A REVIEW ON CAPILLARY ELECTROPHORESIS AND ITS APPLICATION FOR THE SEPARATION OF CHIRAL COMPOUNDS

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Abstract: Capillary electrophoresis (CE) is a highly efficient analytical separation technique. Separation is based upon differences in mobility, which mainly depends on the charge to mass ratio of the molecule. The addition of an enantioselective complexing agent, or chiral selector, can change the charge to mass ratio of the enantiomers in a selective way resulting in mobility difference between the optical isomers which is a prerequisite for (chiral) separation. Optical isomers can be successfully separated if the mobility of the complex differs from the mobility of the free enantiomers and if the complex stability of the diastereomeric complexes is not identical. The stability of complexes between enantiomers and chiral selectors is expressed by their formation constants. The separation of the enantiomers of many compounds of pharmaceutical interest was investigated by CE, employing a soluble neutral β-cyclodextrin polymer as the chiral selector. Both selectivity and resolution were influenced by the concentration of the β-cyclodextrin polymer. CE is applied to determine the optical purity of a pharmaceutical formulation and also for the determination of drug enantiomers in human plasma.

Key words: Capillary electrophoresis, β-cyclodextrin, chiral separation.

1. INTRODUCTION:
A molecule is said to be chiral if it has an identical mirror image and that the two forms are non-superimposable. They are known as enantiomers or optical isomers. Chirality is obtained when a carbon atom possesses four different substituents, like mandelic acid. It can be indicated as levorotatory (Latin: laevus), “L” or “(-)”. The absolute configuration around a chiral center can be noted as D or L.[1] The R/S notation (from Latin; rectus (right) and sinister (left)) has largely replaced the D/L notation.[2] It is well known that the enantiomers of chiral pharmaceuticals can behave very different in the human body. The (-)-enantiomer of the β-blocker propranolol is about 100 times more active than the (+)-form. (R)-(+) -enantiomer of thalidomide possessed the sleep inducing action. The S-(-)-enantiomer possessed teratogenic action, responsible for serious malformation in newborn babies of women who took the drug during pregnancy. These examples underline the need for chiral separation methods.

Fig. 1: Mirror images of mandelic acid.

This carbon is called a stereocenter or chiral center. A 1:1 mixture of the two enantiomers is called a racemic mixture. Enantiomers have identical physical and chemical properties, in an isotropic environment. Its chirality is only observed when the molecule is subjected to a chiral influence. Polarized light is rotated when passing through solutions containing chiral molecules (but not when passing through racemic mixtures). Optical isomers rotate the light in an equal degree but in opposite direction. If the enantiomer rotates the light to the right, it will be indicated as dextrorotatory (Latin: dexter), “d” or “(+)” and to the left, it will be indicated as levorotatory (Latin: laevus), “L” or “(-)”.

2. PRINCIPLES OF CAPILLARY ELECTROPHORESIS:
Electrophoresis is the separation principle in which charged particles or molecules are separated under the influence of an external electric field. Capillary electrophoresis (CE) has proven to be a highly efficient, analytical separation tool, not only for the separation of
The principle mainly involves:

i. Electrophoretic mobility and Electroosmosis

ii. Factors influencing resolution

2.1 Electrophoretic mobility and Electroosmosis:
The velocity of solute molecules (v) will be proportional to the applied electric field (E) and the electrophoretic mobility (μ):

\[ \nu = \mu E \]

The electrophoretic mobility is dependent on charge (q) and the radius (r) of the particle and also temperature. Electrophoretic mobilities increase at a rate of approximately 2%. Electroosmosis is known as the flow of an electrolyte solution caused by an electric field across a capillary. If a fused silica capillary wall is in contact with a solution, the wall will be negatively charged, due to dissociation of the silanol groups. Consequently, the wall will attract cations from the solution, resulting in the formation of an electric double layer adjacent to the capillary surface. If an electric field is applied across the capillary length, the mobile part of this double layer will start migrating towards the cathode. This results in a flow of the electrolyte solution, called the electroosmotic flow or EOF [5].

