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FURTHER INVESTIGATION OF CAPILLARY ELECTROPHORESIS FOR PHARMACEUTICAL ANALYSIS

by

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Declaration

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of PhD is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

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ABBREVIATIONS

ADME - Absorption, Distribution, Metabolism and Elimination,

APCI - Atomic Pressure Chemical Ionization,

BGE – Background Electrolyte,

CAPS - Cyclohexylamino-1-propane sulfonic acid,

CD – Cyclodextrin,

CE – Capillary Electrophoresis,

CEC - Capillary Electrochromatography,

CGE - Capillary Gel Electrophoresis,

CLND- Chemiluminescent Nitrogen Detection,

CMC - Critical Micelle Concentration,

CTAB - Cetyltrimethylammonium bromide,

c-SWNT - carboxylic Single-Walled Carbon Nanotubes,

CZE - Capillary Zone Electrophoresis,

DAU – Daunorubicin,

DI - Direct Injection,

DOX – Doxorubicin,

DS – Degree of Substitution,

DTAB - Dodecyltrimethylammonium bromide,

EA – Ethyl Acetate,

ECL – Eelectrochemiluminescence,

EDC - Endocrine Disrupting Compounds,

ELSD - Evaporative Light-Scattering Detection,

EPI – Epirubicin,

ESI – Electrospray Ionisation

EOF – Electroosmotic Flow,

EP – European Pharmacopoeia,

FIA - Flow Injection Analysis,

FT - Fourier Transform,

GC – Gas Chromatography,

HEPES - N-2-Hydroxyethylpiperazine-N`-2-ethanesulfonic acid,

HP – hydroxypropyl,

HPLC – High Performance Liquid Chromatography,

IDA – Idarubicin,

IF - Isoelectric Focusing,

IS – Internal Standard,

ITP – Isotachophoresis,

IR – Infrared,

LIF - Laser Induced Fluorescence,

LLE - Liquid-liquid extraction,

LOD – Limit of Detection,

LOQ – Limit of Quantitation,

MALDI - Matrix-Assisted Laser Desorption/Ionisation,

MEEKC - Microemulsion Electrokinetic Chromatography,

MEKC - Micellar Electrokinetic Capillary Chromatography,

MES - 2-(N-Morpholino)-ethanesulfonic acid,

MK – Montelukast sodium

MS – Mass Spectrometry,

NIR – Near-Infrared,

NMR - Nuclear Magnetic Resonance,

pcSFC - packed column Supercritical Fluid Chromatography,

PIPES - Piperazine-N,N`-bis(ethanesulfonic acid),

RSD - Relative Standard Deviation,

RTP - Room Temperature Phosphorescence,

SBE - sulfobutylether

SDS - Sodium Dodecyl Sulphate,

SFC - Supercritical Fluid Chromatography,

SPE – Solid Phase Extraction,

TLC – Thin-Layer Chromatography,

Tris - Tris-(hydroxymethyl)aminomethane,

USP – United States Pharmacopoeia,

UV – Ultraviolet.

Thesis abstract

The literature review reveals current analytical methodologies used for pharmaceutical analysis, their advantages and disadvantages. In this context the potential of capillary electrophoresis (CE) is established through a study of the current literature. Capillary electrophoresis method is developed and optimised for alkylxanthines. The achieved separation for these analytes stands out for its high efficiency and especially short analysis time. This work also verifies the potential of CE in the analysis of significant impurities in montelukast sodium – an active ingredient for treatment of bronchial asthma. The obtained method surpasses a chromatographic method currently employed in the pharmaceutical industry in terms of efficiency and time of analysis.

Along with the method development this work shows the capability of CE as a fundamental research tool. The determination of the association constants of steroid hormones with cyclodextrins is successfully achieved from the electrophoretic mobilities. A range of different types of natural and derivatised cyclodextrins is investigated. This straightforward approach marks the first time CE has been used for the assessment of steroid/cyclodextrin interaction. For the first time the versatility of CE is demonstrated in the separation of different types of anticancer drugs – anthracyclines and taxanes. The high hydrophobicity of the drugs requires detailed investigation of suitable buffer composition with additives of miscellaneous component and organic phase. The use of these drugs in combination therapy sparked the interest in this application and this study shows the potential of CE for such a challenge. To demonstrate the potential an attempt is made to apply the developed electrophoretic methods to the analysis of plasma samples.

CHAPTER 1

INTRODUCTION

chromatography, ultrathin layers and ultrafine particle coating has brought TLC to higher resolution and speed of analysis. It has found application in the separation of impurities [9] and degradation products of drugs [10] and in the analysis of herbal extracts [11].

Whilst being an established tool in the analysis of biopolymers, the potential of capillary electrophoresis has not yet been fully realised in pharmaceutical analysis. CE, as a method for drug testing, was introduced for the first time in European Pharmacopoeia (EP)-Supplement only in 2001 [12]. While there is an increasing number of the capillary electrophoretic methods being developed for various drugs and being published in the literature, the number of drug-related method in pharmacopoeias is limited [13]. Different separation options available in CE cover all possible application areas, such as the separation of polar, non polar compounds, charged or neutral, structurally related and optical isomers.

1.1.2 Non-separation techniques

The non-separation methods [14] employed in pharmaceutical analysis include spectroscopic methods, such as nuclear magnetic resonance (NMR), MS, infrared (IR) and near-infrared (NIR) spectroscopy, ultra violet spectroscopy, fluorimetry and Raman spectroscopy. Titrations, electroanalytical methods, flow-injection analysis (FIA), microbiological methods and immunoassays are also used in the analysis of pharmaceuticals. As the focus of this work is on the separation techniques, these methods will not be expanded further.

1.1.3 Combination techniques

The increasing complexity of pharmaceutical research has led to the development and application of multidimensional separation methods and hyphenated techniques. The combination of analytical techniques is especially powerful as it utilises the advantages of each technique in order to determine identity and purity. Figure 1.1 shows the examples of hyphenated techniques. The coupling of GC and MS in the 1960s was the first successful attempt of the new

era of analysis [15]. Using MS or NMR as the detection method potentially affords a higher throughput due to the ability of these techniques to selectively and simultaneously detect multiple components. With the development of soft ionisation methods, such as electrospray ionisation (ESI) and matrix-assisted laser desorption/ionisation (MALDI), the success of MS has spread to the fields of biomedical and biological research [16]. Currently, the combination of MS with different chromatographic methods offers some of the most powerful techniques for pharmaceutical analysis [17]. HPLC-MS has proven to be one of the most valuable techniques and has been applied to the analysis and identification of impurities and degradation products in pharmaceuticals [18], the analysis of chiral impurities [19], the study of drug metabolism [20], high-throughput analysis of drugs and metabolites [21].

HPLC-DAD	HPLC-NMR
CE-DAD	CE-NMR
HPLC-Fluorescence	HPLC-NMR-MS
CE-Fluorescence	HPIC-ICP-SFMS
GC-MS	CE-ICP-SFMS
HPLC-MS*	GC-ECD/ICP-MS
CE-MS*	HPLC/HPLC-MS/MS

*** MS with different ionisation techniques, such as ESI and TSP.**

Abbreviations: DAD – diode array detector, HPIC – high-performance ion chromatography, ICP – inductively coupled plasma, SFMS – sector field mass spectrometry, TSP – thermospray, ECD – electron capture detector.

Figure 1.1. Examples of combination analytical techniques.

1.1.4 High-throughput analysis

The principal aim of the pharmaceutical sector of accelerating drug discovery and ultimately reducing the cost associated with bringing a new medicine to the market determined the expansion of the area of high-throughput analysis [22]. In order to identify the most suitable drug candidates, it has been recognised that investigation of absorption, distribution, metabolism and elimination (ADME) should be initiated at an early stage of the discovery process [23]. This allows unsuitable compounds to be eliminated and thus reduces the development cost. Due to the large number of unsuitable compounds that are now routinely identified from screening compound collections and gene family compound libraries, the industry has recognised the need for high-throughput ADME assays [23]. High-throughput techniques have been employed in the determination of structure, purity and quantitative measurements [24]. Table 1.1 summarises the techniques involved in high-throughput pharmaceutical analysis. The majority of current protocols for high-throughput analysis are based on 96 microtiter well plate technology where a large number of samples can be processed in parallel. For example, only 5 min is required to analyse the entire 96-well sample plate (3.1 sec per sample analysis) by FIA-MS. Assay formats such as 1536 well plate are becoming more acceptable in ultrahigh-throughput analysis [26].

1.1.5 Purity analysis

HPLC is a universal method for high-throughput purity analysis. The increase in throughput is due to the reduction of cycle times and the development of generic analytical methods [24]. The advances in HPLC technology have been in response to the demand of today's pharmaceutical industry for high-speed analysis [27]. Small quantities of impurities and degradation products present in the bulk drug require very sensitive and specific detection; thus, MS detection is often employed in combination with HPLC separation [18, 28]. HPLC-MS is also very powerful for high-throughput purity analysis [28]. A gradient packet column supercritical fluid chromatography (pcSFC) has shown 10 times faster analysis than HPLC [29]. This can be achieved in SFC due to the high flow rates available

with the low viscosity mobile phase (such as supercritical fluid). A pcSFC has been popular for enatiomeric separations. For example, 2.3 min was required for a single pcSFC analysis of R- and S-enantiomers of ketoprofen in a 96-wellplate demonstrated a significant time saving for analysis compared to the HPLC method [30].

Table 1.1. Summary of high-throughput analytical techniques [adapted from 15].

Analytical Technique	Relative throughput[a]	Structural analysis[a]	Purity Analysis[a]	Quantitative Analysis[a]
FIA/DI-MS	+++	+	+	+
MALDI-FT-MS	+	++	N/A	N/A
DI-NMR	++	+++	++	N/A
HPLC-UV	++	N/A	++	N/A
HPLC-UV/MS	++	+	++	N/A
HPLC-ELSD	++	N/A	++	++
HPLC-NMR	+	+++	++	+
FIA-CLND	+++	N/A	N/A	+
HPLC-CLND	++	N/A	N/A	+++
SFC-UV	++	N/A	++	N/A
SFC-MS	++	+	++	N/A
ESI-FT-ICR-MS	+	++	N/A	N/A

[a] An indication of relative throughput and applicability for structural, purity and quantitative analysis of the analytical techniques (+++ = highest).
Abbreviations: CLND, chemiluminescent nitrogen detection; DI, direct injection; ELSD, evaporative light-scattering detection; FIA, flow injection analysis; FT, Fourier Transform; ICR, ion cyclotron resonance; MALDI, matrix-assisted laser desorption/ionisation; SFC, supercritical fluid chromatography.

1.1.6 Quantitative analysis

Quantitative analysis in the pharmaceutical industry can be accomplished in a high-throughput mode. Evaporative light scattering detection (ELSD) in combination with HPLC separation is an attractive quantitation tool [24], because detection depends on the mass of analyte, rather than its absorbance or ionisation efficiency [31]. However, the detection of volatile and low-melting compounds is not possible with ELSD as the detection is performed after the evaporation of the solvent [15]. Accurate quantification at high-throughput scale can be provided by chemiluminescent nitrogen detection (CLND) with FIA or HPLC analysis [24]. CLND response is related to the number of nitrogen atoms in the sample. FIA-CLND provides good linearity but its use is restricted when nitrogen-containing impurities and solvents are present [15].

1.1.7 Miniaturisation

The need for high-throughput analysis and point-of-care testing has determined the interest in miniaturisation of analytical processes [32]. The requirements for minimal space used, portability, ease of manipulation, inexpensive manufacturing cost, minimal sample and reagent consumption have determined the attractive features of miniaturised analytical system. The possibility of high-throughput performance of microscale devices has resulted in the development of high-density arrays of microreactions wells for pharmaceutical analysis [33]. The microplates with large number of smaller wells (from 192 to 20000) have been reported at miniaturised level [33]. An example of a 1536-microwell plate is shown in Figure 1.2.

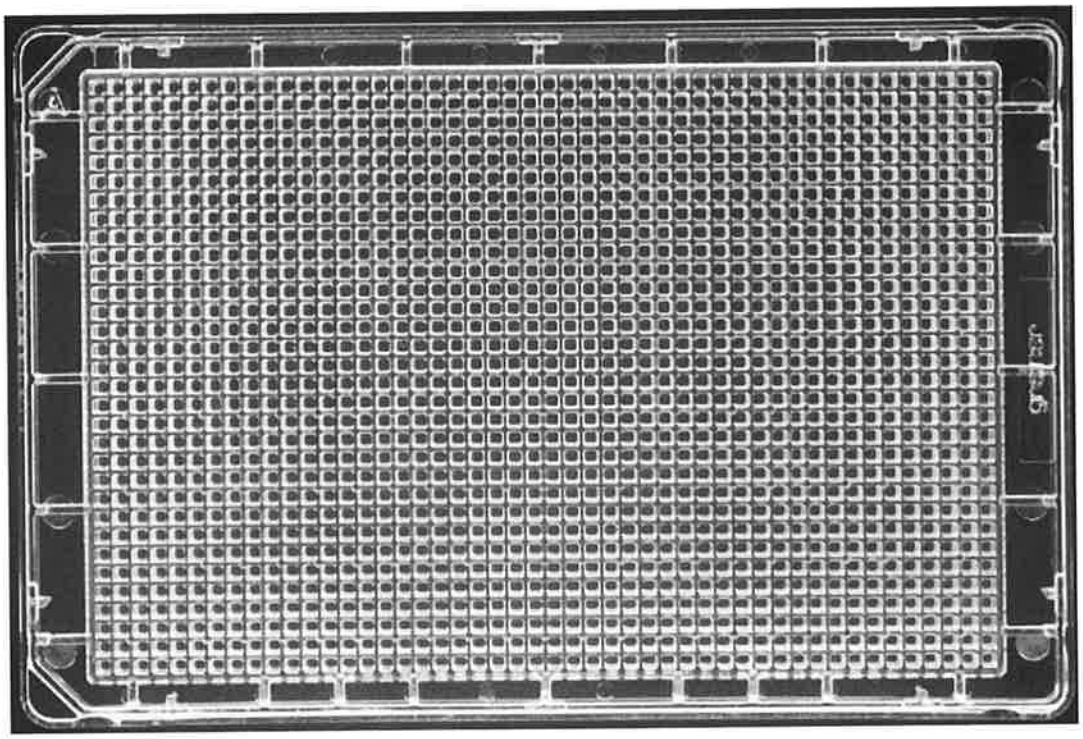

Figure 1.2. 1536-microwell plate [adapted from 33].

The performance of analysis on a microchip platform has shown potential in pharmaceutical analysis [33]. The microchip consists of microchannels and microchambers that are responsible for intra-chip transfer of fluid or electrophoretic separations and function as posts and dams for separation and isolation [15]. A typical size of the microchip is 1.5 cm x 1.5 cm and a few millimetres in thickness. The timesaving benefits of microchip technology in pharmaceutical analysis can be demonstrated by the example of the determination of lincomycin in urine by microchip capillary electrophoresis with electrochemiluminescence (ECL) detection, which was performed within 40 sec [34].

1.1.8 Nanotechnology

Nanotechnology has found its application in pharmaceutical analysis. Nanobiosensors have employed enzymes, antibodies, receptors and molecular imprints in order to recognise analytes [15]. Individual chemical species can be detected using nanoprobes and nanobiosensors [35]. Nanotubes are employed for the extraction of pharmaceuticals prior to analysis. For example, carboxylated

single-walled carbon nanotubes (cSWNT) can be a good alternative to the solid phase extraction (SPE) of non-steroidal anti-inflammatory drugs [36]. Bio-nanotubes utilise the selectivity of an antibody to a single enantiomer and thus can be used in chiral analysis [37]. Various nanotechnologies have been applied in a lab-on-a-chip format [38]. At this scale of analysis it is possible to introduce attoliters of sample and achieve zeptomole detection limits [39]. Nanobiosensors, nanosamplers, cell orienters, nanoanalysers, nanoarrays and nanofluidics are being recognised as biological tools of the future [15].

Faster analysis, higher throughput and minituarised technology are the focuses of modern pharmaceuticals analysis. Only time and experience will tell whether the new technologies and advances have delivered their promises of potential benefits [15].

