Separation of Pharmaceuticals by Capillary Electrophoresis using Partial Filling and Multiple-injections

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Abstract

Different multiple-injection methodologies and the partial filling technique (PFT) have been utilized for separation of pharmaceuticals by capillary elec-trophoresis.

In multiple-injection capillary zone electrophoresis (MICZE), the samples and all standards, used for construction of the calibration curve, are analyzed within a single run. Four different modes of MICZE have been described by means of equations, which were experimentally verified. The developed equations facilitate the transfer from conventional single-injection CZE to one or more of these MICZE-modes, depending on the selectivity between the analyte and the injection marker. The applicability of two of these modes was then demonstrated by quantification of buserelin and salbutamol, re-spectively in commercially available pharmaceutical products. The content of buserelin in an injection solution was determined to 0.94 mg/ml, which only deviated slightly from the declared concentration (1 mg/ml). An alter-native mode of MICZE, offering a higher number of sequential sample injec-tions, was then utilized for single-run determination of salbutamol in 15 tab-lets, with a labelled content of 8 mg. The average content of the tablets was determined to 7.8 mg, with an intra-tablet variation of 3 % or less.

Moreover, UV- and mass-spectrometric detection of enantiomeric amines, resolved by non-aqueous capillary electrophoresis (NACE), was demon-strated. Separation of enantiomeric amines was achieved using the chiral selector (-)-2,3;4,6-di-O-isopropylidene-2-keto-L-gulonic acid, (-)-DKGA. Introduction of the non-volatile (-)-DKGA into the mass-spectrometer was avoided by using the PFT, where the capillary is only partially filled with electrolyte containing the chiral selector.

Keywords: Capillary Coating, Chiral Separation, Enantiomers, Mass-spectrometric Detection, Method Validation, Multiple-injection Capillary Zone Electrophoresis, Non-aqueous Capillary Electrophoresis, Organic Solvents, Pharmaceutical Analysis, Quantitative Analysis

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List of Publications and Manuscripts

This thesis is based on the following papers, to which referrals in the text are made by their roman numerals:

I  **Principles for Different Modes of Multiple-injection Capillary Zone Electrophoresis.** Ahmad Amini, Henrik Lodén, Curt Pettersson and Torbjörn Arvidsson. *In manuscript*


III  **Determination of Salbutamol in Tablets by Multiple-injection Capillary Zone Electrophoresis.** Henrik Lodén, Curt Pettersson, Torbjörn Arvidsson and Ahmad Amini. *In manuscript*


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<tr>
<td>BGE</td>
<td>Background electrolyte</td>
</tr>
<tr>
<td>(-)-DIKGA</td>
<td>(-)-2,3:4,6-di-O-isopropylidene-2-keto-L-gulonic acid</td>
</tr>
<tr>
<td>CE</td>
<td>Capillary electrophoresis</td>
</tr>
<tr>
<td>CEC</td>
<td>Capillary electrochromatography</td>
</tr>
<tr>
<td>CIEF</td>
<td>Capillary isoelectric focusing</td>
</tr>
<tr>
<td>$\Delta \mu$</td>
<td>Electrophoretic mobility difference</td>
</tr>
<tr>
<td>$\Delta t_{mig}$</td>
<td>Migration time difference</td>
</tr>
<tr>
<td>$\epsilon$</td>
<td>Dielectric constant</td>
</tr>
<tr>
<td>EOF</td>
<td>Electroosmotic flow</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray ionization</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>ID</td>
<td>Inner diameter</td>
</tr>
<tr>
<td>IM</td>
<td>Injection marker</td>
</tr>
<tr>
<td>IS</td>
<td>Internal standard</td>
</tr>
<tr>
<td>MICZE</td>
<td>Multiple-injection capillary zone electrophoresis</td>
</tr>
<tr>
<td>MS</td>
<td>Mass-spectrometry</td>
</tr>
<tr>
<td>$\mu_{app}$</td>
<td>Apparent mobility</td>
</tr>
<tr>
<td>$\mu_{eo}$</td>
<td>Electroosmotic mobility</td>
</tr>
<tr>
<td>$\mu_{ep}$</td>
<td>Electrophoretic (effective) mobility</td>
</tr>
<tr>
<td>NACE</td>
<td>Non-aqueous capillary electrophoresis</td>
</tr>
<tr>
<td>$\eta$</td>
<td>Viscosity</td>
</tr>
<tr>
<td>N</td>
<td>Peak efficiency</td>
</tr>
<tr>
<td>$n_{inj}$</td>
<td>Total number of injections</td>
</tr>
<tr>
<td>$n_s$</td>
<td>Sample capacity</td>
</tr>
<tr>
<td>PFT</td>
<td>Partial filling technique</td>
</tr>
<tr>
<td>pH*</td>
<td>Apparent pH</td>
</tr>
<tr>
<td>pI</td>
<td>Isoelectric point</td>
</tr>
<tr>
<td>$pK_a$</td>
<td>Negative logarithm of the acid dissociation constant</td>
</tr>
<tr>
<td>z</td>
<td>Ion charge number</td>
</tr>
<tr>
<td>r</td>
<td>Stokes radius of an ion</td>
</tr>
<tr>
<td>$R_s$</td>
<td>Peak resolution</td>
</tr>
<tr>
<td>RSD</td>
<td>Relative standard deviation</td>
</tr>
<tr>
<td>$\sigma$</td>
<td>Peak standard deviation</td>
</tr>
<tr>
<td>$t_{mig}$</td>
<td>Migration time</td>
</tr>
<tr>
<td>tPE</td>
<td>Time of partial electrophoresis</td>
</tr>
<tr>
<td>$w_{50%}$</td>
<td>Width at half of the peak height</td>
</tr>
</tbody>
</table>
1. Capillary Electrophoresis

Capillary Electrophoresis (CE) is an efficient separation technique with increasing popularity for analyses of small molecules (Altria et al., 2006; Altria and Elder, 2004; Issaq, 2002). The technique has also been used for separation of larger molecules, such as proteins (Dolník, 2008; Dolník, 2006; Huang et al., 2006) and peptides (Kašička, 2008; Kašička, 2006; Righetti, 2001). The unique separation ability offered by CE has been used for chiral separations (Gübitz and Schmid, 2007) and the technique is becoming used more frequently within bioanalysis, e.g., for analysis of DNA and carbohydrates (Kraly et al., 2006). Furthermore, the HUGO (Human Genome Organisation) project of sequencing the human genome was greatly facilitated by utilization of specialized CE-instruments (Dovichi, 2000).

1.1. Basic Principles

Capillary zone electrophoresis (CZE) is the basic mode of CE whereby separation is performed using only a buffer solution as background electrolyte (BGE). CZE in drawn glass tubes of narrow inner diameter (75 μm) was demonstrated by Jorgenson in 1981 (Jorgenson, 1981). However, the CE-technology in a wider bore (6 mm) plexiglass tube was developed much earlier by Hjertén (Hjertén, 1958). Even though CE is a fairly modern technique, the theoretical basis of electrophoresis is much older (Kohlrausch, 1897). In 1948, Tiselius was awarded the Nobel Prize for the introduction of electrophoresis as a separation technique (Tiselius, 1937).

In electrophoresis, separation is achieved within an electrical field owing to differences in the charge over size ratios of the analytes, which will give rise to different electrophoretic mobilities ($\mu_{ep}$), Equation 1:

$$\mu_{ep} = \frac{z}{6\pi \eta r}$$

(1)

Where $z$ is the ionic charge, $\eta$ is the viscosity of the separation medium and $r$ is the Stokes radius of an ion (Janini and Issaq, 1993).
The use of fused silica capillaries in CE enables the formation of an electroosmotic flow (EOF) of solvated ions present in the BGE. A schematic illustration of a fused silica capillary is presented in Figure 1.

Figure 1. Cross section of a fused silica capillary. When the capillary wall is negatively charged, solvated cations in the diffuse layer migrate towards the cathode dragging the bulk electrolyte along, thus forming the electroosmotic flow (EOF).

In bare fused silica capillaries, dissociation of the silanol groups (see section 2.2) results in a negatively charged inner surface of the capillary. Positively charged molecules present in the BGE are attracted to the negatively charged capillary wall thereby forming a stagnant layer which is often referred to as the Stern layer. Outside the Stern layer, more loosely associated positively charged molecules form a diffuse layer, i.e., the Gouy-Chapman layer (Marina et al, 2005). Due to repulsion between these positively charged layers, the diffuse layer can, upon application of an electrical field, move over the stagnant layer almost without friction, which enables a very sharp flow profile (Hjertén, 1967), thereby enabling high separation efficiencies (Marina et al, 2005).