2.2 Factors influencing resolution:
The quality of a separation of two components is described by its resolution (Rs), defined as:

\[ R_s = \frac{t_{m2} - t_{m1}}{2(\sigma_1 + \sigma_2)} \]

In which, \( t_m \) is the migration time and σ is the standard deviation of the peak. The value of the numerator can be increased by increasing the effective mobility difference, which is a measure for the selectivity. Separation of (optical) isomers can be optimized by the addition of the optimum amount of a suitable complexing agent.

3. INSTRUMENTATION:
Basic equipment consists of a high voltage power supply, a separation tube, an injection module, a detector and a data collection system. Generally, for the electrophoretic experiments, a P/ACE 2200 (Beckman, Palo Alto, CA) was used. The Beckman instrument is fully automated, and consists of an auto sampler and a liquid cooled capillary cartridge. The latter allows temperature control between 20°C and 50°C. Fused silica capillaries, with an inner diameter of 50 or 75μm were used as separation tubes. The required minimum length was about 27 cm. Prior to use, new capillaries were rinsed for approximately 30 minutes with a 1 M KOH solution. For some applications, the capillary inner wall was coated with linear polyacrylamide [6]. The coating procedure could be fully automated, using the P/ACE 2200.

The high voltage power supply is capable of delivering voltages ranging from 1kV up to 30kV, and currents up to 250A. In most experiments, electric field strengths were about 500 V/m, resulting in currents of 5-50A. The P/ACE control software allows separations to be

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interaction depends on the stability of the complex formed. Chiral selectors can also be incorporated or bound to a gel matrix \cite{14,15} or capillary wall\cite{16} where the net velocity of the complex will be zero. Complex formation will result in an average velocity of the analyte, which is different from the velocity of the free analyte. As a consequence, a difference in complex stability between two optical antipodes will result in a difference in the average velocity of these compounds. In order to maximize enantioselectivity, one should maximize this difference in average velocity between the two optical antipodes.

5. CHIRAL SELECTORS IN CE:

5.1 Rules for chiral recognition

In order to separate optical isomers it is necessary to introduce a chiral element into the separation process. For CE, this chiral element or chiral selector will be added to the BGE. The addition of a chiral selector to an electrophoretic system does not guarantee the successful separation of all optical isomers. The most important rule for chiral recognition is that the chiral selector must be compatible in size and structure to the racemate; a minimum of three molecular interactions has to occur. These interactions can be either attractive or repulsive. Possible modes of interaction include:

- Ion-ion bonds
- Dipole-dipole bonds like hydrogen bonds
- Van der Waals forces
- Ion-dipole bonds

5.2 Cyclodextrins

Cyclodextrins (CD’s) are by far the most popular chiral selectors used in CE. They are torus-shaped cyclic D-glucosaccharides produced from starch by enzymatic degradation. CD’s containing 6 (α-CD), 7 (β-CD) or 8 (γ-CD) residues are currently used. The interior of the CD cavity is relatively hydrophobic, while the outside rim is more hydrophilic. The rim on the wider side of the CD cavity contains the chiral secondary hydroxyl groups, while the opposite smaller opening is occupied by achiral primary hydroxyl groups. The size of the hydrophobic cavity is such that it can accommodate a single phenyl ring. Interaction between substituents on the asymmetric center of the analyte and the hydroxyl groups on the CD-rim are responsible for chiral recognition.

Fig. 2: Schematic representation of capillary electrophoretic separation system

Fig. 3: Structure of cyclodextrin

Fig. 4: Cyclodextrin
Inclusion complex formation and the size of the analyte’s binding constant to the cyclodextrin are determined by several different factors. The most important factors are the ‘hydrophobic effect’, which induces the apolar portion of the molecule to preferentially reside in the cyclodextrin cavity, and hydrogen bonding between appropriate polar segments of the guest molecule and the secondary hydroxyl groups at the mouth of the cyclodextrin cavity. Other factors which can influence complex formation are Van der Waals interactions, release of high energy water from the CD cavity and a change in ring strain upon complexation.

6. DETERMINATION OF EQUILIBRIUM CONSTANTS OF COMPLEX FORMATION:

The concentration of chiral selector influences the mobility of the optical antipodes \(^{[11,20,21]}\) and also the optical resolution. It is assumed that the concentration of the buffer is much higher than the concentration of the CD, and that the concentration of the CD is much higher than that of the analyte. The consequence of this assumption is that practically all the CD will exist as CD-buffer complex, or in other words, the analytical concentration of CD is practically the same as the concentration of the CD-buffer complex and will improve the mobility difference between the two optical antipodes.