1.2 HPLC IN PHARMACEUTICAL ANALYSIS

It is difficult to underestimate the importance of HPLC for pharmaceutical analysis. It is used in the determination of analgesic, antibiotic, anti-viral, antihypertensive, anti-depressant, gastro-intestinal and other drugs, the classification of which is summarised in Figure 1.3 [40]. In order to explain the predominant position of HPLC it is necessary to reveal features determining its suitability and attractiveness to the pharmaceutical analysis. The separation mechanism in chromatography is based upon the distribution of analytes between two phases, a stationary and a mobile phase [41]. About 75 % of current HPLC analyses are performed in reversed-phase mode [14], where the retention is based on distribution between a nonpolar stationary phase and a polar mobile phase.

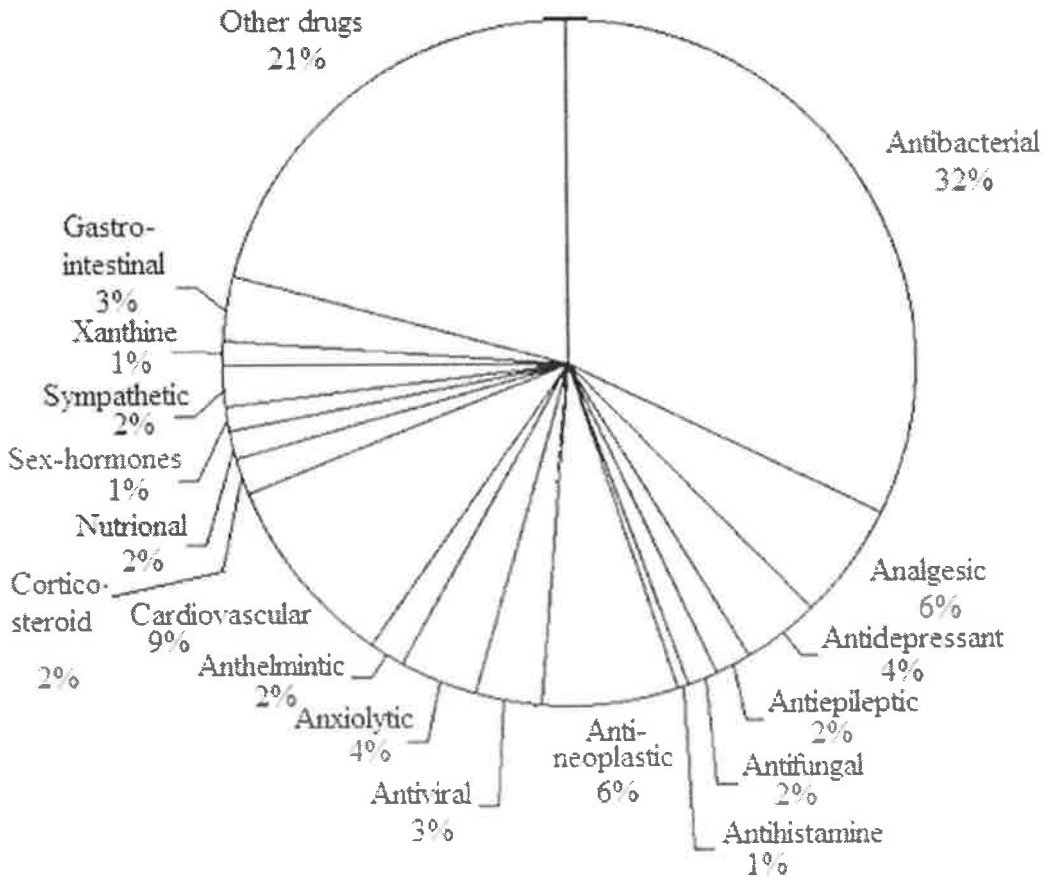

Figure 1.3. Classification (based on analytical abstracts) of drugs analysed by HPLC during 1995-2001 [adapted from 40].

HPLC offers high resolution, good reproducibility, high sensitivity and low limits of detection. The drawbacks of the technique include the relatively long time of analysis, low efficiencies of separation, the generation of excessive amounts of solvents used as mobile phase, large volumes of sample required for analysis and the substantial cost of the equipment. The demand for high quality separation and high sample throughput in the laboratory has determined the development of ways to increase the speed and efficiency of analysis without damaging the resolution. Such advantages of HPLC technology include the use of monolithic columns, small particle columns, high pressure and high temperature [27]. Varying stationary phase, the composition of mobile phase, gradient elution, temperature and the choice of the detector allows the separation to be achieved.

1.2.1 Separation in HPLC

The majority of the methods in pharmaceutical analysis employ reversed phase HPLC with silica-based bonded C_8 and C_{18} alkyl stationary phases [27]. Generally, mobile phase is supplied onto the column with a flow rate of 1 mL min^{-1}. With these conditions, the separation of pharmaceuticals can result in an analysis time of several minutes, such as the determination of the active substance indomethacin and its two impurities which lasted just under 7.5 min [42]. In the case of complicated mixtures, gradient elution is required. This however adds to the complexity of the analysis and also requires a significant amount of time. For example, the analysis of 19 antiretroviral agents on a C_{18} column with gradient of the phosphate buffer and acetonitrile resulted in 50 min of analysis time [43].

1.2.2 Detection in HPLC

The choice of suitable detection for HPLC separations is of great importance in order to ensure that all components are detected. While using UV detection, the multiple wavelength detector or a photodiode-array detector allow the detection of all components. Although the UV detector is widely employed in pharmaceutical analysis [40], it does not allow limit of detection (LOD) values as

low as some other more sensitive detection techniques. For example, the HPLC-UV determination of anti-inflammatory pharmaceuticals (naproxen, ketoprofen, ibuprofen, diclofenac, piroxicam, nimesulide and paracetamol) was characterised by LODs from 36 to 8300 ng mL^{-1} [44]. When employing ESI-MS with the same separation, the LODs were in a range of 7 – 200 ng mL^{-1}. Fluorescence, refractive-index and electrochemical detectors are employed for specific applications [40].

1.2.3 Method validation

HPLC methods are usually highly robust. Thus, precision in the HPLC method for the determination of antiretroviral agents was less than 1% [43]. In the method for the determination of anti-inflammatory pharmaceuticals [44] it was reported to be less than 1.5%. HPLC offers less separation efficiency than other separation techniques such as CE, GC or SFC [14]. The separated peaks are generally characterised by several thousand theoretical plates. For example, in the HPLC method the efficiency of indomethacin was 7655 theoretical plates, while the efficiencies of the two impurities were even lower [42]. The low efficiency of HPLC performance restricts the amount of compounds that can be separated in a single run. Typically, it is difficult to separate more than 20 compounds in HPLC and gradient elution is usually required [45].

1.2.4 HPLC for the separation of chiral pharmaceuticals

One of the main challenges of pharmaceutical analysis is the enantiomeric separation and quantification of drugs [1]. The resolving power of HPLC has been employed in the determination of the enantiomeric purity of therapeutic drugs, which are used as pure enantiomers, and the analysis of racemic mixtures in biological samples. The employment of chiral stationary phases in HPLC columns is one important approach to this problem. Thus, stereoisomers of the non-steroidal anti-inflammatory drug 4-dihydroflobufen lactone were successfully separated on a hydroxypropyl-β-cyclodextrin stationary phase [46]. Additionally, the chiral modifier can be added to the mobile phase. HPLC separation of doxazosin enantiomers was reported using carboxymethyl-β-cyclodextrin as an

additive in mobile phase [47]. Chiral analysis of pharmaceuticals is the area explored extremely in CE, this will be discussed later.

Acknowledging the achievements of the HPLC in the current pharmaceutical analysis and recognising its limitations, i.e. time of analysis and separation efficiency, it is important for alternative methods of analysis to be given an opportunity to utilise their unique advantages in this area. This thesis explores the potential of capillary electrophoresis in the analysis of pharmaceuticals.

1.3 PRINCIPLES OF CAPILLARY ELECTROPHORESIS

1.3.1 Introduction

Capillary electrophoresis is a powerful analytical technique which combines the separation mechanism of electrophoresis and the instrumental concept of chromatography. The outstanding analytical strength of CE is in its vast resolving power, high speed of analysis and high efficiency of separations. Capillary electrophoretic methods have been developed for the separation of compounds ranging from small molecules [48, 49] to macromolecules [50, 51]. The analysis of pharmaceuticals using CE [52-54] has become increasingly popular in recent years.

The powerful mechanism of the technique is based on the principal that charged particles will migrate towards the opposite pole and separate from each other according to their physical characteristics. The differences in the separation mechanisms of CE and those of chromatography makes CE a potential alternative or complementary analytical technique to high performance liquid chromatography. Other attractive features of CE are minimal sample and reagents consumption, a fully automated process of separation and availability of different modes of operation to tackle a wide variety of separation problems.

In order to introduce capillary electrophoresis, the basic principles and instrumentation of the technique along with possibilities of analysis in different modes are revealed. Its application in the analysis of pharmaceuticals is explored.

1.3.2 Components of a CE system

The basic configuration of the CE system is presented in Figure 1.4. The narrow-bore fused silica capillary is the component of the system where the actual separation of analytes takes place. The internal diameter of the capillary of 10 – 100 μm provides efficient heat dissipation, which allows separation to be performed at high field strength. The ends of the capillary are placed in two buffer reservoirs along with two electrodes, which make electrical contact between the high voltage power supply and the capillary. After filling the capillary with buffer, a small amount of sample is introduced at one capillary end by an electrokinetic or hydrodynamic technique. Separations are performed in an electric field by applying high voltages of between 5 and 30 kV along the capillary, typically followed by the detection on-column with a sensor placed at the outlet end [55].

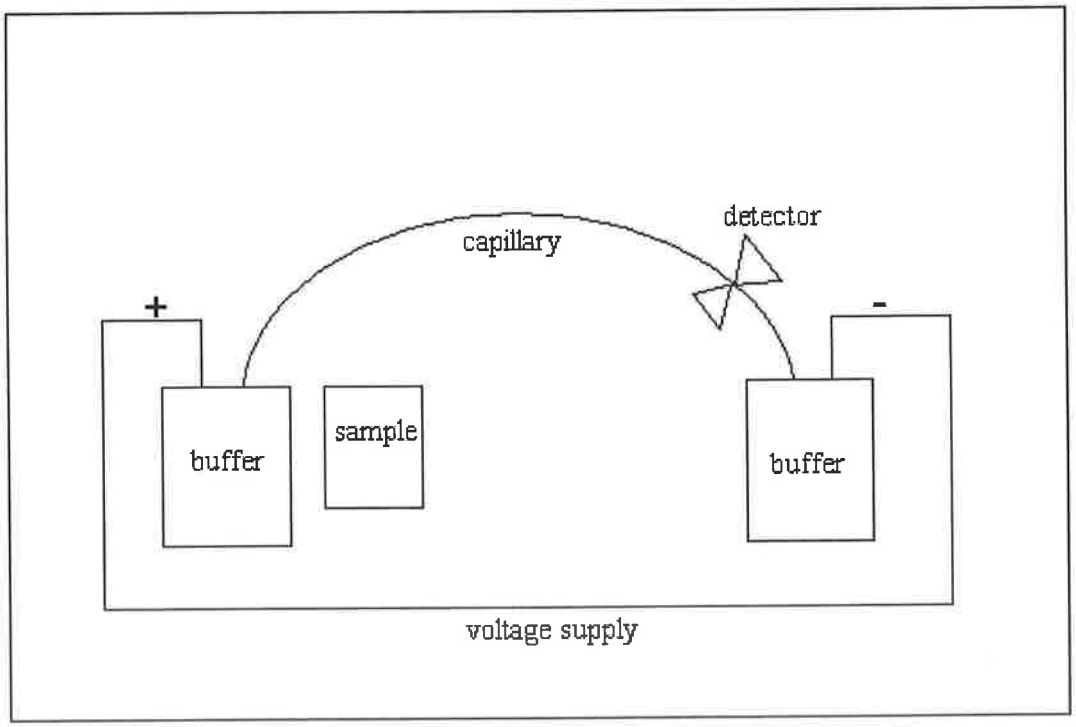

Figure 1.4. Basic configuration of the CE system.

1.3.3 Electrophoretic migration

The difference in the velocities of analytes in an electric field is the reason for their separation in capillary electrophoresis. The velocity of the solute ion can be described by Equation 1.1 [55]:

$$\mathbf{v} = \mu_e \mathbf{E} \qquad \textbf{Equation 1.1}$$

where $\mathbf{v}$ = ion velocity, μ_e = electrophoretic mobility, and $\mathbf{E}$ = applied electric field.

Mobility is related to physical parameters by the following equation:

$$\mu_e = \frac{q}{6\pi\eta r} \qquad \textbf{Equation 1.2}$$

where $\mathbf{q}$ = ion charge, η = solution viscosity, $\mathbf{r}$ = ion radius.

This equation shows that small, highly charged species have high mobilities; at the same time large, minimally charged species have low mobilities. The electrophoretic mobilities can be found in tables as physical constants, which determine when the analyte is fully charged and infinite dilution is assumed. Mobilities that are determined experimentally – effective mobilities – are different and depend on pH and the composition of the buffer.

1.3.4 Electroosmotic flow

Electroosmotic flow (EOF) is a central point of CE separation. The walls of a fused silica capillary contain silanol groups, which are ionised above pH 4 [55]. The deprotonated capillary walls have a net negative charge and when in contact with aqueous solution, an excess of counterions is immobilised in the Stern layer next to the charged surface. At the same time, a mobile excess of counterions forms the diffuse layer (Gouy layer) [56]. These two layers represent an electric double layer as shown in Figure 1.5. The border line between Stern layer and Gouy layer is called the shear plane. The electric potential at the shear plane is defined to be zeta potential [57].

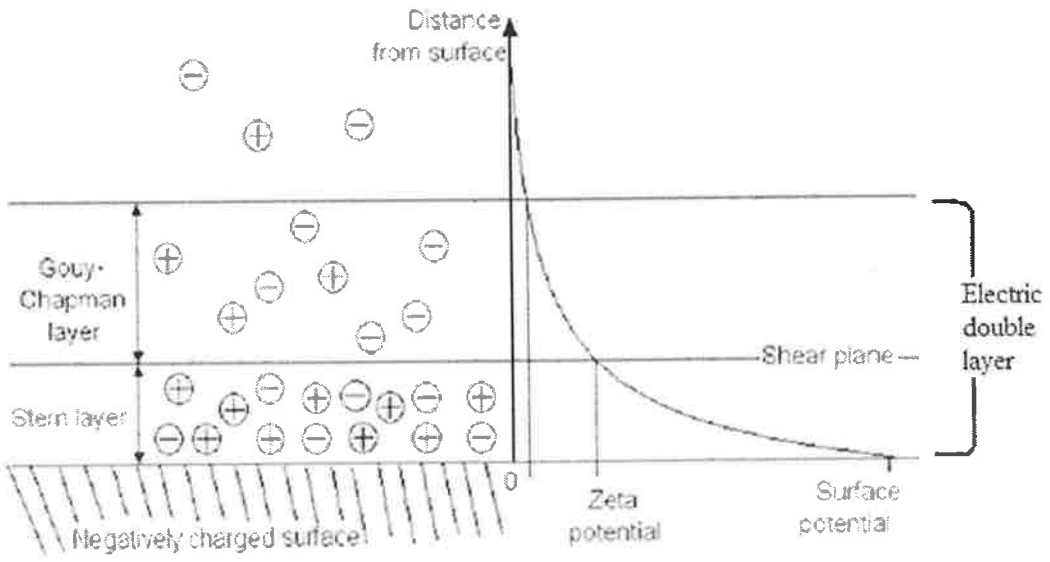

Figure 1.5. Schematic representation of the electrical double layer [adapted from 58].

When an electric field is applied, the positive ions at the capillary wall migrate towards the cathode. This motion of the excess of counterions of the electric double layer results in a bulk flow within the capillary – electroosmotic flow (Figure 1.6). This bulk flow has a flat profile formed by the uniform distribution of the migrating solute ions along the capillary and in conventional systems moves towards the cathode [59]. EOF causes movement of all particles in the capillary: neutral species and ions are all drawn to the cathode regardless of charge. This phenomenon makes possible the resolution of charged and neutral species.