The potential between the stationary silanol groups and the diffuse layer is called the $\zeta$ (zeta)-potential. The magnitude of this potential, which determines the velocity of the EOF, is highly affected by the composition of the BGE, e.g., type and concentration of buffer components as well pH (Berli et al., 2003). As shown in Equation 2 (Hjertén, 1967), the apparent mobility ($\mu_{\text{app}}$) is a combination of the electroosmotic mobility ($\mu_{\text{eo}}$) and the electrophoretic mobility ($\mu_{\text{ep}}$).

$$\mu_{\text{app}} = \mu_{\text{eo}} \pm \mu_{\text{ep}}$$  \hspace{1cm} (2)

The large impact of the charge number ($z$) on the $\mu_{\text{ep}}$ (Equation 1), enables a high separation selectivity of charged analytes, which has been reviewed in (Boček et al., 2000). However, since neutral analytes lack charges, separa-
tion of those is less straightforward. Direct separation of uncharged species was permitted by introduction of a surface active substance into the BGE at concentrations above the critical micelle concentration, CMC, (Terabe et al., 1984).

As shown in Equation 3 (Giddings, 1969), the resolution \( R_s \) in CE depends on the peak efficiency \( (N) \) as well as the relative mobility difference (i.e., \( \Delta \mu/\mu_{\text{app}(\text{av})} \)).

\[
R_s = \frac{1}{4} \sqrt{N_{av} \frac{\Delta \mu}{\mu_{\text{app}(\text{av})}}}
\]  

(3)

where the peak efficiency, in turn, is proportional to the peak width and the migration time \( (t_{\text{mig}}) \) of the solutes. Since the resolution only increases by the square root of the efficiency, the relative mobility difference has a higher impact on the resolution (Paper II).

The setup of a modern CE-instrument, which is basically unchanged since the pioneering work by Hjertén (Hjertén, 1967), is shown in Figure 2.

**Figure 2.** Schematic illustration of a CE-instrument. 1 – Capillary, 2 – High voltage power supply, 3 – Buffer reservoirs, 4 – Electrodes, 5 – Detector, 6 – Computer.

As illustrated in Figure 2, the capillary (1) and the electrodes (4) from the high voltage power supply converge in the buffer reservoirs (3), thus forming a closed electrical circuit. Upon separation, the analytes are detected near the opposite end of the capillary (5).
1.2. Non-aqueous Media

Non-aqueous capillary electrophoresis (NACE) was introduced by Jorgenson in 1984 (Walbroehl and Jorgenson, 1984). In non-aqueous solvents, donor-acceptor and electrostatic interactions dominate, whereas hydrophobic interactions are less pronounced than in aqueous media (Cottet et al., 2001). In addition to reducing adsorption of analytes, the organic solvents used in NACE are less polar than water, enabling analysis of compounds with poor solubility in aqueous BGE:s (Cottet et al., 2001). Since the degree of solvation differs among organic solvents, altered separation selectivities may be obtainable (Fillet et al., 2003).

Different dissociation constants (pKₐ) apply in organic solvents as compared to those in water. Dissociation of the silanol groups are suppressed in organic solvents as compared to water. Hence, decreased velocities of the EOF are generally observed in organic solvents (Porras and Kenndler, 2005; Valkó et al., 1999). The degree of ionic dissociation increases proportionally with the dielectric constant (ε) and most of the organic solvents utilized in NACE have lower ε than water (Lämmerhofer, 2005). The electrophoretic mobility and the electroosmotic mobility are, apart from the ε, also affected by the viscosity (η) of the solvent. Hence, the dielectric constant over viscosity ratio (ε/η) has a major impact on the electrophoretic as well as the electroosmotic mobilities (Jansson and Roeraade, 1995). The popularity over the years of methanol and acetonitrile as BGE-solvents might be the magnitude of their ε as well their relatively low UV-absorptivity and toxicity as compared to other organic solvents (Porras and Kenndler, 2005).

The conductivity of non-aqueous solvents is generally lower than that of water, which enables application of higher field strengths and utilization of wider-bore capillaries in NACE than in aqueous CE without generation of excessively high currents. Separations of aromatic acids at 60 kV, resulting in a field strength of 2000 V/cm, has been demonstrated using a custom-made instrument (Palonen et al., 2002). In a 1-propanolic BGE, reduced migration times were obtained upon increasing the separation voltage from 30 to 60 kV, whereas the separation efficiencies were increased almost linearly. However, in commercially available instruments the highest applicable voltage is often restricted to 30 kV due to safety reasons. Alternatively, the field strength may be increased by the use of a shorter capillary. However, the minimum capillary length is limited by the instrumental design (~20 cm). The disadvantage of using a shorter capillary is that the part of the capillary without thermostating will be proportionally increased by a reduction of the capillary length.
1.3. Chiral Separation

Molecules which exhibit different three-dimensional structures, while containing the same atoms and the same sequence of bonds, are called stereoisomers (Souter, 1985). In most cases, stereoisomerism occurs due to the presence of one or more asymmetrical carbon atom. Depending on their number of asymmetric carbons, these stereoisomers are called enantiomers or diastereoisomers, respectively. A pair of enantiomers, which are non-superimposable mirror images of one another (Figure 3) are said to be chiral (Souter, 1985). Mixtures containing the enantiomeric forms in equal parts are said to be racemic.

![Figure 3. Schematic illustration of chirality. A pair of enantiomers contain the same constituents, but are non-superimposable mirror images of each other.](image)

These different molecular forms often have quite different pharmacological activities and, in some cases, one of the forms may even display toxicity. Hence, a number of different approaches for chiral separation have been developed to determine the amount of the wanted and unwanted enantiomeric forms, respectively, upon synthesis or in formulations. Since the biotransformation of enantiomeric drugs often is stereoselective (Caldwell, 1995), there is also a need for chiral separations within bioanalysis (Caldwell, 1996; Scriba, 2002). Chiral separations have been performed using different techniques, including high performance liquid chromatography (HPLC), gas chromatography as well as CE (Gübitz and Schmid, 2001).

Separation of chiral compounds may be achieved either directly or indirectly. Indirect chiral separation is based on derivatization of the enantiomers with a chiral reagent prior to the separation. The diastereomeric derivatives thus formed are then separable in a symmetrical environment using an achiral stationary phase and without the presence of a chiral additive in the
mobile phase (HPLC). As in HPLC, diastereoisomers are separable in CE without a chiral additive in the BGE, provided they have different electrophoretic mobilities. In order to obtain direct chiral discrimination through complexation in the BGE, it is generally assumed that the chiral selector should promote a so called “three-point interaction” with at least one of the enantiomers, cf. (Dalgliesh, 1952; Davankov, 1997). However, direct chiral separation is also possible without discriminating complexation, provided that the diastereomeric complexes have different electrophoretic mobilities (Chankvetadze et al., 2004).

Indirect chiral separations have rarely been performed by CE (Vandena-beele-Trambouze et al., 2000; Wan et al., 1995), whereas direct chiral separations have gained much more interest (Amini, 2001). When performing direct chiral separation, a chiral selector is added to the BGE and laborious derivatization is therefore not necessary. Chiral separation by CE was first demonstrated by Gassman, who demonstrated separation of racemic amino acids using a copper (II) complex of L-histidine as the selector (Gassman et al., 1985). Since then, CE and capillary electrochromatography (CEC) have been used for separation of a large number of chiral compounds, as reviewed in, e.g., (Gübitz and Schmid, 2004; Gübitz and Schmid, 2007; Vespalec and Boček, 2000). The most commonly used chiral selectors in CE are proteins (Busch et al., 1993), cyclodextrins (Snopek et al., 1988; Vespalec and Boček, 2000), crown ethers (Kuhn, 1999; Snopek et al., 1988) and chiral counter-ions (Bjørnsdottir et al., 1996; Carlsson et al., 2001). Applications of chiral CE with a special emphasis on pharmaceutical analysis have also been reviewed (Amini, 2001; Ha et al., 2006).

1.4. The Partial Filling Technique

The partial filling technique (PFT) was developed by Hjertén in order to circumvent detection problems when using cellobiohydrolase I (CBH I) as a chiral selector for separation of β-adrenergic receptor blocking drugs (Valtcheva et al., 1993). The impairment of the detection sensitivity, caused by the high UV-absorbance of CBH I, was thereby avoided. A schematic illustration of the PFT is presented in Figure 4. As shown, the capillary is partially filled with the high UV-absorbing BGE (zone 1) before the sample is injected. Upon application of an electrical field, the enantiomers are separated in zone 1 which contains the chiral selector. The separated enantiomers then migrate towards the detector through zone 2, with does not contain the chiral selector.
Since its invention, the PFT has been used for chiral separations using different proteins as selectors (Amini et al., 1997; Kilár and Fanali, 1995; Tanaka and Terabe, 1995) and a number of applications utilizing the PFT have been reviewed (Amini et al., 1999). Furthermore, the technique has found a more recent application within mass-spectrometric (MS) detection of CE-separations using non-volatile chiral selectors (Jäverfalk et al., 1998; Shamsi, 2002; Tanaka, 2002; Paper IV). However, a drawback of the PFT is the reduction of separation efficiency upon passage of the analytes through the zone interface, see (Amini et al., 1999) and references therein.