### Table 1: Physicochemical properties of cyclodextrins

<table>
<thead>
<tr>
<th>Dimensions (\text{Å} )</th>
<th>Cavity volume (\text{Å}^3 )</th>
<th>Molecular mass (g/mol)</th>
<th>Specific optical Rotation ([\alpha] )</th>
<th>Solubility In water at 25°C (g /100 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>b</td>
<td>c</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α</td>
<td>5.7</td>
<td>13.7</td>
<td>7.8</td>
<td>174</td>
</tr>
<tr>
<td>β</td>
<td>7.8</td>
<td>15.3</td>
<td>7.8</td>
<td>262</td>
</tr>
<tr>
<td>γ</td>
<td>9.5</td>
<td>16.9</td>
<td>7.8</td>
<td>427</td>
</tr>
</tbody>
</table>

**Fig. 5:** Schematic representation of the mechanism of inclusion complexation with neutral cyclodextrins in CE

Besides the native cyclodextrin, a wide range of modified cyclodextrins \(^{[17]}\) are commercially available, such as dimethylated (DIME-β) or trimethylated (TRIME-β)-cyclodextrin. For DIME-β and TRIME-β, the hydroxyl groups on the CD-rim are replaced by methoxy groups. Modified cyclodextrins show improved solubility.

Hydroxyl groups on the cyclodextrin rim can also be substituted by charged or chargeable groups \(^{[18,19]}\). The introduction of chargeable groups will result in an increased solubility and also allows the separation of uncharged species. Furthermore, the separation mechanism is altered by the introduction of electrostatic interactions. Finally, the use of chiral selectors carrying a charge opposite to that of the analytes can greatly improve the mobility difference between the two optical antipodes.
remain more or less constant, whether the analyte is present or not. Three different types of chiral interaction can be discriminated:

**Type 0 or non-selective interactions;** neither the charged nor the non-charged enantiomers interact selectively with the chiral selector;

**Type I or desionoselective interactions;** only the non-dissociated optical antipodes interact selectively with the chiral selector;

**Type II or ionoselective interaction;** only the charged forms (the dissociated solutes) interact selectively with the chiral selector;

**Type III or douselective;** both the charged and the non-charged species interact selectively with the chiral selector.

### 7. ENANTIOMERIC SEPARATION OF DRUGS BY CE USING A SOLUBLE NEUTRAL β-CYCLODEXTRIN POLYMER:

Cyclodextrins have been widely applied as chiral selectors in CE for many applications [22-25]. When cyclodextrins (CD’s) or their derivatives are used for the separation of optical isomers, the chiral resolution is based on selective inclusion complexation with analytes. Hydrophobic interactions between analytes and the CD cavity and hydrogen bonding between analytes and the hydroxy (or modified) groups on the CD rim can lead to the formation of labile diastereomeric complexes with different stability constants. The optical isomer that forms the most stable complex with the neutral CD will migrate with the lowest effective mobility.

Enantiomeric resolution of trimetoquinol and related substances was studied using uncharged β-cyclodextrin polymer (EP-β-CD). This polymer consists of β-cyclodextrin cross-linked with epichlorohydrin. In this study, the use of chiral β-CD polymer for the separation of enantiomers of several basic compounds of pharmaceutical interest was investigated by CE.

#### 7.1 Experimental:

**7.1.1 Chemicals:**

Soluble β-cyclodextrin polymer (EP-β-CD) was used. The characteristics of the polymer were: molecular weight $m = 3000-5000$; cyclodextrin content: 58.2 %; solubility in water: 40-50%; cross linking agent: epichlorohydrin. All standards were of analytical grade.

**7.1.2 Apparatus:**

A P/ACE 2200 capillary electrophoresis system (Beckman, Fullerton, CA) was used for all the experiments in the temperature range from 20°C up to 50°C. The Beckman instrument used an untreated fused-silica capillary, 370 mm x 50 micrometre I.D., with an effective length of 300 mm. The UV-detector was operated at 214 nm.