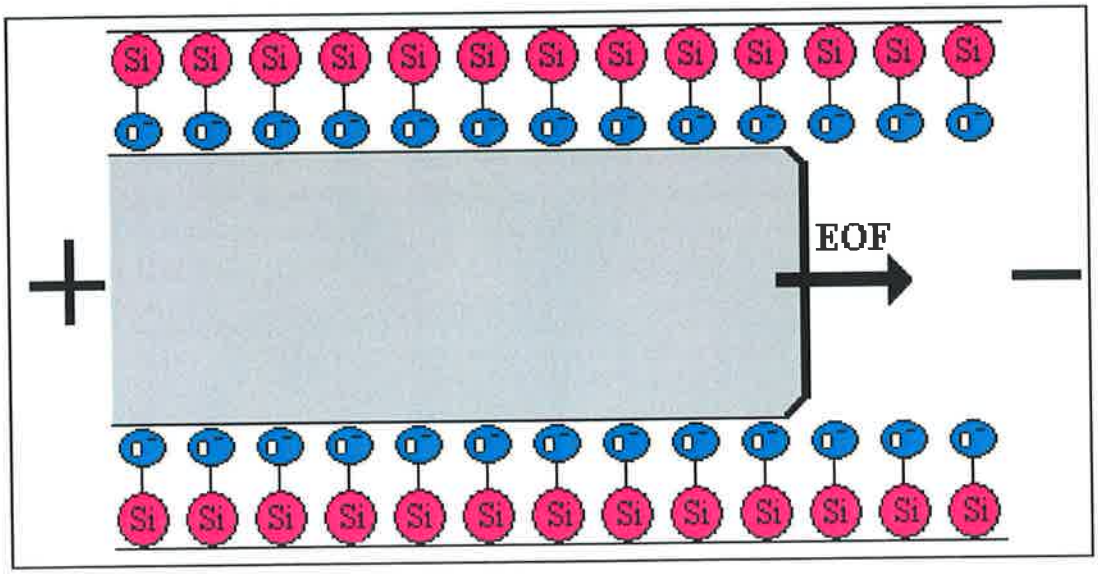

Figure 1.6. Representation of EOF in normal polarity with the detection at the cathode.

The magnitude of the EOF is related to the zeta potential and can be defined in terms of velocity or mobility [55]:

$$v_{EOF} = \frac{\varepsilon\zeta}{\eta} E \qquad \textbf{Equation 1.3}$$

then

$$\mu_{EOF} = \frac{\varepsilon\zeta}{\eta} \qquad \textbf{Equation 1.4}$$

where $v_{EOF} = \mu_{EOF} E$, v_{EOF} = EOF velocity, μ_{EOF} = EOF mobility, ζ = zeta potential, ε = dielectric constant.

While EOF is usually beneficial, it is still essential to control it to realise the full potential of the technique. In order to decrease EOF, several methodologies can be employed, such as decreasing the pH of the run buffer and/or the applied voltage, increasing the ionic strength of the buffer and/or viscosity and modification of the run buffer with additives [55].

1.3.5 Analytical parameters

CE analytical parameters can be described in terms similar to those of column chromatography [55]. The solute mobility can be calculated using migration time and other experimental parameters:

$$\mu_a = \frac{l}{tE} = \frac{lL}{tV} \qquad \textbf{Equation 1.5}$$

where $\mu_a = \mu_e + \mu_{EOF}$, V = applied voltage, t = migration time, l = effective capillary length (to the detector), L = total capillary length.

The basis of separation in CE is the difference in solute mobility. This difference is essential to resolve zones from each other and depends on the length of the zones. Dispersive processes that occur in the capillary determine zone length. Dispersion of discrete zones in CE results from differences in solute

velocity within that zone. Dispersion of a peak due to molecular diffusion is described by Equation 1.6 [55].

$$\sigma^2 = 2Dt \qquad \textbf{Equation 1.6}$$

where σ = standard deviation of the peak (in time, length or volume), **D** = diffusion coefficient of the analytes.

The cause of the dispersion can be Joule heating, injection plug length and analyte adsorption to the capillary wall. The undesirable effect of Joule heating on a CE separation includes EOF variation and the resulting reproducibility problems and also the formation of a parabolic flow profile, which can impact upon peak separation efficiency. Application of lower voltage and capillaries with smaller internal diameter will reduce the amount of heat produced and induce heat dissipation in the system.

A flat flow profile in CE results in high separation efficiencies. The efficiency, expressed in numbers of theoretical plates is:

$$N = \frac{\mu_e V l}{2DL} = \frac{\mu_e E l}{2D} \qquad \textbf{Equation 1.7}$$

From this equation, it is evident that at high field, the solute spends less time in the capillary and has less time to diffuse. This equation shows that the dispersion of large molecules (such as protein) that have a low diffusion coefficient is less than that of small molecules.

The theoretical plate number can be determined from the electropherogram:

$$N = 5.54\left(\frac{t}{w_{1/2}}\right)^2 \qquad \textbf{Equation 1.8}$$

where $w_{1/2}$ = peak width at half height.

Resolution of analytes is the main goal of a separation method. Peak size and shape may affect the resolution [60]. Resolution is determined from electropherogram as:

$$R = \frac{2(t_2 - t_1)}{(w_1 + w_2)} \qquad \textbf{Equation 1.9}$$

where **w** = baseline peak width (in time).

The effect of EOF on resolution can be described as

$$R = \left(\frac{1}{4\sqrt{2}}\right)(\Delta\mu)\sqrt{\left(\frac{V}{D(\bar{\mu} + \mu_{EOF})}\right)} \qquad \textbf{Equation 1.10}$$

where $\Delta\mu = \mu_2 - \mu_1$ and $\bar{\mu} = (\mu_2 + \mu_1) / 2$.

Resolution is shown to be better with longer capillaries and higher voltage applied. But generation of Joule heating and long times of analysis should be taken into account [60]. High EOF provides better resolution of peaks migrating in the opposite direction to EOF [61].

1.4 VERSATILITY OF CE

One of the advantages of CE is that it is a very flexible technique; it can be adjusted for the separation of a variety of compounds due to its numerous modes of operation. Different modes of CE separations can be performed using a standard CE instrument.

The distinct capillary electroseparation methods include capillary zone electrophoresis (CZE), capillary gel electrophoresis (CGE), micellar electrokinetic chromatography (MEKC), capillary electrochromatography (CEC), capillary isoelectric focusing (IF), capillary isotachophoresis (ITP) [62]. Some of these and other method development options that are of particular interest for this work are explored below.

1.4.1 Capillary zone electrophoresis

Capillary zone electrophoresis is the simplest mode of CE mainly because the capillary is only filled with a buffer. Separation occurs because analytes migrate in discrete zones at different velocities due to the difference in their mass-to-charge ratio. Separation of small and large molecules is performed, including the smallest molecules with slight mass-to-charge ratio differences. Cationic and anionic analytes can be separated in CZE. Separation of neutral analytes from each other is not allowed, they all comigrate with the EOF.

A wide range of buffers can be employed in CE. The most common are shown in the Table 1.2 [55]. The role of the buffer is to provide precise control of pH. This is important due to the sensitivity of mobility and electroosmosis to changes in pH [63]. The buffer also gives ionic strength, which is essential for electrical continuity. The run buffer should meet several criteria, such as good buffering capacity at chosen pH, low absorbance at the wavelength of detection, low mobility (implying large, minimally-charged ions) to minimize current generation [55].

Table 1.2. Commonly used buffers [55].

Buffer	pKa
Phosphate	2.12 (1)
Acetate	4.75
2-(N-Morpholino)-ethanesulfonic acid (MES)	6.15
Piperazine-N,N`-bis(ethanesulfonic acid(PIPES))	6.80
Phosphate	7.21 (2)
N-2-Hydroxyethylpiperazine-N`-2-ethanesulfonic acid (HEPES)	7.55
Tris-(hydroxymethyl)aminomethane (Tris)	8.30
Borate	9.24
Cyclohexylamino-1-propane sulfonic acid (CAPS)	10.4
Phosphate	12.32 (3)

The pH value of the run buffer controls the degree of ionisation of the silica and this is why it influences the magnitude of EOF in CE. The net negative charge on the capillary wall increases at high pH. The EOF generated by alkaline pH values will therefore be greater than that at lower pH values, resulting in a faster separation. In normal polarity, cations will possess the highest velocity due to electrophoretic attraction to the cathode and the same direction as the EOF. The velocity of anions is lower than that of EOF as their electric attraction in the opposite direction to the EOF [64].

The pH range where buffer shows maximum capacity depends on its pKa value [65]. Generally the working range of a buffer is limited to ±1 pH of its pKa [55]. Operation outside of that range must be accompanied by frequent buffer replacement to avoid pH changes [66]. Table 1.2 contains pKa values of some buffers typically used in CE. Phosphate and some other buffers are polybasic and thus they possess a greater working range. It should be noticed that there has been a strong preference in CE to use buffers with pH either below 3 or above 8 [66]. EOF is very low below pH 3 and a change of electroosmotic flow with pH is minimal above pH 8. Between these pH values electroosmotic flow changes

extensively with pH and the design of a stable reproducible system using uncoated capillaries is very problematic.

The typical buffer concentration ranges from 10 mM to 100 mM [63]. Separation is faster when dilute buffer is employed, but the sample loading capacity is reduced. An increase in the concentration, and therefore ionic strength, of the run buffer, leads to compression of the double layer. In this situation, the zeta potential decreases and therefore the EOF decreases also [55], resulting in longer analysis time due to increased migration times.

The advantage of zwitterionic buffers (CAPS, MES, Tris) is low conductivity and thus, low current generation upon application of high voltage and reduced Joule heating that allows use of buffers with higher concentration.

1.4.2 Micellar Electrokinetic Chromatography

Micellar electrokinetic chromatography presents a hybrid of electrophoresis and chromatography. Terabe [67] introduced this approach in 1984 and now it is the most widely used mode of CE technique. The main advantage of MEKC is that it can be used to separate neutral solutes and charged solutes in a single run. The separation is achieved by the addition of surfactants to the buffer system.

1.4.2.1 Micelles

Surfactants are surface-active agents; they contain groups of opposite polarity that have solubilising tendencies in an aqueous solution [57]. In an aqueous solution at a concentration above the critical micelle concentration (CMC) [68], surfactant molecules spontaneously organise into aggregates called micelles. This form of molecular organisation occurs due to hydrophobic and electrostatic effects and leads to lowering of the free energy of the system. The hydrophobic tails of the surfactant are oriented towards the centre and the polar heads are oriented towards the buffer [69].

1.4.2.2 Mechanism of separation

In the CZE, neutral compounds simply move with EOF. When anionic or cationic surfactant is added to the run buffer, another separation mechanism comes into play. This is shown in Figure 1.7. Hydrophobic analytes solubilise in the hydrophobic part of the micelle. This encapsulation – de-capsulation process is very rapid and in the time of separation, may be viewed as an instantaneous molecular equilibrium [66]. The greater the time that analyte is incorporated with micelle, the longer the migration time will be. The time that molecules of analyte spend with micelles depends on the strength of their interaction with the micelle: the interaction is stronger in case of more hydrophobic compounds. Anionic surfactants migrate toward the anode (the opposite direction to the EOF). Generally EOF is faster than the migration velocity of the micelles (at neutral or

basic pH) and thus the movement of all the particles in the capillary is toward the cathode [55].

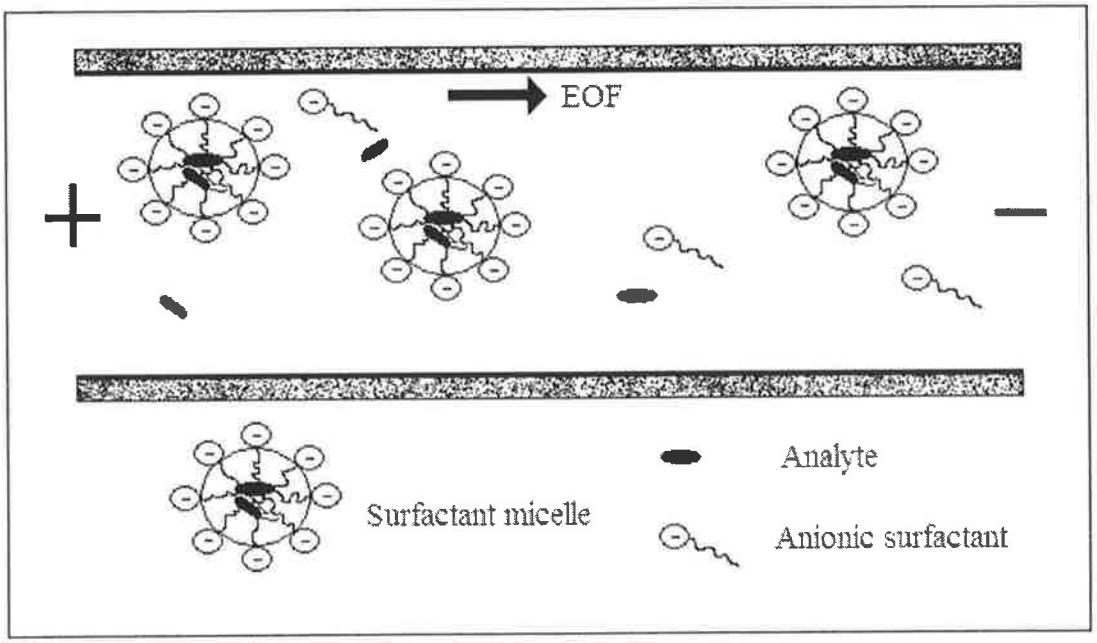

Figure 1.7. Mechanism of separation in MEKC.

By modifying the nature of the micelles, the possibilities of capillary separation can be widened [70]. Increasing the partition of hydrophobic compounds in the aqueous phase [71] helps the separation. The use of cyclodextrins frequently leads to success but at the same time adds some complexity, especially in interpretation. Organic solvents in a buffer affect the stability of micelle-analyte agglomerates [72]. Some publications have reported separation with polymeric pseudostationary phases [73].

1.4.2.3 Surfactants

The most common anionic surfactant used in MEKC is sodium dodecyl sulphate (SDS); its chemical structure is shown in Figure 1.8. It is very water-soluble and has a high degree of lipid-solubilising power. SDS does not absorb in the low UV range of spectrum [74] and gives a stable baseline. The CMC of SDS in water is 8.2 mM, but this magnitude is different in a buffer solution. SDS forms

molecular aggregations from 63 molecules [75]. The aggregate surface has a net negative charge. When the analyte is negatively charged and the concentration of anionic surfactant is high, the solubilisation of analyte may be difficult due to the possibility of charge repulsion from the negatively charged micelles [67].

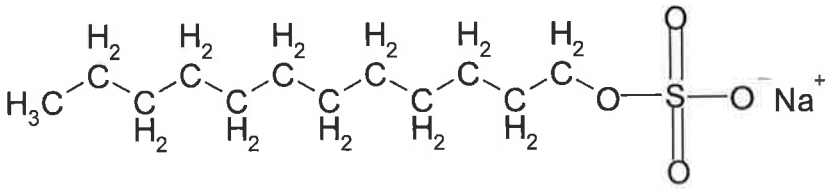

Figure 1.8. Chemical structure of SDS.

Dodecyltrimethylammonium bromide (DTAB) and cetyltrimethylammonium bromide (CTAB) represent the cationic surfactants used in MEKC. Their CMC are 14 mM and 1.3 mM, respectively [55]. When a cationic surfactant is added to the buffer, the migration time of analyte will be short due to micelle-analyte attraction to the cathode by both electrophoresis and EOF. High concentration of cationic surfactant might neutralise the negative charge on the capillary wall. Increasing surfactant concentration leads to the appearance of a bilayer of charge, which gives the capillary wall a positive charge. This might reverse the direction of EOF [55]. In this situation, detection must be allowed at the anodic end of the capillary. Reversal of charge takes place at the concentration of surfactant well below the CMC [62]. The use of surfactants molecules with long alkyl chains gives a narrow elution window [76].

The separation of alkylxantines was reported using SDS surfactant [77, 78]. CTAB was employed in the separation of pesticides [79].

1.4.3 Chiral separation in CE

Chiral separation is an active area of research in gas chromatography, liquid chromatography and CE. It is important for organic chemistry, medical research, environmental control, and drug and food industries. Often pharmacological activity of enantiomers can be different because many biological target sites are stereoselective.

1.4.3.1 Chiral selectors

In CE the role of chiral selectors can be played by cyclodextrins [80, 81], derivatized cyclodextrins [81, 82], optically active micelles [83], macrocyclic antibiotics [84], ligand exchangers [85], glycosaminoglycans [86], peptides [87] and crown ethers [88]. They are usually added straight to the run buffer [80-82] or in some cases they can be bound to the capillary wall [89] or included into gel [90].