1.5. Multiple-injection Methodologies

The multiple-injection methodology may enable increased throughput of analyses in CE through sequentially performed sample injections. Multiple-injections in CE were first utilized for identification of different mutations by separations of DNA (Ren et al., 1997; Ulvik et al., 1997; Ulvik et al., 1998). The technique was then used to illustrate the effect of different lengths of β-cyclodextrin-containing plugs on the chiral separation of terbutaline enantiomers (Amini, 2001). Later applications include: determination of association constants between different antibiotics (e.g., vancomycin) and peptides (Chinchilla et al., 2005; Zavaleta et al., 2006; Zavaleta et al., 2007) and quantitative analysis of buserelin and salbutamol in Papers II and III, respectively. Besides in conventional CE-systems, multiple-injections have been performed in microfluidic systems (Fu et al., 2003) and the technique has been utilized for separation of DNA by capillary array electrophoresis, CAE (Minarik et al., 2003).
Alternatives to multiple-injections for improving the throughput of analysis in CE include; short end injection (Altria et al., 1996) and capillary array electrophoresis, CAE, (Huang et al., 1992). However, even though the actual separation times are reduced by the use of short-end injection, the throughput of separations, which require elaborate preconditioning steps, remain quite low. The increased throughput of analysis in CAE, where special instrumentation is required, is achieved through utilization of a large number of parallel capillaries. Advantages of multiple-injections, as compared to these alternative techniques are: (i) the impact of time-dependent drifts of migration times and peak areas are minimized, since the standards and the sample are analysed within the same run, and (ii) the separations are performed in only one capillary, which reduces the impact of possible deviations in the fused silica (Gómez and Sandoval, 2008; Mayer, 2001; Nawrocki, 1991a,b).

When performing multiple-injections, either partial electrophoresis (Minarik et al., 2003; Ren et al., 1997; Ulvik et al., 1997; Ulvik et al., 1998; Papers I-III) or pressure (Amini, 2001; Chinchilla et al., 2005; Zavaleta et al., 2006; Zavaleta et al., 2007) may be applied in order to separate the sequentially injected samples from each other. A disadvantage of applying pressure between the injections is that the effective capillary length will be shortest for the sample injected first and will then gradually increase for the later injected samples. Hence, as illustrated in Figure 5A, different resolutions are obtained for the injected samples. Furthermore, when utilizing pressure there is an obvious risk of inducing zone broadening due to turbulence caused by the hydrodynamic pressure (Peng and Chen, 1997). On the contrary, when partial electrophoresis is applied the effective length of the capillary will be approximately the same for all injected samples, resulting in a constant peak resolution in all sample plugs (Figure 5B).

If pressure is to be used, the necessary time of applied pressure between the injections \( t_p \), in order to avoid overlap of the injected plugs, may be calculated by Equation 4 (Unpublished results).

\[
t_p = \frac{t_{BT} \left( \Delta t_{mig} + 12\sigma \right)}{t_{mig2}}
\]  

(4)

Where \( t_{BT} \) is the breakthrough time, i.e., the time required for the sample to reach the detection window at the same pressure as during the \( t_p \). The variables \( \Delta t_{mig} \) and \( t_{mig2} \) are obtained from a preceding electrophoretic separation of the sample in question. The peak standard deviation (\( \sigma \)) may be calculated.
from the width at half of the height of the broadest peak, i.e., \( \sigma = \frac{w_{50\%}}{2.35} \) (Mills et al., 1997).

**Figure 5.** Comparison of (A) partial pressure and (B) partial electrophoresis (Unpublished results). Sequential injections of phenylpropanolamine and moxonidine for 15 s at 0.5 psi. Between injections, the samples were subjected to a pressure of 1.0 psi for 7.40 minutes (A) or partial electrophoresis for 9.00 min at +30 kV (B). Final separation at +30 kV and 20°C in 50 μm ID capillary with an effective length of 106 cm. BGE: 10 % (v/v) ACN in 100 mM phosphoric acid adjusted to pH 2.6 with triethanolamine. Detection was performed at 200 nm. Peaks: (1) — phenylpropanolamine, (2) — moxonidine.
2. Pharmaceutical Analysis by Capillary Electrophoresis

The variety of available separation modes has made CE a useful method for analysis of various pharmaceutical compounds, ranging from small ions to macromolecules with diverse structures and physiochemical properties (Suntornsuk, 2007). Applications within pharmaceutical analysis using different modes of CE include: assays of drug components (Altria et al., 2006; Morzunova, 2006; Suntornsuk, 2007), determinations of log P and pKₐ (Altria et al., 2006), and evaluation of dissolution profiles (Altria et al., 1995a). Furthermore, CE and NACE have been used for chiral separations (Ha et al., 2006; Morzunova, 2006) and chiral purity determinations (Holzgrabe et al., 2006; Suntornsuk, 2007) of numerous pharmaceuticals.

CE is able to compete with well established techniques for determination of drugs in fairly simple matrices (Ackermans et al., 1992). Moreover, CE offers advantages over HPLC, such as increased separation efficiency (Issaq, 2002; Natishan, 2005) and reduced consumption of samples and solvents (Natishan, 2005). Often mentioned disadvantages of utilization of CE for pharmaceutical analysis are the low concentration sensitivity when employing UV-detection (Holzgrabe et al., 2006; Suntornsuk, 2007) and unsatisfactory reproducibility (Holzgrabe et al., 2006). Due to the lower reproducibility in CE than in HPLC, reported results are often difficult to repeat in interlaboratory trials (Ali et al., 2006). Factors which affect the reproducibility in CE include; selection of BGE and capillary, the applied electrical field and the mode of injection.

2.1. Injection Precision

The injection precision is generally not as good in CE as in HPLC. The main reason for this is the nanoliter sample volumes that are generally injected in CE (Mayer, 2001). In CE, either electrokinetic or hydrodynamic injections may be performed. The precision and robustness are generally lower in electrokinetic injection mode than in hydrodynamic pressure injection mode (Schaeper and Sepaniak, 2000). However, electrokinetic injections are nec-
ecessary in CEC and capillary gel electrophoresis (CGE) despite the reduced precision (Mayer, 2001).

When performing injections by hydrodynamic pressure, as in Papers I-IV, a number of different factors may affect the precision. The introduced sample volume depends, apart from the capillary dimensions and the sample viscosity, on the magnitude and the duration of the applied pressure for the injection. If the injected volume is very small, the injection precision might be limited by the precision of the injection mechanism of the instrument itself (Shihabi and Hinsdale, 1995). Since integration is facilitated for larger peaks, the peak area variation may be reduced by increasing the injection volume (Friedberg et al., 1997). However, if the injected volume is too large, the separation efficiency and hence the resolution will be impaired due to sample overloading (Wätzig and Dette, 1993; Yin et al., 1996). Therefore, the injected volume should be somewhere between these two extremes.

Another problematic factor, which may result in contamination of the sample and sample carryover, is irregularities on the outside of the capillary (Mayer, 2001). Sample carryover may be reduced by performing a short dip of the capillary ends in water following the sample introduction (Lux et al., 1990). The peak efficiency, as well as the symmetry, can be also be improved by proper straight cutting of the capillary ends (Cohen and Grushka, 1994), as was performed in Papers I and III. Further, removal of the polyimide layer at the capillary inlet (Papers I-IV) may also be beneficial (Mayer, 2001).

The use of an injection marker (IM) for correction of peak areas, i.e., \( \text{Area}_{\text{Analyte}} / \text{Area}_{\text{IM}} \), in CE may be beneficial for improving the precision of determinations (Papers II and III). The precision may otherwise be impaired by, e.g., injection volume variations and evaporation of the sample solutions (Dose and Guiochon, 1991). When employing an IM, however, it is important that its concentration is sufficiently high to enable accurate peak integration. Otherwise, lower precision than without performing the corrections may be achieved (Thomas et al., 1994).

2.2. Reproducibility of Separations

In CE, fluctuation of the EOF has been identified as the factor that contributes most to inter-run variation of the migration times (Jumppanen and Riekkola, 1995; Ross, 1995; Yang et al., 1996). Samples with different mobilities spend different amounts of time within the detection window. Hence, larger peak areas will be obtained for slower migrating analytes, since those spend a longer time in the detection window (Lambert and Middleton, 1990).
Several runs are required to establish steady-state conditions of the capillary surface where the migration times become stable (Smith et al., 1991).