#### 7.1.3 Methods:

All solutions were prepared in demineralized water. A 50 mM phosphate buffer, pH 2.5, was used. The background electrolyte (BGE) containing EP-β-CD was filtered before use with a 0.45m pore size filter. The concentration of the analyzed standards was 5.10^-3 M. The applied voltage was 15 kV. Before every electrophoretic run, the capillary was rinsed with 10mM KOH for two minutes and with phosphate buffer (without EP-β-CD) for two minutes. Before applying the sample, the capillary was rinsed with BGE containing a specific EP-β-CD concentration for 20 seconds. No polymer was present in the electrode vials during separation. In this setup, only a few microlitres of BGE containing the chiral polymer were needed per analysis. The absolute consumption of EP-β-CD per analysis was less than 1 mg. An increase in the concentration of EP-β-CD leads to a general decrease of the mobility of the analytes. All experiments were performed at 20°C.

#### 7.1.4 Results and discussion:

Different basic compounds of pharmaceutical interest, namely α-adrenergic agonists (ephedrine, epinephrine and norepinephrine), β-adrenergic agonists (isoproterenol, terbutaline and clenbuterol), β-adrenergic blockers (atenolol, metoprolol, oxprenolol and propranolol), anaesthetics (ketamine and bupivacaine), anorexic (norphedrine and methamphetamine) and tryptophan methyl, ethyl and butyl esters were selected for the electrophoretic experiments.

### 8. APPLICATIONS OF CAPILLARY ELECTROPHORESIS IN CHIRAL SEPARATIONS:

Capillary zone electrophoresis (CZE) is generally used for chiral analysis [26-28]. The ease of method development might be considered as one of the main advantages of CE over HPLC. It is shown that CE can be applied for the determination of the optical impurity of drugs. Furthermore, it is shown that CE can be applied to determine the metabolism of thiopental and pentobarbital enantiomers, by analyzing plasma samples.

**8.1 Quality control of fenfluramine enantiomers:**

Meta-fenfluramine is a basic compound which has been applied extensively as an anti-obesity drug. First, the pKa and mobility of $m$-fenfluramine were determined at 25°C applying a buffer with an ionic strength of 10 mM. Mobilities were determined by injecting mesityloxide as neutral marker [30]. Subsequently, using an acetate buffer of pH 5 with an ionic strength of 10mM, the effective
mobilities of the optical isomers were determined at different concentration levels of TRIME-β in the range 0-80mM. Under these conditions, m-fenfluramine was completely ionized. Finally, using 10mM sodium/borate pH 9.46 as BGE, effective mobilities were measured at different TRIME-β concentrations. Under these conditions, both the ionic and the non-ionic form of m-fenfluramine were present.

8.2 Determination of thiopental enantiomers in plasma:
Thiopental is commonly employed as an intravenous anesthetic agent, administered by injection for induction of anesthesia. Thiopental is marketed as a racemate although the S(-)-isomer is more potent. The use of CE for the separation of optical isomers in plasma samples has been demonstrated by Prunonosa et al [31]. A polyacrylamide coated capillary [29] of 57cm length was used. Pentobarbital and thiopental were dissolved in deionized water at a concentration of 1mg free acid/ml. Reference plasma was prepared by the addition of thiopental and pentobarbital solutions (5, 10, or 15microlitres). The samples were eluted with approximately 10 ml of diethyl ether. The ether fraction was evaporated to dryness, under a gentle stream of nitrogen. The residuals were dissolved in 100microlitres of deionized water. Thiopental and pentobarbital were well separable at pH 9.0. The capillary electrophoretic chiral separation of thiopental and pentobarbital was done by applying a BGE supported with 30mM β-CD, 50mM sodiumdodecylsulphate (SDS), 60mM l-methoxyacetic acid and 40mM d-camphor-10-sulphonate (d-cam) (final pH 9).

9. CONCLUSION:
The results from this review show that CE can be a suitable technique for the separation of optical isomers in pharmaceutical preparations and in serum. This method can be useful for the analysis and determination of the enantiopurity of real production samples. It can be concluded that CE can be applied for the separation of enantiomers, even in difficult sample matrices. The limited sensitivity is the main drawback of the technique. In this respect, CE is especially suitable for the analysis of production samples and pharmaceutical preparations.

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