1.4.3.2 Cyclodextrins

Cyclodextrins are the most widely used chiral selectors in CE. These compounds are composed of D-glucose units connected to a ring by 1,4-linkages (Figure 1.9). The cavity diameter depends on the number of glucose units: 6, 7 and 8 glucopyranose units are referred to as α-, β-, γ-CD, respectively. The shape of CD's molecule represents a hollow truncated cone (Figure 1.9) with wide and narrow hydrophilic ends delineated by O(2)H and O(3)H secondary and O(6)H primary hydroxyl groups [91, 92]. Table 1.3 contains some physical properties of CDs. The interior of the CD is relatively hydrophobic due to the orientation of the carbon atoms toward the inside space of the cyclodextrin ring while the external surface is hydrophilic due to the sugar's hydroxyl groups which are directed outside of the ring [93]. α-CD is used to separate single-ring aromatic solutes with few side chains [94, 95]. One – to two-ring aromatic compounds are best separated with β-CD [96, 97], whereas γ-CD performs separation of larger molecules [98].

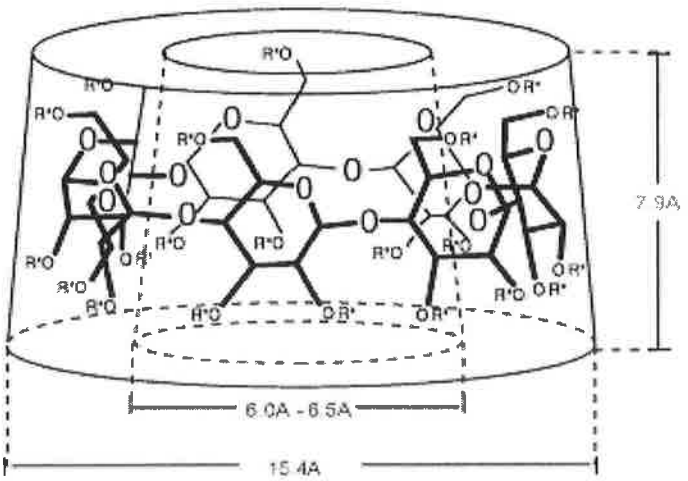

Figure 1.9. Toroid structure of cyclodextrin (β-CD) [99].

Glucose is optically active, that is why the surface of this "ring of hexoses" has the same property and chiral recognition is performed. Hydrophobic groups of aromatic compounds or cycloalkanes can enter the cone and make an inclusion or host-guest complex. The interplay of atomic (Van der Waals), thermodynamic (hydrogen bonding) and solvent (hydrophobic) forces results in the establishment of stable inclusions of analytes and cyclodextrins [99]. It is possible for the solute to sit on the opening of the CD [62]. On the other hand, privileged interaction with one of the enantiomers is facilated by the surface optical selectivity of the cyclodextrin. After such chiral interaction the mobility of that enantiomer has changed and the separation of enantiomers is performed.

Table 1.3. Some characteristics of CDs [100].

Parameter	Type of CD		
	α-CD	**β-CD**	**γ-CD**
M (g/mole)	972	1135	1297
Diameter of cavity (Å)	4.7 – 6.0	8.0	10.0
Volume of cavity ($Å^3$)	176	346	510
Solubility (g/100 ml, 25°C)	14.50	1.84	23.20
pKa assigned to O(2)H and O(3)H [107, 108]	12.33	12.20	12.08

1.4.3.3 Cyclodextrin derivatives

Due to the relatively low solubility of β-CD and inability of native CDs to separate all enatiomers, CDs have been functionalised with additional moieties. The most widely employed substitute groups are hydroxypropyl, succinyl, acetyl, sulfobutyl and sulphate. Any modification at the cyclodextrin ring influences the steric fit [93] and, therefore, the affinity of an analyte to the CD. Differences in the steric fit lead to differences in interaction times of analytes with CD. This in turn leads to different mobilities of enantiomers or isomers, and thus to separation. Charged CDs have been used in CE separations of natural analytes [103]. The sulphoalkyl ether derivatives of β-CD have been used in CE as they exhibit increased solubility in water [104, 105].

The aggregate substitution of additional moieties per glucopyranose unit is called a degree of substitution (DS). For example, a DS of 5 means a distribution of an average of five substitute groups on the CD molecule. Nuclear magnetic resonance is employed to determine the degree of substitution [99]. Molar substitution shows the average number of moles of substitute per mole of glucopyranose.

1.4.3.4 Mechanism of separation

The mechanism of chiral separation is based on differences in the stability of the complexes between the analyte and CD. The model of Wren and Rowe was developed for chiral separation with CDs [106-108]. Separation will not take place if the solutes spend either too little or too much time attached to the CD. In this case, the type of CD or concentration must be changed. The addition of an organic solvent can be useful if solutes are bounded too strongly. Figure 1.10 illustrates the mechanism of separation when CZE is modified with CDs. Neutral solutes can be separated with charged CDs and neutral CDs can be used for the separation of charged analytes. Application of high concentration of charged CDs for the resolution of the same charged solutes may result in charged repulsion of the analytes from the CD, altering the nature of the inclusion. When using a charged CD such as sulfobutylether-β-CD (SBE-β-CD), the anionic CD migrates against the EOF in a similar way to the SDS micelle [62]. In this situation, the CD can represent a slowly moving "phase" in electrokinetic chromatography. However, micelles are much more effective for this purpose.

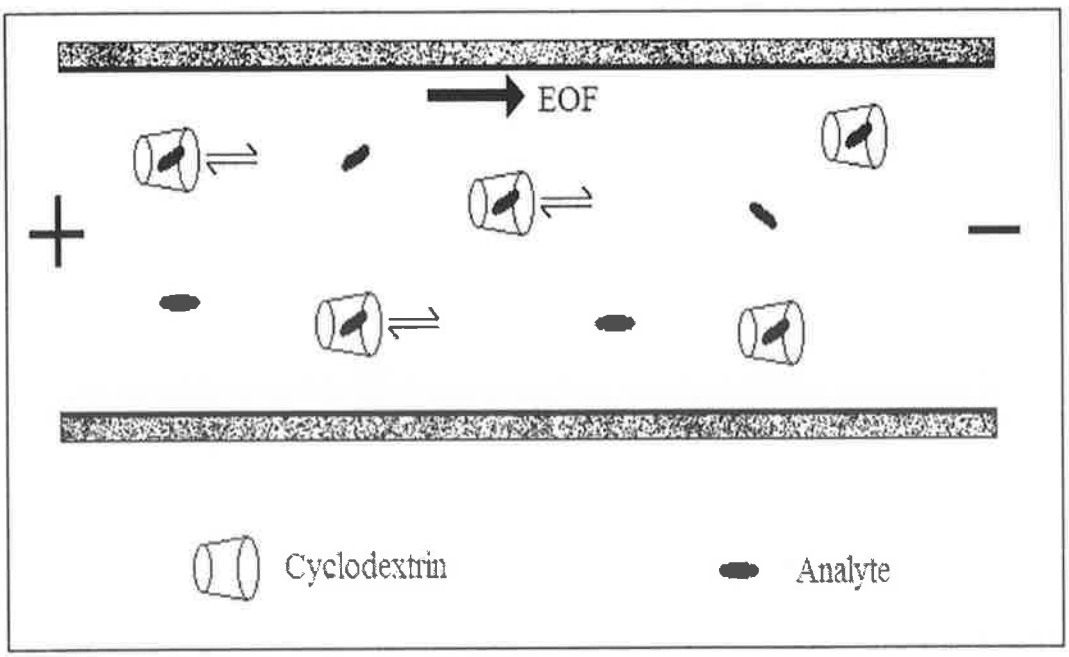

Figure 1.10. Mechanism of separation with cyclodextrin.

1.4.3.5 CD-modified MEKC

Due to the differences in the nature of analyte – micelle and analyte – cyclodextrin interaction, the combination of micelles and cyclodextrins is particularly powerful [62]. The mechanism of CD-MEKC separation is illustrated in Figure 1.11. For the separation to occur the analytes must be sufficiently distributed between the CD-modified micellar phase and the aqueous solution. If the analyte interacts only with micelle or totally included into the CD cones, no separation will be performed [109]. The following separation is based on differences in a solute's partition coefficient between the micelle and the CD. However, nonpolar compounds can demand the addition of high concentrations of surfactants and organic modifiers to control analytes solubility and help resolution of structurally similar molecules. The addition of an organic solvent solubilises the hydrophobic analytes in the aqueous buffer, lowering the interaction of analytes with the micelle and CDs [67]. High concentration of surfactant can result in extensive current generation and time of analysis can increase [110].

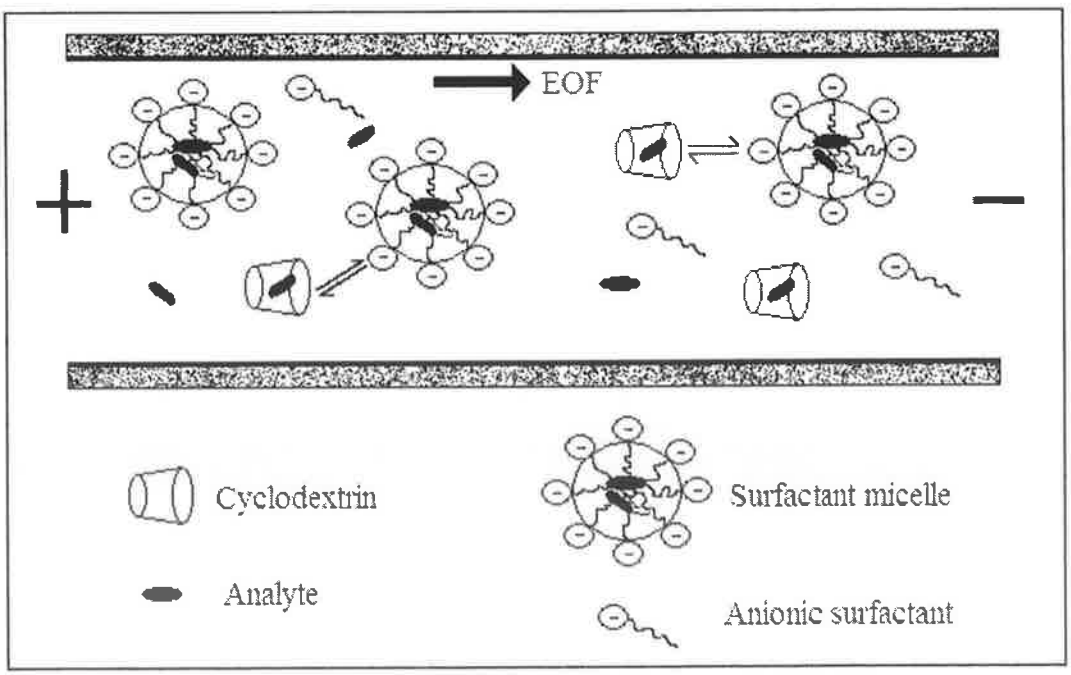

Figure 1.11. Mechanism of CD-MEKC separation.

1.4.4 Microemulsion electrokinetic chromatography

1.4.4.1 Microemulsions

Microemulsions are solutions of nanometre-sized droplets dispersed throughout another, immiscible, liquid [111]. The characteristics of microemulsions include optical transparency, thermodynamic stability and high solubilisation power [112]. Oil-in-water microemulsions are usually employed in microemulsion electrokinetic chromatography. Since it was first introduced in 1991 for the analysis of fluorescent aromatic compounds [113] it has found application in the separations of water soluble and insoluble vitamins [112, 114-116], water insoluble steroids [117], water insoluble pesticides [118] and a range of pharmaceuticals [114, 119-123]. While MEEKC has been principally known to have a great separation capability for highly hydrophobic compounds [113, 124, 125], it also was demonstrated to be a reliable separation tool for hydrophilic analytes [126, 127].

Figure 1.12 represents a schematic diagram of an oil-in-water microemulsion. The oil droplet (heptane or octane) acts as the core phase and is suspended in an aqueous buffer. Surfactant molecules, such as SDS, are added in the concentration greater than their CMC to facilitate droplet formation by lowering the surface tension [111]. A co-surfactant such as small alcohol molecules (butanol) is added in order to create oil-water bridges and lower surface tension, further stabilising the microemulsion system.

The oil-in-water microemulsions are not stable in all proportions of the ingredients and should be kept within a certain narrow range [128]. A typical composition of microemulsion used in CE consists of 0.8% core phase, 3.3% surfactant, 6.6% co-surfactant and 89.3% aqueous buffer [129].

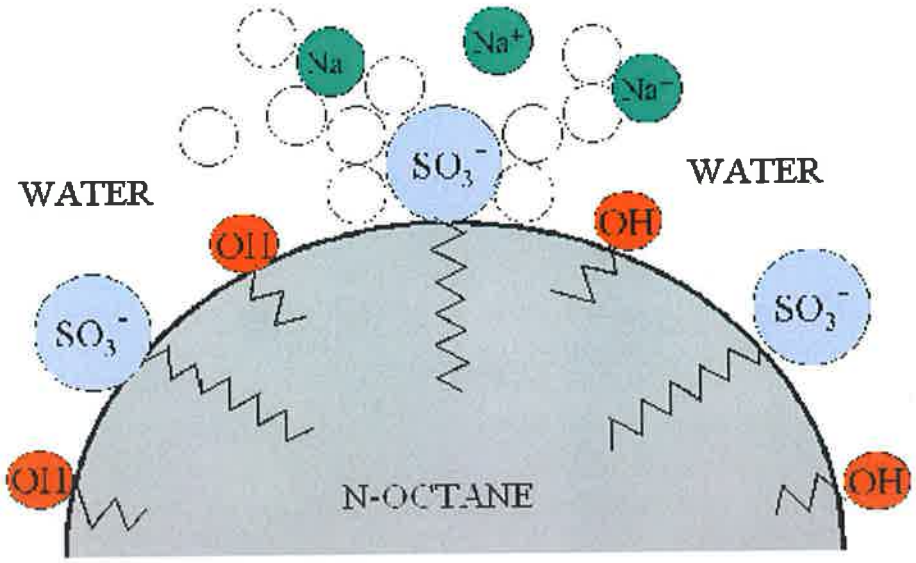

Figure 1.12. Schematic representation of oil-in-water microemulsion [adapted from 69].

1.4.4.2 Mechanism of separation

MEEKC allows separation in a similar fashion to MEKC but provides superior separation efficiency to MEKC, due to improved mass transfer between the microemulsion droplet and aqueous phase [130], the reason being that the structure of the microemulsion is less rigid than that of a micelle [126]. A schematic representation of the separation mechanism in MEEKC is shown in Figure 1.13 where analysis is performed using high pH and anionic surfactant. The separation occurs due to chromatographic partitioning of analytes between the microemulsion droplets and aqueous buffer phase in addition to their mobilities determined by mass to charge ratio. The analyte migrates at a velocity between two extremes, i.e. the electroosmotic velocity and the velocity of microemulsion [119]. Highly hydrophobic solutes will have long migration times as they strongly incorporate into the microemulsion droplets. Cationic positively charged analytes can be separated through partitioning and ion-pairing processes. Negatively charged solutes are charge repelled from the anionic droplets but can still be separated on the basis of their electrophoretic mobilities.

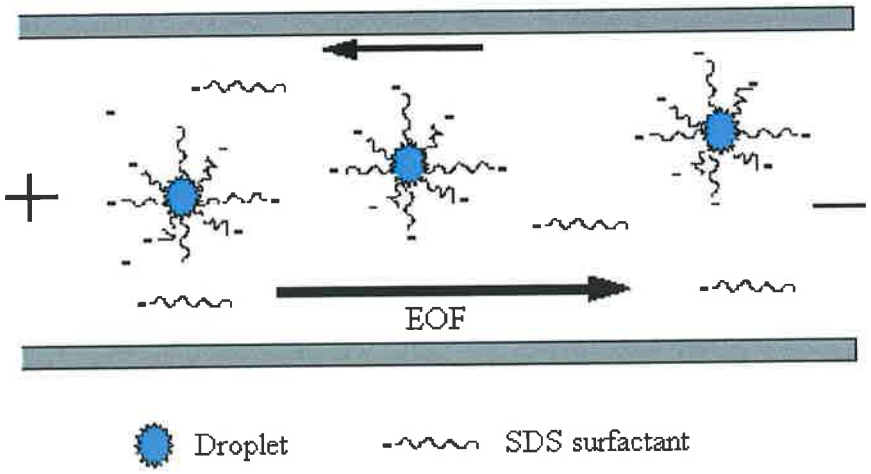

Figure 1.13. Mechanism of MEEKC separation [adapted from 69].