Some of the variation of the EOF is attributed to different distributions of silanol groups on the capillary surface. The acid dissociation constant ($pK_a$) of these silanol groups has been determined to 7.1 (Hair and Hertl, 1970). However, this $pK_a$ might not be a clearly defined value, as there appear to be two kinds of silanol groups, i.e., -SiOH and –Si(OH)$_2$, with $pK_a$-values of 4.5 and 8.5, respectively (Ong et al., 1992; O’Reilly et al., 2005). Besides the pH of the BGE, the magnitude of the EOF is affected by a number of different factors, e.g., the age and history of the capillary, the ionic strength of the buffer, the conditions of preconditioning and the applied electrical field, as well as the external capillary temperature (Smith et al., 1991). Moreover, there are significant differences between fused silica capillaries from different manufacturers and even from batch to batch (Mayer, 2001).

Fluctuations of the EOF may be compensated for by normalization of the peak areas with their corresponding migration times (Altria, 1993a). When performing peak area normalization, it is assumed that the zone concentration profile is symmetrical for all of the peaks (Lambert and Middleton, 1990). However, this is not always the case, since the peak symmetry is a function of the conductivity which, in turn, is affected by the concentration of the sample zone (Hjertén, 1990). Further compensation for EOF-variations may be achieved by injection of marker molecules, with known electrophoretic mobilities, together with the analyte (Jumppanen and Riekkiola, 1995; Lee and Yeung, 1991; Yang et al., 1996). Relative migration times towards the marker, or markers, results in increased precision of the migration times and also facilitates comparison of separations performed in capillaries of different length and inner diameter, ID (Lee and Yeung, 1991).

Optimization of the capillary preconditioning is a crucial, but often overlooked, parameter in CE-method development. Preconditioning of the capillary is performed in order to eliminate adsorbed molecules and to achieve equilibrium conditions between the BGE and the capillary wall, thereby improving the migration time repeatability (Faller and Engelhardt, 1999). The reproducibility of the migration times and the peak resolution depends largely on the selection of proper conditions for preconditioning the capillary (Gómez and Sandoval, 2008; Holzgrabe et al., 2006; Lambert and Middleton, 1990).

Conditioning solely with the BGE, is often not sufficient in order to obtain satisfactory performance of the capillary (Gómez and Sandoval, 2008). Hence, a wash step with sodium hydroxide is often included in the preconditioning, since sodium hydroxide effectively removes adsorbed material from
the surface (Smith et al., 1991). The time-period as well as the pressure during the wash-step has been found to affect the separation performance. Improved stability of the EOF was observed for up to 6 hours of preconditioning with 1 M sodium hydroxide, after which it levelled off (Kaupp et al., 2000). By increasing the pressure during conditioning (as in Papers I and III) significantly improved precision of the EOF has been reported (Gómez and Sandoval, 2008). Although conditioning with sodium hydroxide often is beneficial, impairment of the migration time reproducibility for acidic species has been reported (Ross, 1995). Hence, sodium hydroxide conditioning might be omitted in cases where adsorption is a minor problem.

Etching with sodium hydroxide may be followed by leaching with hydrochloric acid (Papers II and IV). Presumably, hydrochloric acid increases the formation of additional silanol groups through catalysis of the opening of siloxane surface bridges (Gómez and Sandoval, 2008). Hydrochloric acid-leaching also enables removal of surface associated trace-metals, such as Fe, Mg and Ca, while not dissolving the silica substrate itself (Barret et al., 2001). Leaching with hydrochloric acid has been shown to reduce the velocity of the EOF through a “hysteresis-effect” (Lambert and Middleton, 1990) and to improve the inter-run precision of the EOF (Gómez and Sandoval, 2008).

Other conditioning methodologies include etching of the capillary surface by the use of hydrofluoric acid, which generates a smoother silica surface through dissolution and re-deposition of silicic acid (Köhler and Kirkland, 1987). Adsorbed proteins may be efficiently removed from the capillary surface by washing the capillary with a solution containing sodium dodecyl sulphate, SDS (Lloyd and Wätzig, 1995; Paper II).

Positively charged analytes are easily adsorbed to the negatively charged capillary surface. Analyte adsorption reduces the separation efficiency and may induce fluctuations of the EOF, resulting in poor migration time reproducibility. More complex molecules, such as proteins and peptides, may interact with the capillary surface through different intramolecular interactions (Rodriguez and Li, 1999). Different approaches have been developed in order to reduce interactions between analytes and the capillary wall. Apart from modification of the BGE, different coating procedures have been developed. A wide range of these strategies have been accounted for in an excellent review (Rodriguez and Li, 1999).

Without performing any coating, the degree of adsorption may be reduced by careful selection of the BGE. Since the ionization of the silanol groups is suppressed at low pH (Papers I-III), electrostatic interactions between analytes and the capillary surface are reduced. Alternatively, by employment of
a BGE with a pH which exceeds the isoelectric point (pI) of the analytes, adsorption may be decreased through Coulombic repulsion from the capillary wall (Lauer and McManigill, 1986).

The first class of coatings are the so-called “permanent coatings”, which are pre-attached to the capillary surface and are not included in the BGE. These coating agents may be attached to the capillary surface through either adsorption or covalent interactions (Rodriguez and Li, 1999). Hydrophilic polymers, which may be either cationic- or neutral, respectively, are adsorbed to the capillary surface. Examples of cationic polymers used are chitosan (Yao and Li, 1994) and hexadimethrine bromide, Polybrene (Córdova et al., 1997; Yao et al., 1994). Polybrene (Figure 6A), utilized in Paper II, is a hydrophobic polyamine polymer which interacts with the capillary surface through multiple electrostatic interactions. Additional Polybrene may then become associated to the previously attached Polybrene-molecules through hydrophobic interactions (Yao et al., 1994), thereby generating a strongly anodic EOF (Córdova et al., 1997; Yao et al., 1994; Paper II).

\[ \text{Figure 6. Structures of Polybrene (A) and triethanolamine (B).} \]

The other sub-category of permanent coatings is the covalently attached coatings. Typically, covalent coatings are accomplished in a two-step reaction, where the upper hydrophilic layer is attached to the capillary surface via a hydrophobic linkage. These coatings, which may be either neutral or charged, have attracted much interest over the years (Rodriguez and Li, 1999). Neutral covalent coatings such as linear polyacrylamide (Hjertén, 1985) and polyacryloylaminoethoxyethanol, poly-AAEE (Chiari et al., 1995) have been utilized primarily for suppression of the EOF. A neutral covalently coated capillary was utilized in Paper II for comparison with the performed dynamic coatings and during the capillary isoelectric focusing
(CIEF) experiments. Charged covalent coatings have been used to increase the speed of separations or to enable detection of differently charged analytes (Rodriguez and Li, 1999).

The second class of coatings are the “dynamic coatings”, which require less preparation than do permanent ones. A drawback of using dynamic coatings is that the coating agent is included in the BGE which may impair, e.g., MS-detection (Rodriguez and Li, 1999). Generation of a positively charged capillary surface and thereby an anodic EOF has been accomplished by the use of cationic buffer additives, such as triethylamine (Corradini et al., 1994) and triethanolamine, Figure 6B, (Corradini et al., 1994; Papers I-III). Triethylamine and triethanolamine are alkyl-amines which mainly interact with the silanol groups on the capillary surface through their amine functions (Corradini et al., 1994). Neutral polymers, such as propyleneglycol (McNerney et al., 1996 and hydroxypropylmethyl cellulose (Lindner et al., 1995) have also been utilized as dynamic coatings. These compounds shield the silanol groups, thereby preventing adsorption (Rodriguez and Li, 1999).

2.3. Detection Sensitivity

On-capillary UV-absorption, which was used in Papers I-IV, is by far the most commonly used mode of detection in CE. Although UV-absorbance is a versatile mode of detection, the sensitivity is often low due to limited sample injection volumes and the short light path through the capillary (Osborn et al., 2000; Simonet et al., 2003). Hence, different methodologies for increasing the sensitivity in capillary electrophoresis have been developed over the years. These strategies include various on-line preconcentration techniques as well as the use of alternative detectors.

When using conventional online UV-detection, the sensitivity may be enhanced by the use of capillaries fitted with a 3 x bubble at the detection window (Heiger et al., 1994), as in (Lodén et al., 2008) or alternatively, by using capillaries coupled through a Z-cell (Heiger et al., 1994; Kim et al., 1994). Another option in order to increasing the sensitivity may be utilization of rectangular capillaries, which permit an optical path-length up to 1000 μm (Tsuda et al., 1990). However, later use of rectangular capillaries appears to have been limited.

As mentioned, an alternative means to increase the sensitivity may be through employment of on-line preconcentration techniques. These techniques almost exclusively utilize the increased electrophoretic velocity obtainable in a low conductivity BGE to enable zone-sharpening at the boundary of the BGE’s of low and high conductivities (Osborn et al., 2000). This
phenomenon, which was explained in (Mikkers et al., 1979) is utilized, e.g., in field-amplified sample stacking (FASS), where samples are dissolved in a matrix with low conductivity (Papers I-IV). The maximum loadable sample volume in FASS is about 5% of the capillary volume, which limits the sensitivity increase to 10-20 fold (Breadmore, 2007).

