the three molarities of the HP-β-CD solution at 15.5, 18 and 20.5 °C, respectively. ANOVA showed that there is no significant difference between the resolutions obtained with different temperatures ($\alpha = 0.05$; $P = 0.790$ [8 mM], $P = 0.961$ [15 mM] and $P = 0.901$ [22 mM], respectively).
Fig. 6.1 Resolutions between the phenothiazines in various run media and at A) 15.5°C; B) 18.0°C; C) 20.5°C. Trifluoperazine; 1) prochlorperazine; 2) perphenazine; 3) promethazine; 4) promazine; 5) trifluopromazine; 6) acepromazine; 7&8) trimeprazine enantiomer; 9) chlorpromazine; and 10) thioridazine.
Paired two-sample t-tests showed that there was only a significant difference at a 95% reliability level between the resolutions when comparing the resolutions obtained with an 8 mM HP-β-CD solution at 15.5°C, giving the best results, with the resolutions obtained with an 8 mM HP-β-CD solution at 20.5°C (P=0.0442). All the other possible combinations did not indicate a significant difference between the resolutions at a 95% reliability level (0.0819 ≤ P ≤ 0.945). Use of concentrations lower than 8 mM HP-β-CD may result in significant differences between the resolutions at the temperatures used but according to earlier research described in Chapter 4 and the research mentioned in this Chapter, concentrations lower than 8 mM will probably not lead to an increase in resolution. This is also based on the fact that when there are no CDs added, the phenothiazines cannot be fully separated. In Figure 6.2 the electropherograms of the phenothiazines are shown using only run-buffer for the separation, i.e. straight CZE (A) and using an 8 mM HP-β-CD solution for the separation (B) at 15.5°C. As can be seen in Figure 6.2(a) the ten phenothiazines could not be sufficiently separated and the peaks corresponding to perphenazine(3) and promethazine(4) could not be separated. Plate numbers calculated for the peaks in Figure 6.2(b) range from 97000 to 189000 (Rt < 22 min).

The separation between two analytes in the same run can be expressed by a selectivity factor (s). This selectivity factor is calculated by $s = (t_{ri} - t_{ri(0)}) / t_{ri(0)}$, where $t_{ri}$ is the migration time of analyte $i$ in the presence of CDs and $t_{ri(0)}$ is the migration time of the same analyte in a straight CZE system under the same conditions. The $s$-values, calculated from the data shown in Figure 6.2, are presented in Table 6.2. Although there are no changes in elution order, the variation in the calculated $s$-values implies that CDs have the ability to change the selectivity in a capillary electrophoresis system not just by an extension of the separation window but by an increase in selectivity that is dependent on the structure of the phenothiazine, i.e. its variation in complexation with the CDs.

Structure separability relations can be derived from the results shown in Figure 6.2(b) and the molecular structures shown in Table 6.1. The phenothiazines with a piperazine group (C) on the R10 position migrate quickly and the phenothiazine with a piperidine moiety (B) migrates slowly.
When we compare trifluoperazine with prochlorperazine (both belonging to group C) we can see that the substituent on the R2 position also has some influence on the migration. Apparently, the electron withdrawal group and/or its size has a negative effect on the complexation of the analyte with the CDs. The same can be seen when we compare promazine, trifluopromazine, acepromazine and chlorpromazine (group A). The order of

Figure 6.2. Electropherograms of the separation of the phenothiazines at 15.5°C without (A) and with (B) the addition of cyclodextrins to the run buffer. 1) Trifluoperazine; 2) prochlorperazine; 3) perphenazine; 4) promethazine; 5) trifluopromazine; 6) acepromazine; 8&9) trimeprazine enantiomer; 10) chlorpromazine; and 11) thioridazine.
migration \((R2 = \text{H} > \text{CF}_3 > \text{Acetyl} > \text{Cl})\) can be due to a change in, for instance lipophilicity, dipole-dipole interactions, electronegativity, hydrogen-bonding or steric hindrance, and cannot be adequately explained.

**Table 6.2.** Calculated selectivity factors \((s_f)\) of the phenothiazines along with their migration times measured with \((t_{ri})\) and without \((t_{ri(0)})\) the addition of cyclodextrins at 15.5\(^\circ\)C using 8 mM HP-\(\beta\)-CD.