A vast number of published methods have employed high pH buffers [117, 125, 129, 131-133] in order to generate high EOF velocities. The application of high voltage across the capillary results in the migration of negatively charged oil droplets and associated analytes towards the anode due to the influence of electrophoresis. Enhanced electroosmotic flow created at high pH sweeps oil droplets and all components to the detector at the cathode end. MEEKC at low pH has also been investigated in the literature [115, 121, 134-137]. At low pH there is no EOF, therefore the polarity of the separation must be reversed in order to attract the oil droplets towards the detector. In this case the most retained analytes elute first [121, 131]. Suppression of EOF minimises the repulsion of acidic analytes from negatively charged droplets, which could occur at high pH where both solutes and oil droplets are negatively charged.

The addition of organic modifiers to the microemulsion separation buffer has been proven to be useful when water insoluble solutes strongly incorporated into the oil droplets [138]. The organic solvent reduces the degree of partitioning of the analytes into the core phase. It also reduces the electrical current generated in the system, which can reduce the effect of Joule heating. The concentration in which methanol, acetonitrile, ethanol or other solvents can be added, is restricted by a certain amount [111]. Greater concentrations may lead to lower peak efficiencies due to the disruption of the micelle structure.

1.5 IMPROVING SENSITIVITY IN CE

Capillary electrophoresis stands out from other separation techniques for its several very beneficial features. High-voltage separation in a narrow capillary requires only minimal amount of sample for the injection. It uses small volumes of aqueous-based buffer, has numerous features for adjusting a buffer system for any particular separation and is relatively inexpensive. The obtained separations are highly efficient and very well resolved. Yet the lack of sensitivity does not allow the employment of capillary electrophoresis in areas where low limits of detection are essential. The small inner diameter of the capillary, small volumes of injection and the widely used UV-Vis detector result in poor concentration sensitivity. Work is ongoing in the area of improving sensitivity in CE. The main directions of improving sensitivity lead to the improved CE detection and on-line capillary pre-concentration methods [139].

1.5.1 CE detection

The detection techniques combined with CE separation, their detection limits and major concerns are summarised in Table 1.4 [59].

The vast majority of commercially available CE instruments use UV-Vis detection due to its simplicity in instrumentation and flexibility in the detection of wide range of compounds. This type of detection is usually performed on the capillary. Therefore, the attempts that have been made to enhance the sensitivity vary in the way they enlarge the light path: bubble cells [140, 141], Z-shape cells [142, 143] or multi-reflection detection cells [144, 145]. CE with UV detection will always be useful in the method development approach.

Table 1.4. Methods of detection [59].

Method	Concentration detection limit (molar)[a]	Advantages/disadvantages
UV-Vis absorbtion	$10^{-5} - 10^{-8}$	-Universal -Diode array offers spectral information
Fluorescence	$10^{-7} - 10^{-9}$	-Sensitive -Usually requires sample derivatisation
Laser-induced fluorescence	$10^{-14} - 10^{-16}$	-Extremely sensitive -Usually requires sample derivatisation -Expensive
Amperometry	$10^{-10} - 10^{-11}$	-Sensitive -Selective but useful only for electroactive analysis -Requires special electronics and capillary modification
Conductivity	$10^{-7} - 10^{-8}$	-Universal -Requires special electronics and capillary modification
Mass spectrometry	$10^{-8} - 10^{-9}$	-Sensitive and offers structural information -Interface between CE and MS complicated
Indirect UV, fluorescence, amperometry	10-100 times less than direct method	-Universal -Lower sensitivity than direct methods

[a] – for 10 nL injection volume

Fluorescence, especially laser induced fluorescence (LIF), is a very sensitive detector. When LIF is employed with CE separation, its limit of detection can reach attomole to zeptomole levels and even detect a single molecule [146, 147]. Most drugs do not possess native fluorescence and, thus, derivatisation is often required. The fluorescence derivatising agent, 5-iodoacetamidofluorescein, was applied to the determination of anthihypertensive drug captopril [148]. The reported LOD was 0.5 ng mL^{-1}. The analysis of baclofen in plasma was performed using derivatisation agent naphthalene-2,3-dicarboxaldehyde [149]. Cao *et al.* [150] reported determination of amino compounds with 6-oxy-(N-succinimidyl acetate)-9-(2'-methoxycarbonyl) fluorescein. The application of LIF is limited due to the high cost of laser and limited wavelengths.

Mass spectrometry is a very useful detection technique because it offers structural information about the analytes as well as quantitative analysis. Coupled with CE it is very powerful as it combines high resolving power and structural information in one system. The principal advantage of CE-MS is that analytes are identified both by their differential separation and their molecular masses and/or fragmentation patterns [151]. Electrospray ionisation, atomic pressure chemical ionisation (APCI) and matrix-assisted laser desorption/ionisation are among other ionisation techniques in MS. It is very challenging to interface CE to ESI-MS due to unsuitability of some run buffers with the ionisation process. CE-MS methods are characterised by quite low limits of detection. Thus, LODs for the determination of 4-alkyl 2,5 dimethoxy-amphetamine derivatives from urine samples were in the range 3.98 to 4.64 ng mL^{-1} [152]. Different applications of CE-ESI-MS have been recently reviewed by W.F. Smyth [153].

1.5.2 On-line capillary pre-concentration methods

Sample stacking is widely used to enhance the sensitivity in CE [117]. In order to obtain stacking effect the conductivity of the sample should be significantly lower than that of the running buffer. For example, the sample is dissolved in the run buffer with the same composition but at much lower concentration. An electric field in the sample zone, generated upon application of high voltage, will be significantly greater and cause the ions to migrate faster. Reaching the boundary of the run buffer, where the electric field is lower, the ions migrate more slowly. When all the ions reached the run buffer zone the sample is concentrated in a smaller zone. Other mechanisms include pH and viscosity changes to obtain different velocities [139].

A very specific focusing effect of sweeping involves accumulation of analyte by pseudo-stationary phase that enters the sample zone. The stronger the interaction between analyte and the pseudo-stationary phase the better pre-concentration result. Hsieh and Lin [154] obtained 1500-fold improvement in detection sensitivity of the CE method for the determination of trans-resveratrol in red wine in comparison with normal-MEKC mode.

Isotachophoresis employs two buffers with different mobilities: a leading – with faster mobility and terminating – with lower mobility. When the electric field is applied and equilibrium is reached, all ions travel according to their mobility and at the same velocity [155]. Either cations or anions can be analysed. Concentration of each zone is determined by the concentration of the leading electrolyte. More concentrated zones than leading zones get sharper, less concentrated zones get broader. When using ITP concentrations improvement can reach 1000-fold [139]. ITP can be performed in a single capillary [156, 157] or double-capillary [158, 159] mode. The advantage of using double-capillary mode is the possibility of direct injection of the biological samples. In the isotachophoretic method for the determination of fenoprofen in human serum [158], the mixture of hydrochloric acid, 6-aminocaproic acid and polyvinylpyrrolidone was employed as the leading electrolyte, while the

terminating electrolyte was 4-morpholineethanesulfonic acid. The achieved LOD of the method was 0.02 mM.

On-line coupling of micro solid phase extraction cartridges in the inlet area of the capillary have been reported [160]. Employing a UV detector, the detection limit for the model compound enkephalin was as low as 5 ng mL^{-1}. The high affinity and the high selectivity of the antigen-antibody interactions allow the specific extraction and the concentration of the analytes of interest in one step [161]. Other techniques utilise a small bed of packing material (C_4 or C_{18} particles) or hydrophobic membranes directly at the inlet of the CE capillary [162].

1.6 THE ADVANTAGES OF CE IN THE ANALYSIS OF PHARMACEUTICALS

The outstanding selectivity and high speed of analysis of CE makes it competitive to the HPLC technique, which currently dominates in the analysis of pharmaceuticals. Advantages include reduced cost of analysis, reduction in solvent consumption and disposal, the possibility of rapid method development, generic separation conditions for a wide range of analytes and the possibility of coupling to a variety of detector types. Nevertheless, industry, licensing authorities, and the international pharmacopoeias, e.g. the European Pharmacopoeia, do not make use of CE [13]. Exceptions include the analysis of amino acids, peptides, and protein compounds as well as products of recombinant DNA technology and products [163]. The United States Pharmacopoeia (USP 28) [164] makes use of CD-modified CE for the analysis of enantiomeric purity of ropivacain.

The main reasons for the reluctance to use CE are lack of sensitivity and poor precision of the methods. Low sensitivity is usually a factor in the case where UV-Vis detection is employed. As was discussed in section 1.5, this is due to the small amount of sample injected into the capillary and due to the short detection pathway of the light. The inferior analytical performance has been overcome by the introduction of the internal standard, by the capillary wall conditioning procedure and by other methods overviewed by Mayer *et al.* [165]. Now reproducibility below 1% relative standard deviation (RSD) can be obtained for migration time (ratios) and peak area (ratios) [52]. Therefore CE should be given more opportunities in analysis where it can perform at a high level.

1.6.1 Determination of pharmaceutical content

An additional advantage of CE in the analysis of the main component of the pharmaceuticals is that the sample pretreatment step can be reduced as the CE capillary can be washed with NaOH between injections and neutral interfering components do not migrate [53]. CE methods require only 10-20 mL of buffer for the day of analysis, while the HPLC method consumes litres of mobile phases containing expensive and harmful organic compounds. The price of uncoated fused silica capillaries is small compared to the cost of HPLC columns. However, HPLC is still undoubtedly advantageous for a preparative option.

A variety of developed capillary electrophoresis methods have been applied for testing of pharmaceutical formulations including tablets [166-168], infusion solutions [169], syrups [170] and eardrops [171]. Numerous CE methods have been developed for main component assay of pharmaceuticals [172, 173] and were validated with comparable results to HPLC. CE has also been employed in the stability studies of pharmaceuticals [174, 175].

A cyclodextrin modified CZE method has been reported for analysis of raglitazar and its counterion arginine in active pharmaceutical ingredients and low-dose tablets [176]. The method can be used for active ingredient assay and identification of ragaglitazar and arginine, chiral purity of ragaglitazar and purity of ragaglitazar. The precision of the method for peak area (%RSD) of ragaglitazar was found to be 0.63% and 3.5% for arginine, while % recovery was 101-106% for ragaglitazar and 101-125% for arginine. The determination of folic acid in tablets by the MEEKC method has been proposed [177]. The method was characterised by precision of less than 1.2% RSD and recovery of 99.8±1.8% at three concentration levels.

CE methods have been employed for high throughput analysis due to their low cost and easy of performance. The analysis of dissolution test samples of combination formulation of acetaminophenol, phenylephedrine and chloropheniramine has potential for high throughput as it is performed in just 4 min using simple phosphate buffer of pH 6.2 [178].

1.6.2 Determination of drug-related impurities

An important role of CE in pharmaceutical industry could be the determination of drug-related impurities. The structural impurities of a drug possess similar properties to that of the main component and therefore are hard to resolve. The main advantage of CE over chromatographic methods in this case is high separation efficiencies. The extreme peak sharpness can overcome poor selectivity and results in acceptable resolution. A detection limit of 0.1% area/area is widely accepted as a minimum requirement for a related impurities determination method [53]. This can be achieved by CE methods [179, 180]. Toro and colleagues [179] developed a CE method to quantify the impurity profile of a new substance of pharmacological interest LAS 35917. The method allowed the detection and quantitation of impurities above 0.04% and 0.08% level. Chloromethylated, monomethylated and hydroxylated impurities coeluted when analysed by HPLC.

A HPLC method for the determination of impurities in tripeptide glutathione [13] is employed in Japanese pharmacopoeia. This method failed to evaluate the impurities without degradation of the sample during analysis. A CE method in European pharmacopoeia is capable of separating the impurities and limiting them to 0.1% without degradation [180]. In addition the degradation can easily be observed by CE method.

The impurity profile of polypeptide bacitracin is currently being analysed by an isocratic HPLC method in EP 5.0 [163]. Due to the broadened peaks this method is not able to separate all components of bacitracin [13]. A MEKC method was reported [181] which is characterised by improved selectivity and efficiency. The HPLC and MEKC methods are compared in Figure 1.14. These advantages of CE over HPLC method are also true for tobramycin and other aminoglycosides [182]. The related impurities and ranitidine were analysed in bulk drug and pharmaceutical formulations by CE assay [183]. The ionic strength and pH of the buffer affected selectivity the most. The detection limits for impurities were between 0.03% and 0.24%. The CE method was able to resolve several additional peaks, which were not resolved by TLC or HPLC.

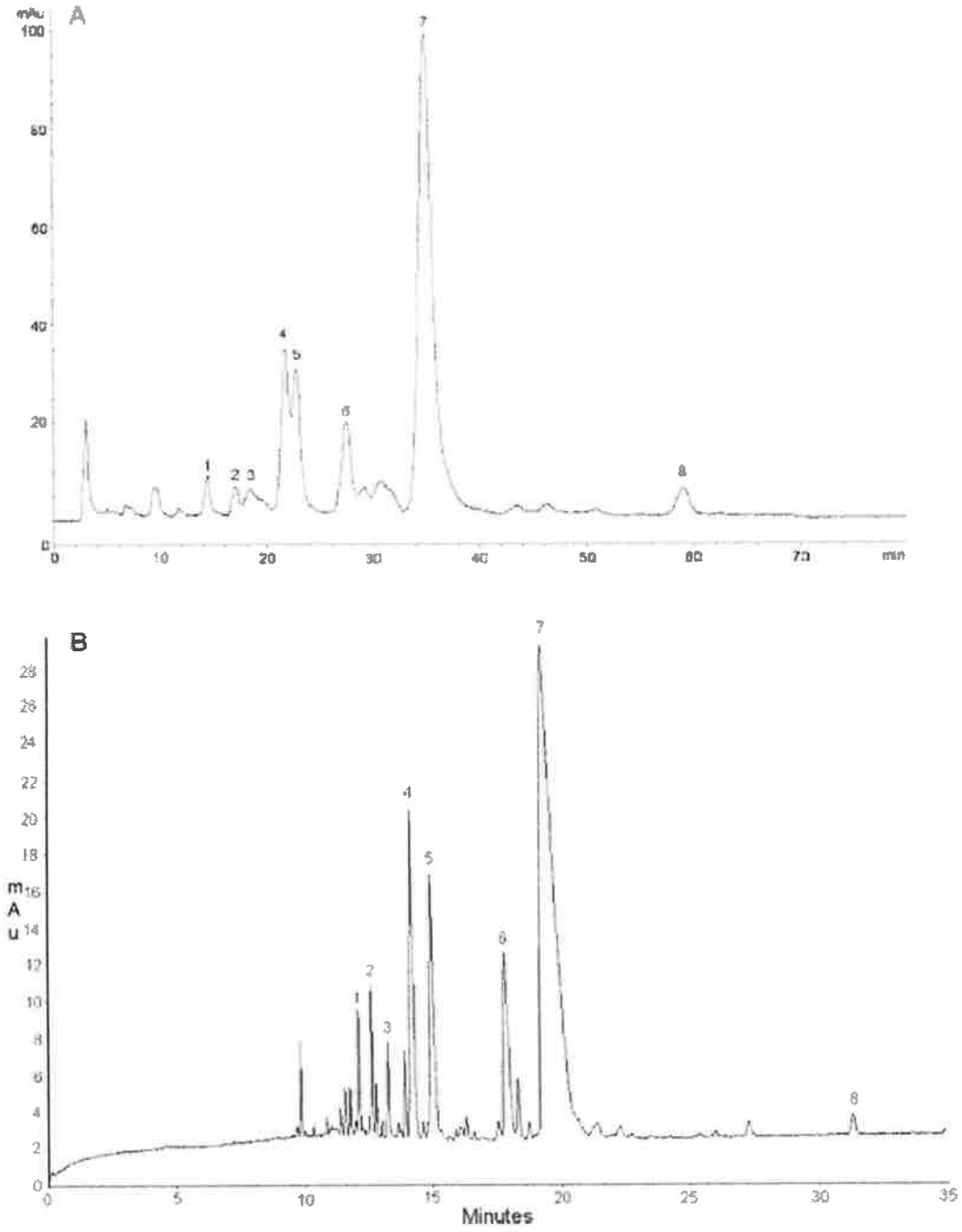

Figure 1.14. A comparison of HPLC (A) and MEKC (B) methods for the analysis of bacitracin [adapted from 13]. Peak 7 – bacitracin.

CE is not always superior to HPLC. There are numerous examples of where CE methods do not outperform methods reported by other techniques [184-186]. It is important to be able to employ the best quality separations offered by CE. If a method superior in all other ways has already been performed by other analytical techniques, it is not recommended to try to achieve the same results by CE.