In large volume sample stacking (LVSS), however, a larger part of the capillary is filled with sample in a low conductivity BGE (Chien and Burgi, 1992). A reversed polarity separation is then performed to remove the sample matrix by reversal of the sample plug out of the capillary, while the analytes accumulate at the BGE boundary. Before the analytes migrate out of the capillary, normal polarity is applied to permit detection of the sample (McGrath and Smyth, 1996; Osbourn et al., 2000). Between 100-500 fold increased sensitivity has been reported using LVSS (Breadmore, 2007).

Isotachophoresis (ITP) is particularly useful for samples with high conductivities, such as biological samples containing salts. In traditional ITP, the injected sample is sandwiched between zones of leading- and terminating ions with higher and lower mobilities, respectively, than the sample. Upon application of an electrical field, the analytes migrate towards the detector as distinct zones according to their respective mobilities. Due to the different field strengths in the adjacent zones, each analyte is held in place and migrates as a distinct band on its way towards the detector. Usually, either anions or cations may be analyzed within the same run (Osbourn et al., 2000). However, in order to be utilized for preconcentration in CE the ITP-step has to be transient, i.e., the preconcentration has to be completed before the electrophoretic separation takes place. In so-called transient-ITP (tITP) this disruption of the preconcentration-step is accomplished by letting the separation electrolyte migrate through either the leading- or terminating electrolyte, respectively. By utilization of tITP, the concentration sensitivity may be enhanced up to 500 000 fold (Breadmore, 2007).

Another option for increasing the sensitivity and selectivity is afforded by mass-spectrometric detection. MS-detection combined with CE was first demonstrated in 1987 (Olivares et al., 1987). Since then, almost all types of MS-instruments have been coupled to CE (Klampfl, 2006) and numerous applications using CE-MS have been reviewed in, e.g., (Gaspar et al, 2008; Klampfl, 2006; Schmitt-Kopplin and Frommerger, 2003). Today, CE-MS is becoming more of a routine technique, e.g., in bioanalysis, and ready-to-use CE-MS systems are available from several manufacturers (Klampfl, 2006).

Several different interfaces are available for transformation of the separated solutes in CE into the gaseous charged ions necessary for utilization of MS-detection. The most commonly used means of ionization for CE-MS, elec-
trospray ionization, ESI (Smith et al., 1988), was utilized in Paper IV for ionization under atmospheric pressure. The ESI-technique may be subdivided into non-liquid supported systems (nanospray), where ionization is accomplished through direct contact with the BGE, or liquid-supported systems, where a sheath liquid is utilized. The liquid-supported systems may be used either without (liquid junction) or with the presence of nebulisation gas (microspray) (Schmitt-Kopplin and Frommberger, 2003). Liquid-supported systems are popular, since the presence of the sheath liquid increases the MS-compatibility without modifying the BGE. However, the sheath liquid may dilute the eluent from the CE (Schmitt-Kopplin and Frommberger, 2003) and also cause suction out of the capillary, which reduces the available time of separation (Huikko et al, 2002; Paper IV). Moreover, a number of parameters which affect the stability of the electrospray need to be controlled. These parameters include: the flow rates of the sheath liquid and the nebulizing gas, the sheath liquid composition and the position of the capillary in the interface (Gale and Smith, 1993).
3. Aims

The overall objective of this thesis has been to increase the use of capillary electrophoresis within the field of pharmaceutical analysis by introduction of novel methodologies. More specifically, the aims were:

- Development of the theoretical basis for different modes of MICZE, followed by experimental verification using small molecules, e.g., salbutamol and moxonidin, as model substances (Paper I).

- Application of different modes of MICZE for assays of pharmaceutical formulations. In Paper II, a straightforward multiple-injection system was used for determination of the synthetic peptide buserelin. Following method validation, a hybrid-mode of MICZE was then utilized in Paper III for determination of salbutamol in 15 tablets within a single-run.

- Evaluation of the effects of different BGE-compositions in NACE on the separation selectivity of enantiomeric amines using (-)-DIKGA as the chiral selector (Paper IV).

- Utilization of the partial filling technique for demonstration of the mass-spectrometric compatibility of the system developed in Paper IV.
4. Multiple-injection Capillary Zone Electrophoresis

Multiple-injection capillary zone electrophoresis (MICZE) offers increased throughput of analyses by permitting sequential sample injections (see section 1.5). In order to enable quantitative analysis by MICZE, some prerequisites must be fulfilled. Firstly, the levels of interfering impurities in the samples have to be sufficiently low in order to avoid a reduction of the sample capacity ($n_s$). The $n_s$ may be defined as the highest number of two-component samples, containing the analyte and an injection marker (IM), which can be introduced within a given MICZE-system. The use of an internal standard (IS) or an IM is required for quantitative analysis by MICZE in order to compensate for injection-volume fluctuations (Papers II and III). The distinction between an IS and an IM is that an IS often is an analogue to the analyte, whereas an IM may be any type of analyte. Secondly, the adjacent analyte peaks must be resolved sufficiently to allow accurate peak integration (Figure 7).

![Figure 7](image)

**Figure 7.** Illustration of the peak resolution through magnification of the base line (Paper III). Sequential injections of 0.25 mM salbutamol and 0.41 mM oxprenolol in 50 μm ID capillary with an effective length of 148 cm. Between injections, the samples were subjected to partial electrophoresis for 2.35 ($t_{PE1}$) and 10.50 minutes ($t_{PE2}$), respectively. Other conditions were as in Figure 5. Peaks: (1) — oxprenolol, (2) — salbutamol.
A final prerequisite is that interferences or interactions between the analytes are negligible, since such would complicate the calculation of the appropriate times of partial electrophoresis ($t_{PE}$) between the sample injections.

4.1. Principles for Different Modes (Paper I)

In Paper I, the theoretical basis for four different modes of MICZE was presented. Selection of a suitable mode, which depends on the migration times ($t_{mig}$) and the migration time difference ($\Delta t_{mig}$) of the analytes, is facilitated by the developed equations. Schematic illustrations of these different modes of MICZE are presented in Figure 8.

**Figure 8.** Schematic illustrations of the developed MICZE-modes (Paper I). In mode I, the analytes from each introduced sample migrate as distinct zones through the capillary (A). In modes II, III and IV, respectively, the faster-migrating analyte from each injected plug migrates through the slower-migrating analyte from all injections (B and C). Mode IV, where sets of samples are injected consecutively, is a hybrid of modes I and II (C).

As illustrated in Figure 8, the sample plugs are introduced consecutively into the capillary. By application of an electrical field for a short period of time
(t_{PE}) between injections, the injected samples are isolated by plugs of BGE. Depending on the respective solute mobilities, different migration patterns are observed. Mode I, used in Paper II, may be considered as the basic mode where the analytes from each injection migrate close to each other. The distinction between modes II and III, respectively, is that the migration time of the faster migrating analyte (t_{mig1}) is larger than the $\Delta t_{mig}$ in mode II, whereas the opposite applies for mode III. By utilization of mode IV, as in Paper III, it is possible to increase the sample capacity for analytes for which mode II would normally apply. In those cases, the $\Delta t_{mig}$ between the analytes from each sample is sufficiently large to allow detection of the slower migrating analyte from a number of following injections. Hence, sets of samples may be consecutively injected according to the principles of mode II. Following the last sample injection in each set, a longer time of partial electrophoresis (t_{PE2}) than between the samples within each set (t_{PE1}) is required in order to avoid overlapping of peaks from the different sets.

Further, Paper I contains equations for calculation of suitable times of partial electrophoresis (t_{PE}) between the injections in the different modes of MICZE, based on an initial separation of two analytes by conventional CZE. Otherwise, selection of the appropriate t_{PE} may be time-consuming when developing a MICZE-method. In order to verify the validity of these developed equations, separations of different analytes were initially performed in the single-injection mode (Table 1).

### Table 1. Migration of the analytes used for illustration of the four modes of MICZE.

<table>
<thead>
<tr>
<th>Mode</th>
<th>Analyte 1</th>
<th>Analyte 2</th>
<th>t_{mig1} (min)</th>
<th>$\Delta t_{mig}$ (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>oxprenolol</td>
<td>salbutamol</td>
<td>56.28</td>
<td>4.20</td>
</tr>
<tr>
<td>II</td>
<td>PPA a)</td>
<td>salbutamol</td>
<td>39.33</td>
<td>17.70</td>
</tr>
<tr>
<td>III</td>
<td>salbutamol</td>
<td>benzoic acid</td>
<td>13.85</td>
<td>38.50</td>
</tr>
<tr>
<td>IV</td>
<td>PPA</td>
<td>moxonidine</td>
<td>40.57</td>
<td>8.50</td>
</tr>
</tbody>
</table>

a) Phenylpropanolamine

The separations for demonstration of modes I, II and IV were performed using 10 % (v/v) acetonitrile in 100 mM phosphoric acid adjusted to pH 2.6 with triethanolamine as BGE. The presence of triethanolamine (Figure 6B) in the BGE generates an anodic EOF, which increases the t_{mig} and the $R_s$ of the analytes and thus contributes to increasing the sample capacity ($n_s$). However, a BGE composed of 50 mM phosphoric acid adjusted to pH 7.0 with sodium hydroxide was necessary in order to enable demonstration of mode III.