<table>
<thead>
<tr>
<th>Phenothiazine group</th>
<th>Phenothiazine</th>
<th>group</th>
<th>(t_{ri})</th>
<th>(t_{ri(0)})</th>
<th>(s_f)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Trifluoperazine</td>
<td>C</td>
<td>11.07</td>
<td>9.19</td>
<td>0.204</td>
</tr>
<tr>
<td>2</td>
<td>Prochlorperazine</td>
<td>C</td>
<td>11.64</td>
<td>9.46</td>
<td>0.231</td>
</tr>
<tr>
<td>3</td>
<td>Perphenazine</td>
<td>C</td>
<td>11.98</td>
<td>9.54</td>
<td>0.256</td>
</tr>
<tr>
<td>4</td>
<td>Promethazine</td>
<td>A</td>
<td>12.42</td>
<td>9.54</td>
<td>0.303</td>
</tr>
<tr>
<td>5</td>
<td>Promazine</td>
<td>A</td>
<td>13.84</td>
<td>10.83</td>
<td>0.278</td>
</tr>
<tr>
<td>6</td>
<td>Trifluopromazine</td>
<td>A</td>
<td>14.34</td>
<td>11.15</td>
<td>0.286</td>
</tr>
<tr>
<td>7</td>
<td>Acetopromazine</td>
<td>A</td>
<td>14.61</td>
<td>11.38</td>
<td>0.284</td>
</tr>
<tr>
<td>8</td>
<td>Trimeprazine ((-/+))</td>
<td>A</td>
<td>15.04</td>
<td>11.70</td>
<td>0.285</td>
</tr>
<tr>
<td>9</td>
<td>Trimeprazine (+/-)</td>
<td>A</td>
<td>15.33</td>
<td>11.70</td>
<td>0.310</td>
</tr>
<tr>
<td>10</td>
<td>Chloorpromazine</td>
<td>A</td>
<td>16.22</td>
<td>11.70</td>
<td>0.386</td>
</tr>
<tr>
<td>11</td>
<td>Thioridazine</td>
<td>B</td>
<td>20.81</td>
<td>12.82</td>
<td>0.623</td>
</tr>
</tbody>
</table>

Extension of the side chain (promethazine and trimeprazine, group A) shows an increase in the migration time, probably due to the increase in lipophilicity or to one of the other factors mentioned above.

The effect of the side chain \((R10)\) and the substituent at the \(R2\) position on the migration time can be calculated with a simple factorial design [20]. Four phenothiazines are chosen to visualize this effect; two different side chains at the \(R10\) position (a piperazine (A) and a propyl-[dimethyl]amine (B) side chain) and two different substituents at the \(R2\) position (a trifluoromethyl group (C) and a chloride atom (D)). The four phenothiazines are trifluoperazine \((A+C)\), prochlorperazine \((A+D)\), trifluopromazine \((B+C)\) and chloorpromazine \((B+D)\). The effect on the migration time is calculated by taking the average migration time of the
phenothiazines with the piperazine chain minus the average migration time of the phenothiazines with the propyl(dimethyl)amine chain, giving $\text{Eff} = 3.93$. In the same way, the effect of the substituent at the R2 position on the migration time can be calculated. For this, the average migration time of the phenothiazines with de -CF$_3$ group is deducted with the average migration time of the phenothiazines with de -Cl atom on the R2 position, resulting in $\text{Eff} = 1.22$.

It appears that a change in the side chain at the R10 position has the greatest effect on the migration time of the phenothiazines. The latter is visualized in Figure 6.3.

![Figure 6.3. Effects of the chain at the R$_{10}$ position and the substituent at the R$_2$ position of the phenothiazines on the migration time. 1) Average migration time with piperazine chain; 2) average migration time with propyl(dimethyl)amine chain; 3) difference in migration time; 4) average migration time with CF$_3$ group; 5) average migration time with Cl atom and; 6) difference in migration time.](image)

### 6.4 Conclusion

The use of cyclodextrins as a non-chiral selector dissolved in the run-buffer appears to be an adequate method to change the selectivity in the separation of phenothiazines by capillary electrophoresis. Although there are no changes in elution order, the calculated $\xi$-values imply a small non-unilateral change in selectivity that can be significant for full baseline
separation. A concentration of 8 mM HP-β-CD led to a minimum resolution of 1.5 between the examined phenothiazines, where one-way analysis of variance does not confirm an increase in resolution when higher concentrations are used.

A decrease in temperature led to an increase of the analysis time but also improved the resolution. Of the three chiral phenothiazines only the enantiomers of trimeprazine could be successfully separated. These data confirm our hypothesis that the addition of complexation agents is a more straightforward way to obtain the required selectivity in capillary electrophoresis than by pH-adjustment and/or addition of organic modifiers.

6.5 References


14 S.K. Poole and C.F. Poole. (1996) Separation of pharmaceutically important estrogens by micellar electrokinetic chromatography, J.Chromatogr.A. 749, 247-255


16 R. Kuhn, F. Stoecklin and F. Emi. (1992) Chiral separations by host-guest complexation with cyclodextrin and crown ether in capillary zone electrophoresis, Chromatographia. 33, 32-36