1.6.3 Chiral separations

Chiral purity is an important aspect of pharmaceutical safety. Chiral compounds can be synthesised as racemates or single enantiomers. A final product should contain less than 0.1% of undesired enantiomers [14]. To quantify these amounts can be a challenge for the sensitivity and selectivity of any technique. As was discussed in section 1.4.3 capillary electrophoresis can choose from a large range of chiral selectors to achieve the required aim. Certain issues may arise if randomly substituted CD derivatives are employed [187]. Most international pharmacopoeias use optical rotation or chiral HPLC methods for the determination of enantiomeric purity of drugs. Since HPLC sometimes cannot offer the required selectivity and because of the similar molecular weights, structural isomers are not specifically detected using HPLC-MS. Capillary electrophoresis, on the other hand, offers high separation efficiency and can be applied as an adjunct to HPLC. Therefore, a set of highly selective CE methods is used orthogonally in the specificity assessment of HPLC methods [188].

A generic approach to chiral separations by CE has recently emerged in publications by pharmaceutical companies [189, 190]. Knowing the structure or even functional groups of the compound, initial separation conditions can be selected and then variation of the potential components of the buffer can be run in a sequence. Other parameters of separation can be further optimised. The development of the same method with HPLC can be quite a time-consuming process because stationary and mobile phases combinations have to be investigated. The total cost of the capillary and the cyclodextrin is still much lower than the cost of a chiral HPLC column with specialised stationary phases.

Sänger-van de Griend *et al.* [191] reported a method for the enantiomeric separations of adrenaline in high-concentrated local anaesthetic injection solution. No sample preparation was required for this simple and robust chiral CE method to detect racemisation amount of adrenaline in the concentration of 0.1 μg mL^{-1} (0.002-0.003% of the local anaesthetic). An α-CD was employed in a new chiral CE separation of galantamine hydrobromide enantiomers [192]. A limit of detection of 0.04% (w/w) was achieved and the method was successfully included in a new drug application by Janssen Pharmaceuticals. The method was fully validated according to International Conference on Harmonisation guidelines and was successfully transferred to laboratories in Europe, US, Japan and China [53]. A CE method for determining ephedrine enantiomers [193] could replace the current optical rotation method in the European Pharmacopoeia as it provides required LOD (0.1%) for (+) ephedrine and linearity in a range 0.1% to 1%.

1.6.4 Bioanalysis of pharmaceuticals

The determination of pharmaceuticals in biological fluids is essential for therapeutic drug monitoring, forensic and clinical toxicology and pharmacological research. The issues of sample clean up and preconcentration of sample arise due to the complexity of biological matrices and small quantities of analytes. Biological samples consist of many components, such as macromolecules of proteins, lipids, carbohydrates and small molecules of different nature, which may prohibit the performance of the capillary or can interact with analytes. The pharmaceutical contents have been investigated in the variety of matrixes. For example, anthracycline antibiotics were analysed in human plasma [194], dexamethazone in tears [195], amphetamine and related compounds in urine [196, 197].

Direct sample injection is possible with biological samples if the analytes do not coelute with the matrix components. The main problem with the direct injection of plasma or serum is the high concentration of proteins [198], which can absorb to the capillary walls or bind to analytes. The high concentration of salts present in urine causes high conductivity of the sample and less efficient

separations [162]. A direct sample injection was employed for the determination of six organic acids in cerebrospinal fluid [199]. In this work, adverse effects of proteins, which frequently compromise the CE performance, could be effectively minimised by the triple layer coating in combination with rinses of 0.1 M hydrochloric acid. %RSDs for migration times and peak areas were <2% and <7%, respectively. Direct sample injection is also possible when the sample is diluted with the background electrolyte, but it often compromises the sensitivity.

Protein removal by ultrafiltration [200] or precipitation [201] is often employed prior to CE analysis of biological samples. Microdialysis sampling was coupled on-line to micellar electrokinetic chromatography to monitor extracellular dopamine concentration in the brains of rats [202]. The detection limit for dopamine was as low as 2 nM when sampling by dialysis. Tagliaro *et al.* [203] reported the analysis of theophylline in serum using simple methanol precipitation of proteins. The method was characterised by good linearity range of 2-120 μg mL^{-1} and sufficient LOD (2 μg mL^{-1}), while the efficiency was 20 times higher than the HPLC method.

Liquid-liquid extraction (LLE) or solid phase extraction are attractive sample pretreatment methods as selective and simultaneous concentration of analyte usually takes place [161], but the methods are time consuming. Various approaches have been made in on-line sample pretreatment methods for CE [204], which significantly minimise time and effort.

A unique advantage of CE is the possibility to apply electrophoretic preconcentration. Charged analytes can be concentrated by ITP or by sample stacking [205]. A combination of SPE and ITP method with conductivity detector for the analysis of diuretics and β-blockers in serum and in urine [206] resulted in low LODs 32-46 ng mL^{-1}. Sweeping preconcentration of a CE method for the traces amount of anthracyclines provided an excellent detection limit of 10^{-9} mol L^{-1} even with the UV detection [194]. Improvement of the sensitivity of CE is also possible in the detection step by coupling CE with LIF [148-150], electrochemical detection [207] and mass spectrometry [153]. A Z-type detection

cell with UV detection was also employed for lower concentration limits of methotrexate in plasma and urine [92].

The development of high speed technology in CE, which allow separations on the millisecond time scale, have opened new areas of application such as real time chemical monitoring and detection of short-lived species (protein conformers or non-covalent complexes), rapid multi-dimensional separations and high-throughput assays for clinical laboratories [208]. An automated system was developed for a competitive immunoassay for insulin [209]. The separation of bound and free-labelled insulin was performed in 7 sec. The throughput was significantly improved and the system allowed continuous monitoring of the sample stream as shown in Figure 1.15.

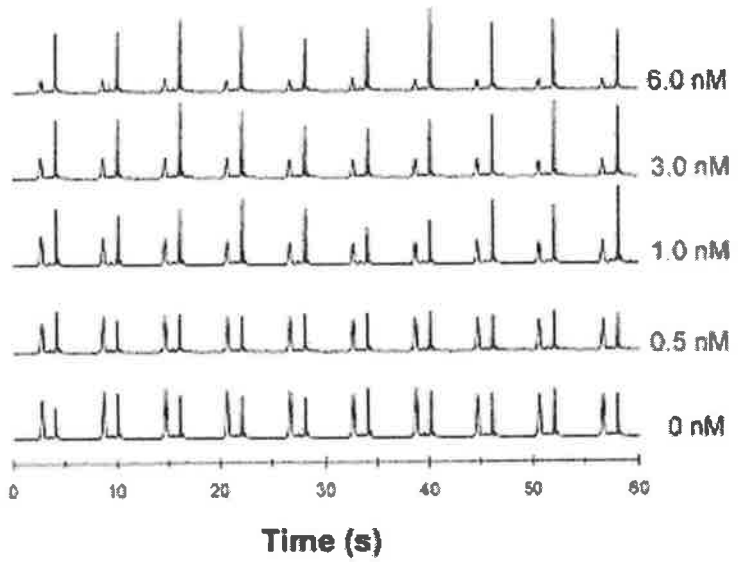

Fig. 1.15. Series of electropherograms obtained in the on-line system for a competitive immunoassay of insulin [adapted from 208].

1.6.5 Physicochemical measurements

The success of CE has not only been in the separation and determination areas, but also in the measurement of physicochemical properties. CE has been used in the study of various equilibrium constants, such as acid-base, complex-forming, ion association and inclusion. The knowledge of the separation mechanism and model parameters including the respective equilibrium constants enables the behavior of analytes during the separation to be predicted and the separation to be optimized [210]. Equilibrium constants are estimated from the changes in migration behavior while changing the composition of background electrolyte.

The determination of pKa values by CZE is based on the observation of the effective mobility of an ionisable compound in a series of electrolyte solutions of constant ionic strength at different pH [211]. The effective mobility is plotted as a function of pH within a suitable model for the number of ionisable groups. The main benefit of employing CE in this procedure is that it can handle impure samples (unlike potentiometric titration or UV-Vis spectroscopy). In addition, purity of electrolyte is not essential and little adjustment of the methods is required for high throughput applications; only mobilities are used in calculation and the concentration is not required (unlike titration) [211]. The pKa values of several pharmaceuticals have been calculated [212, 213] and successfully compared to literature values [214]. By adding organic solvents to the electrolyte, the dissociation constants of water-insoluble and sparingly soluble compounds can be determined [212]. Only a small amount of material is needed for CE analysis and this can be very useful for screening novel pharmaceutical compounds when only restricted amounts of sample are available [215]. This is also advantageous when analysing small quantities of radiopharmaceuticals [216] or unstable compounds [217].

Another example of the estimation of the stability constant by CE is the determination of constants of inclusion complexes with cyclodextrins. In the pharmaceutical field the different interaction of optical isomers with cyclodextrins is the main way of performing their separation [218-220]. The degree of interaction of compounds with cyclodextrin affects its electrophoretic behavior.

Assumption of 1:1 stoichiometric interaction in combination with several mathematical models [106] leads to relatively simple calculations of the stability constant. Knowing this constant it is possible to calculate the optimum concentration of chiral selector [106-108]. Inclusion complexes of some pharmaceuticals have been investigated in literature [221-223].

The possibility to employ buffers at extreme pH (<2 and 12) has been used in CE to measure the partitioning coefficient $\log P_{ow}$ of acids and bases in their uncharged state [224]. Such an opportunity may not arise in the HPLC method where the stability of the stationary phase has to be taken into account. The partitioning of compounds into MEKC micelles [225] and MEEKC oil droplets [226] has been related to their solubility measurements. The octanol-water partitioning coefficient is determined from a calibration graph of known $\log P_{ow}$ of a neutral marker against migration time. The $\log P_{ow}$ of analyte can then be calculated by its own migration time through the graph. Longer migration times of compounds correspond to higher $\log P_{ow}$ values. The octanol-water partitioning coefficient has been successfully measured for pharmaceuticals [120, 227].

1.7 CONCLUSIONS AND THESIS OUTLINE

The fundamental aim of this thesis was to further investigate the advantages of capillary electrophoresis in the analysis of several pharmaceuticals and, thus, making a contribution to the establishment of CE among other analytical methods.

The basic principles behind the superior resolving power of the capillary electrophoresis were outlined in the Literature review. Different ways of optimising separation in CE were demonstrated. Revealing the advantages of the technique while acknowledging its drawbacks, the wide application of the CE and comparison to other analytical techniques in the analysis of pharmaceuticals was explored.

In conclusion to this literature review it should be mentioned that the real value of CE is not in those areas where significant additional work is required to overcome the drawbacks, but in those where it can make the analysis easier, faster and more straightforward.

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CHAPTER 2

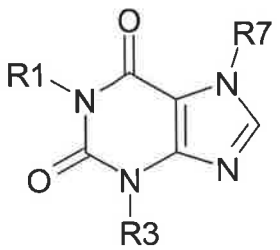
RAPID SIMULTANEOUS DETERMINATION OF ALKYLXANTHINES BY CZE

2.1 INTRODUCTION

The demand for fast, minimal cost and sample preparation analysis comes from a busy environment of modern industries. Nevertheless, the acceptable parameters of analysis, such as peak resolution, efficiency, applicable LODs and reproducibility of the analysis should be met in the method development process. A simple and fast capillary electrophoretic method for the simultaneous determination of alkylxanthines and its application for the analysis of real samples are reported in this chapter.

2.1.1 Alkylxanthines

The family of compounds alkylxanthines includes caffeine, theophylline and theobromine. The parent compound of all alkylxanthines is xanthine (2:6 - dihydroxypurine); found in potatoes, coffee beans, etc., it is a very weak base [1]. The chemical structures and pKa values of some compounds are presented in Figure 2.1 and Table 2.1.



	R1	R3	R7
Theophylline	$-CH_3$	$-CH_3$	-H
Theobromine	-H	$-CH_3$	$-CH_3$
Caffeine	$-CH_3$	$-CH_3$	$-CH_3$
Enprofylline	-H	$-CH_2CH_2CH_3$	-H
Dyphylline	$-CH_3$	$-CH_3$	$-CH_2CHOHCH_2OH$

Figure 2.1. Chemical structures of some alkylxanthines.

Table 2.1. pKa values of some alkylxanthines [2, 3].

	pKa
Theophylline	8.8
Theobromine	10.0
Caffeine	14.0
Enprofylline	8.4
Dyphylline	11.6

The family of compounds alkylxanthines occur naturally in plants (tea leaves, coffee beans, kola nuts). Their chemical structures arise from replacement of the protons at position 1, 3 and 7 in xanthine with various substituents (Figure 2.1). The substitutions lead to a similar chemical structure and slightly different pharmacological activity that has determined their use in pharmaceuticals [3].

Alkylxanthines are very well known in pharmaceutical science due to the following properties [4]: antiasthmatic, analgesic adjuvants, antitussives, bronchodilators, cardiac stimulants, cognition enhancers, diuretics, lipolytic agents, cancer chemotherapy adjuvants, treatment of cerebral ischemia, treatment of Parkinson's disease, renal failure. The frequency of human consumption of this type of pharmacologically active compound speaks for itself. Xanthines are present in all kinds of tea, coffee, carbonated beverages, chocolate and chocolate products and chewing gum [5]. Adding all these factors together, the necessity for quality control of pharmaceuticals and alkylxanthine contents of food becomes very important.

2.1.2 Alkylxanthines in treatment of asthma

Over 3% of all people in most countries are affected by asthma [6]. It is increasing in severity, prevalence and mortality despite a big improvement in the treatment of asthma. Asthma is commonly attributed to exposure to environmental agents: house dust mite, animal dander, pollen and certain chemicals. During asthma attack the bronchi and bronchioles become partially or completely blocked, which results in breathing difficulties. The epithelial layer of cells in asthmatic subjects is often inflamed and damaged. Foreign particles penetrate lung lining more easily. Antibodies recognise the foreign particles and signal to mast cells, which release more signalling chemicals. Histamine is one of the signaling chemicals that cause the airway's smooth muscle to contract, narrowing the diameter of the airway. This signalling cascade sparks results even in further inflammation and damage.

Alkylxanthines belong to the bronchodilator category of antiasthmatic drugs. Theophylline is the one most widely employed in clinical medicine (at least in the USA [7]). It can be used as well as theophylline-ethylenediamine, also known as aminophylline. Theophylline "works" as an adenosine receptor (A_{2B}) antagonist. It "blocks" adenosine receptors on the mast cells, and that prevents following asthma syndromes. But this hypothesis is in doubt because it was shown [8-10], that enprophylline, having very similar structure to theophylline, is five times more potent a bronchodilator as theophylline but does not inhibit adenosine receptors. Several authors have shown that caffeine has properties of weak bronchodilator [11, 12]. In addition to its potent bronchodilator action theophylline also inhibits the release of mediators from mast cells [13].

A certain level of theophylline must be maintained in the blood stream at all times for effectiveness [12]. The dosage should be individualised; the initial dose should be light, rising slowly until the therapeutic plasma level is reached (20 mg mL^{-1} is significant). There are some interactions with other drugs and physiological states that may lead to enhanced clearance low concentration of theophylline in serum, and, therefore, less efficiency [14]. Side effects of theophylline include nausea, vomiting, stomach cramps, diarrhoea, increase in heart rate, shakiness, restlessness, headaches, and occasionally an increase in urination may take place [15].

2.1.3 Determination of alkylxanthines in real samples

Due to various pharmacological and toxicological effects of alkylxanthine it is important to control them in pharmaceuticals, food and beverages. For example, the physiological effects produced by many non-alcoholic drinks like tea, coffee and cola depend mainly on their natural xanthine content.

A vast amount of articles in the literature has been dedicated to methods of determination of the alkylxanthines in various samples such as bodily fluids [16-25], pharmaceutical formulations [3, 24-30], non-alcoholic beverages and food [14, 27, 31-43]. It is essential for an analytical method to be reliable, simple and fast to determine the alkylxanthines from different sources in order to find a more precise relationship between the amounts of consumed alkylxanthines and their physiological effects [44]. The most common analytical techniques employed in the analysis of alkylxanthines are chromatography, capillary electrophoresis and spectroscopy.

2.1.3.1 Chromatographic methods

In general, chromatographic methods for the separation of alkylxanthines require a clean-up step prior to the analysis itself and at least 20 min for the last compound to elute. The separation time is less in the case of the analysis of a single compound [45]. A UV detector is widely employed for quantification of analytes at 214, 230, 270-275 or 280 nm [16] and with it the limit of detection is often around 50 ng mL^{-1}.