As shown in Table 1, different t_{mig1} and $\Delta t_{mig}$ were obtained for the pairs of analytes, which were used for calculation of the times of partial electropho-
resis ($t_{PE}$) as well as the sample capacities ($n_s$) for the different modes. However, if the desired number of injections ($n_{inj}$) is smaller than the $n_s$, a longer $t_{PE}$ could be chosen. In turn, this also improves the resolution between the sample plugs further. The calculated values of the $t_{PE}$ and $n_{inj}$ were verified by MICZE-analyses of the analytes (Table 1) by the modes I through IV.

As mentioned, in order for MICZE to be a valid alternative for quantitative analysis, one of the prerequisites is that the levels of interfering sample impurities are sufficiently low, i.e., below their detection limits. This requirement was fulfilled by the injection solution of buserelin (Paper II) as well as the salbutamol tablets (Paper III), since the amounts of impurities in these formulations are low and the content of the active substances were sufficiently high to permit dilution of the analyte samples.

4.2. Determination of Buserelin (Paper II)

Due to the high specificity of peptides, their use as drugs has increased (Loffet, 2002). As a consequence, there has evolved a need for methods for analysis of these peptides. As mentioned in section 1, the high separation efficiency of CE has made the technique popular within peptide analysis. In Paper II, the peptides buserelin and goserelin were used as model substances. These peptides are anti-androgenic analogues used for treatment of prostate and breast cancer (Chander et al., 2005; Emtage et al., 1990).

Owing to the high $pI$-value of 9.6 for buserelin and goserelin (Paper II), adsorption of these basic peptides to the capillary surface through molecular interactions tends to occur even at pH 3.0. Hence, different separation conditions were evaluated, Figure 9.

As shown in Figure 9B, the resolution of the peptides was slightly increased by addition of 10 % (v/v) acetonitrile to the BGE. As mentioned in section 2.2, the presence of triethanolamine (Figure 6B) in the BGE generates a positively charged capillary surface and thus an anodic EOF (Amini et al., 2006; Corradini et al., 1994; Papers I-III). Hence, increased migration times and reduced adsorption of the peptides onto the capillary surface was achieved (Figure 9C). Adsorption of the peptides was decreased further by introduction of a preconditioning step with a 0.25 % (w/v) aqueous solution of Polybrene (Figure 9D). Polybrene (Figure 6A) generates a positively charged capillary surface and thus an anodic EOF by interacting with the capillary surface through multiple electrostatic interactions, see section 2.2 (Córdova et al., 1997; Paper II). Due to the reduced stability of the Polybrene coating at low pH (Amini and Olofsson, 2004), the capillary was flushed with the Polybrene solution prior to each run. The Polybrene treat-
ment enhanced the peak efficiencies and resolution between the peptides by 50 and 30 %, respectively, as compared to those in the BGE with triethanolamine (Paper II).

![Figure 9](image.png)

**Figure 9.** CZE-separation of buserelin and goserelin under different conditions (Paper II). The separations were performed in 65 mM phosphate buffer at pH 3.0 (A), 10 % (v/v) acetonitrile in 65 mM phosphate buffer at pH 3.0 (B) and 10 % (v/v) acetonitrile in 100 mM phosphate-triethanolamine buffer at pH 3.0 (C and D). Non-coated fused silica capillaries were used in (A, B and C) whereas the capillary was coated with Polybrene in (D). Capillaries with 50 μm ID and an effective length of 50 cm were used. The applied voltage was +25 kV in (A-C), and -25 kV in (D). Detection was performed at 220 nm. Peaks: (1) — buserelin, (2) — goserelin.

The structural similarity between the peptides resulted in a low $\Delta \mu$ (1.8 x 10^{-10} m/Vs), and subsequent close migration of buserelin and goserelin (Figure 9D). Therefore, mode I (Paper I) of MICZE could be employed. From the separation in the Polybrene-coated capillary, the migration times of goserelin and buserelin were 27.64 and 28.46 minutes, respectively, which resulted in a migration time difference of 0.82 minutes. These variables were then used for designing the MICZE-method.

In order to obtain a sufficiently high resolution between the peaks at the boundaries between the injected plugs, the distance between these peaks should be $12\sigma$, where $\sigma$ is the peak standard deviation. From the width at half of the height ($w_{50\%}$) of the broadest peak, i.e., goserelin, the $\sigma$ was calculated to 0.077 minutes. The shortest necessary time of partial electrophoresis ($t_{PE}$) was then calculated by Equation 5 (Paper I) to be 1.74 minutes.
$$t_{PE} \geq (\Delta t_{mig} + 12\sigma) \quad (5)$$

However, in order to further increase the resolution between the boundary peaks, a $t_{PE}$ of 2.00 minutes was selected. The number of possible injections ($n_{inj}$) at the selected $t_{PE}$ was then calculated to be 14 injections, Equation 6 (Paper I).

$$n_{inj} = \frac{t_{mig2}}{t_{PE}} \quad (6)$$

As is shown in Figure 10, matrix peaks originating from the multiple injections migrated with the EOF, thereby reducing the number of possible sample injections. However, there was still enough space to permit 6 consecutive injections, which was considered sufficient to perform quantitative analysis of buserelin.

The obtained linearity was $(R^2 \geq 0.996, n = 6)$ upon injections of six standards containing 50-300 μg/ml buserelin and 50 μg/ml goserelin (IS). However, the inter-run variation of the slopes of the quantification graphs was significant (Paper II). The reason behind the variation of the slopes (±395 %) might be a minute inter-run variation of EOF, as mentioned in section 2.2. However, since the sample is analyzed within the same run as the standards in MICZE, the determinations were not impaired by this inter-run slope variation (Paper II).

As mentioned further in section 2.2, it may be possible to enhance the precision by normalization of the peak areas by their $t_{mig}$ (Altria, 1993a). However, when using peak area ratios, i.e., $\text{Area}_{\text{Analyte}} / \text{Area}_{\text{IS}}$, no further reduction of the peak area variation was observed through normalization. This might indicate that the migration time alterations affected the analyte and the IS from each injection similarly.

In order to increase the accuracy, a narrower concentration range, i.e., 50-150 μg/ml buserelin was used for determination of buserelin in a pharmaceutical formulation with a declared concentration of 1.0 mg/ml. Besides buserelin, the six standards contained 50 μg/ml goserelin (IS). The sample containing buserelin was diluted to 90 μg/ml before the concentration of buserelin was determined to be $0.94 \pm 0.05$ (n = 12) and $0.98 \pm 0.05$ mg/ml (n = 3), respectively, using two different brands of CE-instruments.
Figure 10. MICZE-analysis of goserelin standards containing buserelin (Paper II). The standards contained 300, 190, 150, 100, 75 and 50 μg/ml goserelin and 50 μg/ml buserelin (IS). Time of partial electrophoresis (t_{PE}) was 2.00 min. Separation at -25 kV in a Polybrene-coated capillary. Other conditions were as in Figure 9. Peaks: (1) — buserelin, (2) — goserelin.

4.3. Determination of Salbutamol (Paper III)

The hybrid-type of MICZE which was developed in Paper I, called mode IV, might be an interesting approach for simultaneous analysis of a large number of samples in, e.g., dissolution testing or quality control assays of pharmaceuticals. In Paper III, this mode of MICZE was applied for determination of salbutamol (Figure 11) in Ventoline® Depot tablets.

![Salbutamol and Oxprenolol structures](image)

Figure 11. The structures of salbutamol (analyte) and oxprenolol (injection marker).

Salbutamol, which is a β2-receptor agonist used for alleviation of bronchoconstriction, and with a pK_a of 9.3, had previously been analyzed by conventional single-injection CZE (Ackermans et al., 1992; Altria, 1993b; Altria et al., 1995b) and micellar electrokinetic chromatography (Mälkki-Laine and Hartikainen, 1996).
As mentioned above, the hybrid mode of MICZE developed in Paper I (Figure 8C) was employed in Paper III in order to obtain a high sample capacity \( n_s \). Oxprenolol (Figure 11), with a \( pK_a \) of 9.5, was selected as the injection marker (IM) in order to obtain a suitable migration time difference for application of the selected MICZE-mode. Aiming at increasing the \( n_s \) further, a capillary with an effective length of 148 cm was utilized. The use of such a long capillary was made possible by connecting two standard tubes for the cooling-liquid with Epoxy glue from Biltema AB (Helsingborg, Sweden).

An initial separation performed by single-injection CZE revealed that the long capillary, together with the anodic EOF generated by the presence of triethanolamine in the BGE (Amini et al., 2006; Corradini et al., 1994; Papers I-III), did indeed result in long migration times for the IM and salbutamol, i.e., 103.60 and 111.04 minutes, respectively (Figure 12A).

As shown in Figure 8C, sets containing a number of two-component samples are repeatedly injected in this mode of MICZE. The transfer of a single-injection system to this hybrid MICZE-mode requires calculation of the times of partial electrophoresis between the injections within each set \( (t_{PE1}) \) as well as between the different sets \( (t_{PE2}) \). These calculations are greatly facilitated by the equations developed in Paper I. Hence, the duration of \( t_{PE1} \), which is determined by the standard deviation of the peak, i.e., \( \sigma = w_{50\%} / 2.35 \) (Mills et al., 1997), was calculated to be 2.35 minutes (Equation 7).

\[
t_{PE1} \geq 12\sigma
\]  
(7)

By using Equation 8, the number of possible injections in each set \( (n_1) \) at the selected \( t_{PE1} \) was determined to be 3 samples.

\[
n_1 = \frac{\Delta t_{mig}}{t_{PE1}}
\]  
(8)

In order to obtain complete separation between the sequentially injected sample sets, the time of partial electrophoresis between the sets \( (t_{PE2}) \) has to be longer than \( t_{PE1} \) (Paper I). The minimum value of \( t_{PE2} \) was calculated to 9.79 minutes by use of Equation 9, using the migration time difference \( (\Delta t_{mig}) \) of 7.44 minutes between oxprenolol and salbutamol.

\[
t_{PE2} \geq \Delta t_{mig} + 12\sigma
\]  
(9)
Following adjustment of the $t_{\text{PE2}}$ to 10.50 minutes, the maximum number of applicable sets ($n_2$) was calculated to be 7 (Equation 10).

$$n_2 = \frac{t_{\text{mig2}}}{t_{\text{PE2}} + (n_1 - 1) \cdot t_{\text{PE1}}}$$  \hspace{1cm} (10)

The total number of injections at the selected $t_{\text{PE}}$ ($n_{\text{inj}}$), which is the product of $n_1$ and $n_2$, was then calculated to be 21 using Equation 11.

$$n_{\text{inj}} = n_1 \cdot n_2$$  \hspace{1cm} (11)

As shown in Figure 12B, the number of injections ($n_{\text{inj}}$), predicted by Equation 11, was confirmed by MICZE analysis of 21 standards containing salbutamol and oxprenolol.

The developed MICZE-system was then validated regarding linearity, precision, accuracy and robustness. The validation was performed in the MICZE-mode, except the robustness-study, which was performed in the single-injection mode in order to facilitate interpretation of the electropherograms. The linearity was studied over the range of the standard concentrations, i.e., 0.199 – 0.300 mM salbutamol. Besides salbutamol, the 6 standard solutions contained 0.409 mM of the IM (oxprenolol). Peak area ratios, i.e., $\text{Area}_{\text{salbutamol}} / \text{Area}_{\text{oxprenolol}}$, were used for construction of the standard curves. The obtained linearity was ($R^2 \geq 0.996$) and the agreement between the slopes was good ($n = 6$). As in Paper II, the peak areas were not normalized by their migration times.

In the robustness-study, the impact of increasing the pH from 2.6 to 3.1 by addition of triethanolamine and changing the content of acetonitrile in the BGE from 10 to 8 and 12 % (v/v) were investigated. Moreover, the results obtained using a capillary from a different batch were compared to those from the original capillary. The obtained migration times and peak widths at 50 % of the height of the broadest peak ($w_{50\%}$) were then used for re-calculation of the required times of partial electrophoresis ($t_{\text{PE1}}$ and $t_{\text{PE2}}$) in order to enable 21 consecutive injections. The system was found to be robust towards these introduced variations in the composition and pH of the BGE, although slightly increased migration times were observed upon increasing the pH of the BGE from 2.6 to 3.1. This may have been due to generation of a stronger anodic EOF as well as to an increase in the viscosity of the BGE. On the contrary, slightly reduced migration times were obtained in the second capillary. This was probably caused by incomplete coverage of the si-
lanol groups on the capillary wall with triethanolamine, resulting in a weaker anodic EOF. The results from the robustness-study underline the importance of performing an initial separation of the analytes in the single-injection mode upon changing the separation conditions. Thereafter, it is possible to perform necessary re-adjustments of the $t_{PE1}$ and the $t_{PE2}$.

Figure 12. Separations of salbutamol and oxprenolol in the single-injection mode (A) and in the MICZE-mode (B) (Paper III). The BGE consisted of 10 % (v/v) ACN in 100 mM phosphoric acid adjusted to pH 2.6 with triethanolamine. Samples were injected at 0.5 psi for 15 seconds. In the MICZE-mode, the $t_{PE1}$ and $t_{PE2}$ were 2.35 and 10.50 minutes, respectively. Separation was performed at +30 kV in a 50 μm ID fused silica capillary with an effective length of 148 cm. UV-detection was performed at 200 nm. Peaks: (1) — Oxprenolol, (2) — Salbutamol.

The MICZE-method was then applied for single-run determination of salbutamol in 15 tablets of Ventoline® Depot with a labelled content of 8 mg. The average content of the tablets was determined to be 7.8 mg. The inter-sample precision from the 90 determinations was 4.2 % (RSD), whereas the intra-sample precision from the six determinations was between 1–3 % (RSD).
5. Chiral Non-aqueous Capillary Electrophoresis

As mentioned in section 1.2, organic solvents affect the electrophoretic mobility as well as the EOF through differences in, e.g., viscosity, $pK_a$ and solvation (Sahota and Khaledi, 1994). A higher degree of ion-pair formation is possible in organic solvents than in water (Schill, 1965), which favors an increased degree of complex formation. This, in turn, facilitates chiral separation using ion-pair selectors (Lämmerhofer, 2005; Wang and Khaledi, 1996; Wang and Khaledi, 2000). Such chiral selectors, which have been utilized in NACE, include: quinine (Stalcup and Gahm, 1996), (+)-(S)-camphor-sulphonic acid (Bjørnsdottir et al., 1996), N-benzyloxy-carbonylglycyl-$L$-proline, $L$-ZGP, (Hedeland et al., 2003) and (-)-2,3:4,6-di-$O$-isopropylidene-$2$-keto-$L$-gulonic acid, (-)-DIKGA, (Carlsson et al., 2001; Paper IV).

5.1. Separation of Pharmaceutical Amines (Paper IV)

In Paper IV, separations of different amino-alcohols were performed using (-)-DIKGA as chiral selector. The aims of Paper IV were to increase the enantioresolution by adjusting the composition of the BGE and to develop a system which was compatible with on-line MS-detection.

The chiral selector, (-)-DIKGA, contains a carboxylic acid moiety which needs to be in its carboxylate form in order to increase the ionic interactions towards the solutes (Carlsson et al., 2001; Pettersson and Gioeli, 1993). Therefore, besides the chiral selector, an apparent pH (pH*) where both the selector and the enantiomers are charged is required in order to improve the enantioselectivity of the NACE-system (Carlsson et al., 2001). At the beginning of the study, 40 mM sodium hydroxide was present as the base in the methanolic BGE containing 100 mM (-)-DIKGA. Experiments were then carried out in order to increase the resolution of racemic pronethalol and atenolol, respectively, by additions of other organic solvents to the BGE.

As mentioned in section 1.2, different solvents exhibit different $\varepsilon/\eta$-ratios, where $\varepsilon$ and $\eta$ are the dielectric constant and the viscosity, which affect the
magnitude of the EOF (Wright et al., 1997). The highest resolution of the pronethalol enantiomers was obtained upon addition of 2-propanol, even though the mobility difference ($\Delta \mu$) was larger in the acetonitrile-containing BGE. The reason for this discrepancy was found to be the increased EOF in the presence of acetonitrile, which decreased the effective time of separation in the capillary. On the contrary, the low EOF generated by addition of 2-propanol to the BGE afforded a longer time of separation, which resulted in increased resolution of the amines.

In order to increase the MS-compatibility of the system, sodium hydroxide was replaced by ammonium acetate. No reduction of the mobility difference ($\Delta \mu$) or the resolution ($R_s$) of the enantiomers were observed following the exchange, which suggested that ammonium acetate was equally efficient as sodium hydroxide for deprotonation of (-)-DIKGA.

Generally, the enantioresolution reaches a maximum at a certain selector concentration (Wren and Rowe, 1992). Hence, separations of rac-pronethalol were performed at different concentrations of (-)-DIKGA and ammonium acetate, respectively, in the BGE. The $\Delta \mu$ of rac-pronethalol was reduced by increasing the concentration of ammonium acetate. This reduction was probably due to either increased interactions between the negatively charged acetate ions and the cationic solutes or, alternatively, increased competition between the solutes and the ammonium ions for binding the negatively charged (-)-DIKGA. An increase in the selector concentration beyond 50 mM also caused a decreased $\Delta \mu$. However, owing to the decreased velocity of the EOF upon increasing the concentration of (-)-DIKGA, the resolution actually increased up to 70 mM (-)-DIKGA. These experiments led to the choice of 80 mM (-)-DIKGA and 40 mM ammonium acetate in the BGE.