Holland *et al.* [46] developed a reversed-phased HPLC method for the simultaneous determination of caffeine and paraxanthine in human serum. Serum proteins were precipitated with perchloric acid. A simple composition of mobile phase involved phosphate buffer at pH 4.9 mixed with methanol (85:15, v/v). Even at a high flow rate of 1.75 mL min^{-1} the elution time of the last compound caffeine was 18 min. A UV detector was employed in this method and the limit of quantitation (LOQ) of the method was reported to be 50 ng mL^{-1} for each drug. Very similar time (19 min) was required to separate 13 methylxanthines in the work of Georga *et al.* [19]. However, this involved a linear gradient elution of acetate buffer and methanol. Another example of the HPLC separation of caffeine

and 11 metabolites with gradient elution was reported by Schneider *et al.* [47]. In this case a gradient elution consisted of three different mobile phases and resulted in over 25 min of analysis time.

It is possible to achieve shorter elution time if only one component is analysed and the separation is performed on a short column. Such as in work of Horie *et al.* [45] caffeine was determined in less than 3 min using 75 mm x 3 mm i.d. column. The temperature of this separation was raised to 40°C, which also shortened the time of analysis. Since caffeine was the only compound in the system, multiple samples could be injected within a 2 min interval. The determination of artificial sweeteners, preservatives and caffeine, theophylline and theobromine on anion-exchanged column was also performed at 40°C [27]. An isocratic elution with aqueous NaH_2PO_4 (pH 8.2) solution containing 4% (v/v) acetonitrile resulted in 45 min separation. The time of this analysis was very long, however this method had advantage of having quite low LODs (4 and 30 ng mL^{-1}) as wavelength-switching UV detection was employed which provided the optimum sensitivity for each anlyte.

A study by Aranda and Morlock [48] demonstrated a high-performance thin-layer chromatography in combination with electrospray ionisation mass spectrometry (HPTLC/ESI-MS) method for the quantification of caffeine in pharmaceutical and energy drink samples. The quantification by MS was performed in the positive ion single ion monitoring mode. The resulting LOD was 5.8 ng mL^{-1}, which was a factor of 13 lower than with HPTLC-UV. Meyer *et al.* [33] achieved even lower limits of detection for caffeine and theobromine (2.5 ng mL^{-1}), theophylline (1 ng mL^{-1}) using amperometric detection in combination with HPLC separation.

2.1.3.2 Capillary electrophoresis

While capillary electrophoresis has been reported to show successful separation of this group of compounds [2, 3, 16, 20, 21, 24, 28, 38, 41, 49-52], lengthy capillary rinse times and pre-rinses make total analysis time long. Table 2.2 represents the comparison of the analysis time of different CE methods. Due to the fact that most alkylxanthines are weak acids (Figure 2.1, Table 2.1), the alkali pH of several buffers (such as borate [3, 41], phosphate [24, 28], glycine [21, 38] or a mixture of them [20]) is employed for the CE separation. Some methods use the MEKC mode of separation [2, 3, 22, 52], thereby increasing the necessity for lengthy rinses between the separations and irreversible modification of the capillary takes place. MEKC is often necessary in order to determine the neutral compounds in this group [2, 3, 21]. A UV detector is widely employed in CE methods for alkylxanthines, but sensitivity is 10-100 times lower than HPLC [35, 36]. CE offers a distinct advantage in terms of time required for analysis; it can be as short as 2 min [24, 38]. Some biological samples and beverages can be injected directly onto the capillary without the need for the sample pretreatment step [23, 24]. Sombrab *et al.* [53] concluded that investigated CE and HPLC methods for the analysis of caffeine in seed powder and commercial tablets were equal in terms of sensitivity and precision. Furthermore, the analysis time of the CE method was up to two times shorter than that of HPLC and solvent consumption was more than 100-fold less.

Table 2.2. Comparison of analysis time of different CE methods.

Analytes	Method	Time of run (min)	Rinsing between runs (min)	Total time of analysis (min)
Caffeine, theobromine, paraxanthine, theophylline	MEKC [24]	2	not reported	-
Caffeine, theophylline, dyphylline	MEKC [3]	4	8	12
Theophylline, its metabolites	MEKC [22]	30	3-6	33-36
Caffeine, aspartame, asp-phe, benzoic acid, phenylalanine	CZE [38]	2	two volumes	-
Theophylline, ephedrine, phenobarbital	CZE [28]	9	4	13
Theophylline, enprofylline theobromine, caffeine, theophylline-7-acetic acid, dyphylline, proxyphylline, pentoxifylline	MEKC [2]	8	6	14

A very rapid CZE-UV determination of caffeine was reported in the presence of aspartame and benzoic acid in carbonated beverages in just 2 min. The preconditioning of the capillary involved two volumes of run buffer [38]. Blanco and Valverde [2] have demonstrated the behavior of 8 alkylxanthines under CZE and MEKC conditions. CZE did not allow separation of the neutral xanthines, but that was achieved by MEKC with 20 mM tetraborate at pH 8.5 with 120 mM SDS. The last xanthine migrated after 8 min. Employment of surfactant in the background electrolyte required quite intensive conditioning of the capillary before the analysis (6 min with BGE, 0.1 N NaOH and water). MEKC for the determination of fewer compounds can be quite short. Thus, Huang *et al.* [3] simultaneously determined theophylline and dyphylline in 4 min. However, the rinsing between runs with 0.1 N NaOH, deionised water and run buffer added another 8 min to the times of analysis.

Lai and Dabek-Zlotorzynska [54] described a capillary electrochromatography separation of caffeine, theophylline, theobromine and four related drugs on a normal phase column with PDA detection. Excellent resolution of all drugs was achieved using ternary mobile phase composition of isopropanol/hexane/1 mM Tris (52:40:8, pH 8). However, it resulted in long (30 min) time of analysis. The method was characterised by the detection limits at low $\mu g\ mL^{-1}$ level.

Microchip CE separation of alkylxanthines was reported in several papers [55-57]. Thus, Zang e*t al.* [57] used poly(dimethylsiloxane) (PDMS) CE microchip for the separation of caffeine and theophylline with electrochemical detection. Microscale separation offered extremely short analysis times of 40 sec, while simple composition of a buffer was employed (5.0 mM borate at pH 9.2 containing 10% (v/v) methanol). However, the device had to be flushed for 15 min with the run buffer prior the injection. The detection limits were 4 μM for caffeine and theophylline.

2.1.3.3 Spectroscopic methods

Spectrophotometric determination was reported to allow accurate and reproducible results to be obtained from sample analysis using a relatively simple and inexpensive procedure when compared to a chromatographic technique [58]. López-Martinez *et al.* [58] described a method of simultaneous determination of caffeine and theobromine in coffee and tea samples using partial least squares. Sample preparation was required to eliminate strongly interfering components. The electronic absorption spectrum was recorded from 200 nm to 450 nm of samples with Britton-Robinson buffer (pH 12) and diluted with water. The method did not show statistically significant difference with an HPLC standard technique.

A UV spectrophotometric method [30] was successfully used to determine caffeine and other active ingredients in tablets. The linearity range for caffeine was in a very narrow interval of 0.012-0.028 mg mL^{-1}. This method was also compared to LC procedure and both methods showed excellent precision and accuracy.

Bouhsain *et al.* [41] developed a flow injection Fourier Transform infrared determination of caffeine in coffee. A fully automated procedure involved on-line extraction of caffeine with chlorophorm, measuring absorption as a function of time at 1659 cm^{-1}, with a baseline established between 1900 and 830 cm^{-1}. The limit of detection was relatively high at 9 mg mL^{-1}.

The paper substrate room temperature phosphorescence (RTP) of theobromine, caffeine and theophylline was investigated in a work of Chuan *et al.* [31]. The method was based on fast speed quantitative filter paper as substrate and KI-NaAc as heavy atom perturber. Various factors affecting their RTP are discussed. The method allowed very low limits of detection, e.g. 1.14 ng per spot for theobromine, 0.78 ng per spot for caffeine and 1.8 ng per spot for theophylline.

2.1.4 Observations

Different analytical techniques for the analysis of alkylxanthines have been examined. Each of them demonstrated advantages and disadvantages. Thus, both chromatographic and spectroscopic methods require a sample pretreatment step, while some of the CE samples can be injected directly. They can achieve very low limits of detection; and this is advantageous when the concentration of target compounds is low. A disadvantage of the chromatographic technique is a long time of analysis, up to 20 min, while CE methods can take just 2 min [24, 38], and sometimes methods are complicated with the necessity for gradient elution and employment of several mobile phases [19]. Chromatographic methods require expensive equipment compared to CE. The problem with spectroscopic methods is that methylxanthines give high spectral overlap [58] when determined simultaneously and that is why this technique is poorly developed. All the methods are characterised by high reproducibility.

According to all the reasons listed above capillary electrophoresis methods have great potential in the separation of alkylxanthines and, in the opinion of the author, there is more potential for further improvement.

2.1.5 Aim of this study

The aim of this study was to develop a method of rapid simultaneous determination of alkylxanthines using capillary zone electrophoresis. To select the optimum conditions the following factors were investigated: buffer composition, the effect of pH, buffer concentration, voltage of separation, length of the capillary, temperature of the separation and hydrodynamic and electrokinetic types of injection. The application of the proposed method in the quality control of theobromine in chocolate samples and theophylline in commercial tablets was studied.

2.2 EXPERIMENTAL

2.2.1 Instrumentation

All separations were performed using an Agilent Technolodies ^{3D}CE system (Agilent Technologies). Data acquisition and signal processing were performed using Agilent Technolodies ^{3D}CE ChemStation (rev. A.09.03, Agilent Technologies). The fused silica capillaries (Composite Metal Services ltd., The Chase, Hallow, Worcs. WR2 6LD, UK) were 63.3 cm in length (55.3 cm to the detector) with an internal diameter 50 μm, unless otherwise stated.

2.2.2 Reagents

All analyte compounds investigated in the separations had purity 99% and were used without further purification. Caffeine, theophylline, dyphylline (7-[2,3-Dihydroxypropyl]-theophyllin), enprofylline (3-propylxanthine), theobromine (3,7-dimethylxanthine), sodium phosphate (monobasic and dibasic), HEPES (N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]), CAPS (cyclohexylamino-1-propane sulfonic acid), Tris ([hydroxymethyl]aminomethane hydrochloride and Trizma base), HCl, NaOH were purchased from Sigma-Aldrich, Dublin, Ireland. Methanol (HPLC grade) was from Lab-Scan, Dublin Ireland. Boric acid was obtained from Riedel-de Haën, Seelze, Germany. Buffers were prepared using distilled water and adjusted using 1 M and 0.1 M NaOH and 0.1 M HCL. Tris buffer was adjusted using Tris acid or Tris base.

2.2.3 Standards

Stock solutions of 10 mM caffeine, theophylline, dyphylline, 2 mM stock of theobromine and 1 mM stock of enprofylline were prepared in distilled water.

2.2.4 Sample preparation

2.2.4.1 Chocolate

The sample preparation technique was taken directly from the literature [59] and was not further optimised in this work.

Samples of chocolate were placed into a mortar and pestle. Liquid nitrogen was poured over the chocolate to freeze it, allowing it to be ground into a fine powder. One gram of this chocolate powder was measured using an analytical balance, and 5 ml of chloroform was added in a conical flask. This sample was placed in a sonicator for 50 min until the solution became a suspension of very small particles. A syringe and a 0.22 μm filter were used to remove any large or precipitated particles. 2.5 ml of the filtered extracted solution was transferred into another vial. The chloroform was evaporated by placing the vial into a warm water bath and blowing an air stream over it. 10 ml of ethanol was added to the vial following the evaporation, and a gentle swirling action was applied to dissolve the crystals of the compounds in the ethanol. The dissolved ethanol solution was placed into an HPCE vial using a micropipette, while taking care to avoid transferring any of the cocoa butter (insoluble in ethanol) into the vial. The vial was placed into the auto sampler for analysis.

2.2.4.2 Pharmaceutical tablets

Each of 10 commercial tablets containing 300 mg of theophylline was weighed and finely powdered. Three samples of 0.01 g of the tablet powder were placed in 100 ml volumetric flasks, filled with water and then sonicated until dissolved (40 min). The solutions were filtered through a 0.22 μm filter and transferred to the vials that were placed into the autosampler for CE analysis.

2.2.5 Procedure

All buffers were sonicated for 15 min and filtered through a 0.2 μm filter before use. Test mixtures of analytes were diluted in the separation buffer. Xanthines were detected at 200 nm, where all exhibited maximum absorption. Separations were carried out at 30 kV electrophoretic voltage and a temperature of 23°C, unless otherwise stated. Injections were hydrodynamic at 50 mbar for 4 sec, unless otherwise stated. Conditioning of the CE capillary between runs was performed with 0.1 M NaOH for 2 min, water for 5 min and run buffer for 5 min, unless otherwise stated. All separations were repeated at least three times.

2.3 RESULTS AND DISCUSSION

2.3.1 Buffer study

2.3.1.1 Buffer selection

For the purpose of this study caffeine and theophylline were employed as test analytes. In order to perform a separation in CE the mobility of analytes should be different. To ensure the separation of caffeine and theophylline at least one of the compounds should possess its own mobility. Since pKa value of caffeine (14) is much higher than pKa of theophylline (8.8) the pH of the separation buffer should at least provide the ionization of theophylline. At the pH near 8.8 theophylline is partially negatively charged (due to 50% dissociation at R7) and moves along the capillary with its own velocity, while caffeine is neutral and migrates with EOF, according to the CZE theory. Four buffers meeting the conditions above were studied (Table 2.3).

Table 2.3. Working pH range of several buffers [60].

Buffer	Useful pH range
Borate	8.14 – 10.14
Phosphate	6.20 – 8.20
HEPES	6.55 – 8.55
Tris	7.30 – 9.30

The separation of caffeine and theophylline with all buffers is shown in Figure 2.2. Separation with borate and Tris buffers was found to give the shortest migration times for analytes of the choice at given pH, that is why they were chosen for further investigation.

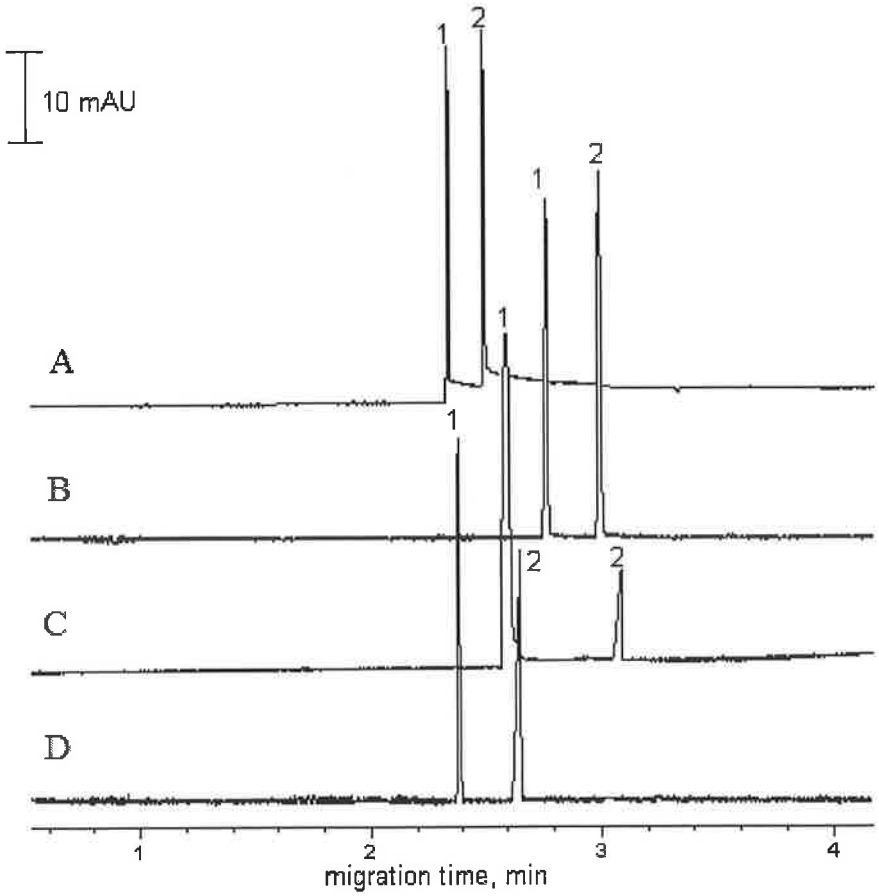


Figure 2.2. Separations of caffeine and theophylline with different buffers. Separation conditions: A – 10 mM borate pH 8.5, B – 10 mM phosphate pH 8, C – 10 mM HEPES pH 8.5, D – 10 mM Tris pH 8.5; separation voltage 30 kV, capillary 63.3 cm (55.3 cm - effective length), 50 μm i.d.; 23°C; hydrodynamic injection 4 seconds; UV detection 200 nm. Peak identification: 1=caffeine, 2=theophylline. Concentration of analytes 0.5 mM.