Partial filling (see section 1.4) was then utilized in order to avoid introduction of the non-volatile chiral selector into the electrospray ionization (ESI) interface in the forthcoming experiments with MS-detection. The effective migration direction of the negatively charged (-)-DIKGA was anodic. However, owing to the presence of a minute EOF, the net migration of the selector zone was still towards the cathode. In order to delay the arrival of the non-volatile (-)-DIKGA at the detector, attempts were made to delay the migration of the selector zone by reducing the EOF in the zone without selector. The effect of 30 mM acetic acid in the zone without selector is illustrated in Figure 13. As shown, addition of acetic acid significantly delayed the arrival of the selector zone at the detector, probably by reducing the pH*, which, in turn, decreased the velocity of the EOF.
Figure 13. Effect of acetic acid on the selector zone migration (Paper IV). Separation of 0.1 mM rac-pronethalol. Conditions: Partially filled capillary (28 cm) with \( L_{\text{eff}} = 50 \) cm. BGE with selector: 80 mM (-)-DIKGA and 40 mM ammonium acetate in methanol. BGE without selector: Methanol containing 40 mM ammonium acetate (A) or 40 mM ammonium acetate and 30 mM acetic acid (B). Separations were performed at +25 kV and the peaks were detected at 214 nm.

The developed PFT-NACE-system was then coupled to a quadrupole-time of flight MS. The sheath liquid was composed of 0.25 % (v/v) acetic acid in a 50:50 (v/v) mixture of methanol and water. MS-MS-detection of the resolved daughter ions of rac-pronethalol at m/z 153.4, [M+H]\(^{+}\), was demonstrated (Figure 14).

When employing MS-detection, a faster migration of the selector zone was observed than in the preceding experiments with UV-detection. This was probably due to a minute hydrodynamic suction induced by the ESI. In turn, this increased migration rate of the selector zone necessitated the use of a shorter selector plug than was applicable in the UV-mode. As is evident from Figure 14, much of the resolution obtained by the NACE-separation was lost. The reasons for this were most likely the occurrence of a turbulent flow inside the capillary as well as mixing of the separated zones with the sheath liquid in the ESI.
Figure 14. MS-MS product ion electropherogram of 10 μM rac-pronethalol (Paper IV). Detection of resolved daughter ions at m/z = 153.4 [M+H]^+ (parent ion m/z = 230). Conditions: Partially filled 50 μm capillary (55%) with L_{eff} = 50 cm. BGE with selector: 80 mM (-)-DIKGA and 20 mM ammonium acetate in methanol with 25 % (v/v) 2-propanol. BGE without selector: 20 mM ammonium acetate and 20 mM acetic acid in methanol with 25 % (v/v) 2-propanol. Separation performed at +30 kV.
6. Conclusions

By utilization of the powerful separation ability of capillary electrophoresis (CE), novel methods for separation and determination of pharmaceuticals have been developed within the work of this thesis.

In Paper I, four different modes of multiple-injection capillary zone electrophoresis (MICZE) were described and experimentally verified. The equations developed facilitate the transfer from conventional single-injection CZE to one or more of these modes, which are available depending on the selectivity between the analytes.

In Paper II, quantification of the synthetic peptide buserelin was performed by MICZE using goserelin, an analogue of buserelin, as an internal standard. The strong anodic EOF, provided by the Polybrene-coating, provided high separation efficiency and increased peak resolution. It was demonstrated that the MICZE-methodology can be used to increase the speed of quantitative analysis without impairment of the accuracy and precision.

In Paper III, determination of the salbutamol content of 15 individual tablets within a single run was demonstrated. This was accomplished by the use of an extra long (148 cm) capillary and a background electrolyte containing triethanolamine, which increased the sample capacity by generation of an anodic EOF.

In Paper IV, a partial filling method was developed for chiral separations by non-aqueous CE (NACE) using (-)-DIKGA as the chiral selector. Initially, different concentrations and solvent compositions of the BGE were evaluated in order to tune the separation of enantiomeric amines. Then, the compatibility of the developed system with mass-spectrometric (MS) detection was increased by replacing sodium hydroxide in the background electrolyte (BGE) with ammonium acetate and by utilization of the partial filling technique. Addition of acetic acid to the zone without selector delayed the selector zone, thereby enabling the use of a longer selector plug, which increased the separation ability of the system. Finally, MS-detection of pronethalol separated by the developed chiral partial filling NACE system was demonstrated.
7. Populärvetenskaplig sammanfattning

Huvudtemat för detta avhandlingsarbete har varit utveckling av ny metodik för separation och bestämning av farmakologiskt verksamma substanser med hjälp av kapillärelektrofores (CE).

Kapillärelektrofores är en teknik där olika ämnen separeras med hjälp av hög spänning (upp till 30000 V) i en tunn kapillär av kiseldioxid (glas). Då kapillärer av kiseldioxid fylls med ett separationsmedium (bakgrundselectrolyt) och en spänning appliceras, så uppstår ett så kallat elektroosmotiskt flöde, vilket för de injicerade provmolekylerna mot detektorn. Under molekylernas vandring genom kapillären mot detektorn separeras de elektroforetiskt beroende på sina laddningar. Den elektroforetiska separationen baseras på principen att positivt laddade molekyler strävar mot den negativt laddade polen och vice versa.

Till skillnad mot då en vätska drivs framåt genom pumpning, så bidrar inte det elektroosmotiska flödet i samma utsträckning till utvidgning av provzonen i sidled. Detta ger i sin tur upphov till smala toppar med hög s.k. effektivitet. Den höga effektiviteten möjliggör i sin tur att separation av likartade substanser, som skulle vara svåra att separera med andra metoder, kan uppstå. Då hastigheten av det elektroosmotiska flödet vanligtvis är större än molekylernas elektroforetiska vandringshastigheter leder detta till att samtliga provmolekyler förs mot detektorn oberoende av deras laddning.

I traditionell CE analyseras endast ett prov i taget. För att minska analysiden, och inflytandet av eventuellt förändrade betingelser mellan analyserna, är det möjligt att använda sig av multipla injektioner (MICZE). Vid MICZE åtskiljs de sekventiellt injicerade proverna från varandra genom att spänning appliceras en kort tid mellan injektionerna.

I delarbete I beskrivs principerna för fyra olika sätt (modes) av MICZE. I arbetet utvecklades även ekvationer för att underlätta övergången till MICZE från ett konventionellt system baserat på enkelinjektion. Vid kvantitativ analys är det lämpligt att använda en injektionsmarkör för att kompensera för varierade injektionsvolymer. Därför användes olika blandprover innehållande två substanser för att verifiera de utvecklade ekvationerna.
I delarbete II beskrivs utvecklingen av ett system med multipla injektioner (MICZE) för haltbestämning av den farmaceutiska peptiden buserelin i en produkt. Som intern standard användes den analoga peptiden goserelin. Då peptider ofta tenderar att interagera med kapillärens yta genom adsorption, utvärderades trietanolamin och Polybren för s.k. dynamisk coating av kapillären. Det fanns att en kombination av trietanolamin och Polybren minskade adsorptionen av peptiderna mest, samtidigt som den omvända riktningen på det elektroosmotiska flödet möjliggjorde fler provinjektioner efter varandra. Genom att utnyttja MICZE i detta system utfördes sedan kvantitativ analys av buserelin ca 6 ggr snabbare än vid konventionell CE.

I delarbete III beskrivs utvecklingen av ett MICZE-system med hög provkapacitet, vilket kan vara fördelaktigt vid exempelvis studier av upplösning respektive kvalitetskontroll av läkemedel. För att erhålla den höga provkapaciteten användes en extra lång kapillär samt en bakgrundselectrolyt innehållande trietanolamin. Efter metodvalidering demonstrerades simultan haltbestämning av salbutamol i 15 individuella tabletter.

I delarbete IV beskrivs utvecklingen av ett enkelinjektionssystem för kiral separation i vattenfri miljö (metanol med tillsatser) av ett antal farmaceutiska substanser. Som kiral selektor användes (-)-DIKGA, vilken tidigare framgångsrikt använts för kiral separation med vätskekromatografi (HPLC) och CE. För att möjliggöra mass-spektrometrisk detektion utnyttjades s.k. partial filling, där endast en del av kapillären fylldes med lösning innehållande den icke-flyktiga kiral selektorn. Partial-fillingsystemet optimerades för att fördjöja ankomsten av lösningen innehållande selektorn till detektorn, varefter mass-spektrometrisk detektion av läkemedelssubstansen pronetahalol demonstrerades.
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