2.3.1.2 Buffer pH.

The pH study was carried out to investigate the mechanism of the separation of target analytes and find out the optimum pH. Figure 2.3 and Figure 2.4 show the effect of the pH of borate buffer and Tris on the effective mobility of analytes. The effective mobility of caffeine was zero as it was neutral within investigated pH range and migrated with EOF at all times. At the pH below 8, it was not possible to separate the two components as the mobility of theophylline was very close to that of EOF. At pH 8 separation occurred with the resolution 1.3 as theophylline is partially negatively charged and its mobility was different from EOF. Since it migrated towards the anode its mobility was negative and it exited the capillary after neutral caffeine. With further increases in pH, theophylline was fully charged and the difference of the mobility of two analytes became more significant, so as the migration times.

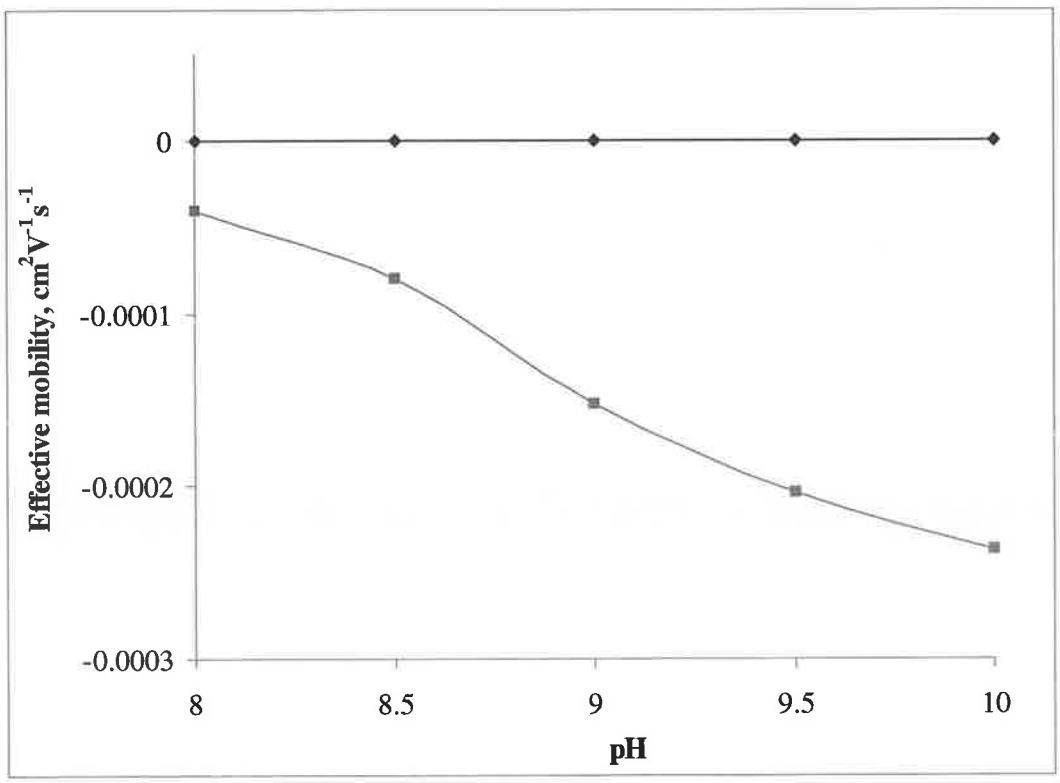


Figure 2.3. Effect of borate buffer pH on the effective mobility of analytes. Buffers concentrations 10 mM, other separation conditions as in Figure 2.2. —◆— - caffeine, —■— - theophylline.

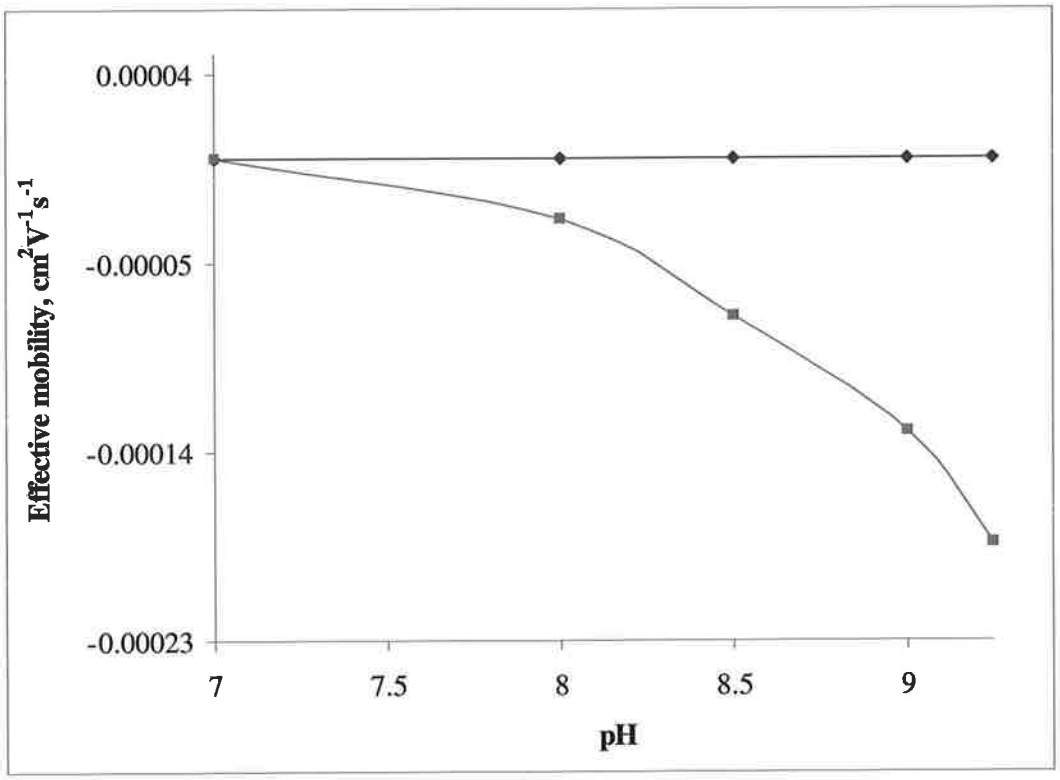


Figure 2.4. Effect of Tris pH on the effective mobility of analytes. Buffers concentrations 10 mM, other separation conditions as in Figure 2.2. —◆— - caffeine, —■— - theophylline.

Taking into account resolution and migration time, the optimum pH for the separation of caffeine and theophylline was found to be pH 8.5 for borate buffer and pH 9.25 for Tris.

2.3.1.3 Buffer concentration

The increase in buffer concentration from 10 mM to 100 mM led to similar effects in the case of borate buffer and Tris. The effect of the buffer concentration on the mobility of EOF and effective mobility of theophylline is shown in Figure 2.5 and Figure 2.6. The migration time of both analytes increased with the increase in concentration of buffer. A decrease in the magnitude of EOF with increasing buffer concentration was correlated to a decrease in the zeta potential due to the compression of double layer at the capillary wall [60]. The effective mobility of theophylline was not significantly affected by the concentration of both buffers. Enhancing the concentration of a buffer might provoke excessive Joule heating, which can have undesirable effects on resolution and analyte stability. In this case resolution of caffeine and theophylline was greater than 10 at all concentrations. All separation factors were considered, therefore the buffer concentration 10 mM was chosen as the optimum for borate and Tris buffers.

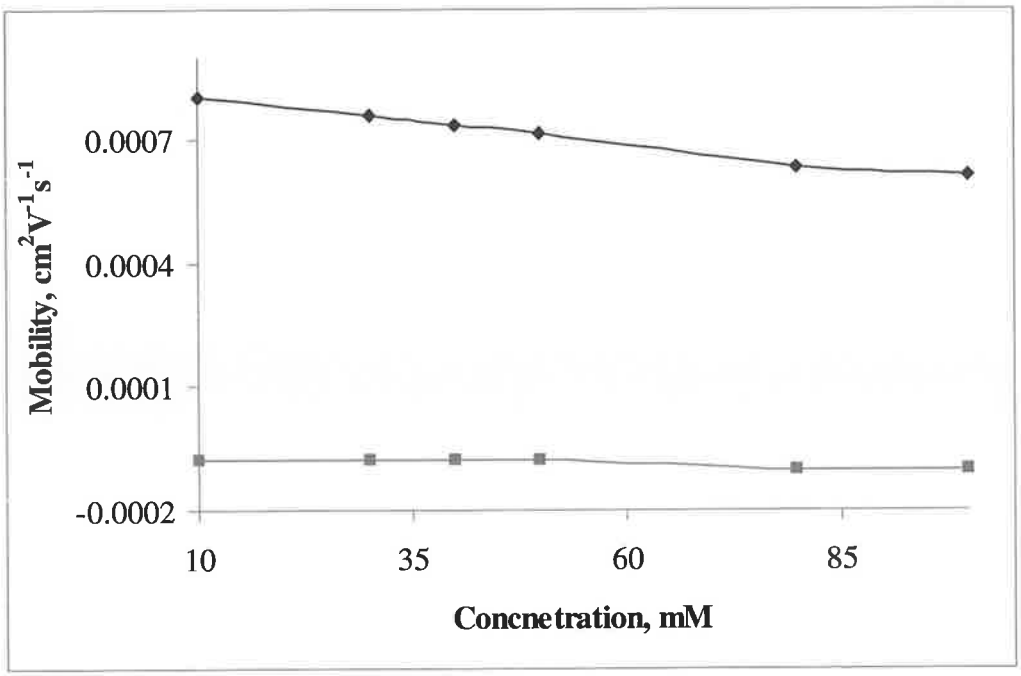


Figure 2.5. Effect of the concentration of borate buffer on the mobility of EOF (caffeine) and effective mobility of theophylline. Buffers pH 8.5, other separation conditions as in Figure 2.2. —◆— - EOF (caffeine), —■— - theophylline.

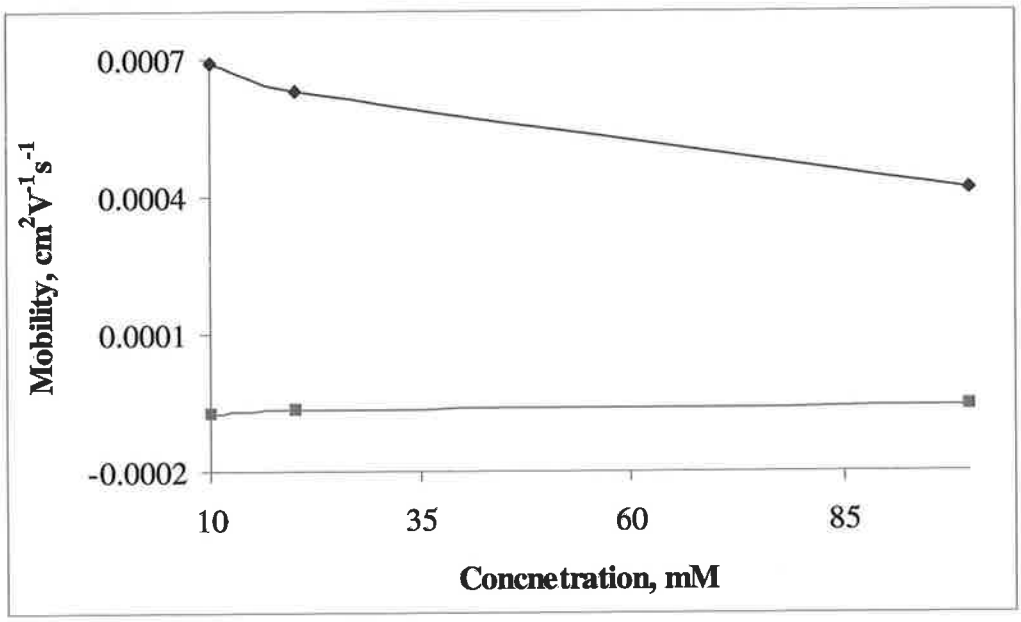


Figure 2.6. Effect of the concentration of Tris buffer on the mobility of EOF (caffeine) and effective mobility of theophylline. Buffers pH 8.5, other separation conditions as in Figure 2.2. —◆— - EOF (caffeine), —■— - theophylline.

2.3.1.4 Ohm's law plot

An Ohm's law plot of current (mA) versus voltage (kV) was plotted. Figure 2.7 shows current generated in CE system, depending on applied voltage which is usually used to indicate excessive heat generation [61]. As can be seen, the increase in current was proportional (R^2=0.9996) to the voltage applied, so there was no temperature increase observed within the whole voltage interval using 10 mM borate pH 8.5 and 10 mM Tris pH 9.25. The separation at higher voltage is more desirable as it provides a shorter migration time and sharpness of peaks and resolution [61]. Thus, 30 kV was found to be the optimum for both buffers.

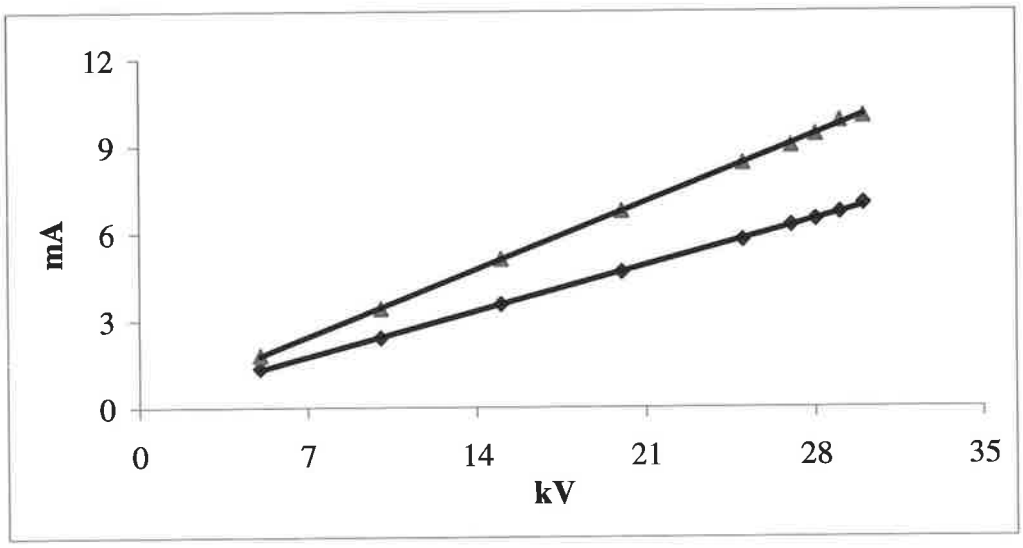


Figure 2.7. Ohm's law plot representing current generated as a function of applied voltage. ◆ - 10 mM borate pH 8.5, y = 0.331x + 0.1244, R^2 = 0.9997. ▲ - 10 mM Tris pH 9.25, y = 0.2247x + 0.1814, R^2 = 0.9996. Other separation conditions as in Figure 2.2.

2.3.1.5 Optimising the separation

Comparison of the parameters (Table 2.4) of the optimal separations with borate and Tris buffers shows that both buffers have a very good resolution of the two peaks, given similar times of analysis. The difference is in the efficiency of peaks: with borate, the efficiency is much higher and it is preferential for quantitative analyses.

Table 2.4. Comparison of separation data for caffeine and theophylline. n = 3

	Analyte	**t_m (min)**	**%RSD (t_m)**	**N**	**R**
10 mM Borate	caffeine	2.39	0.06	150275±14425	10.3
pH 8.5	theophylline	2.66	0.02	164287±7445	
10 mM Tris	caffeine	2.12	0.75	90766±13879	12.5
pH 9.25	theophylline	2.66	0.99	34390±4856	

2.3.2 Study of capillary conditions

This study was carried out with caffeine, theophylline and dyphylline as target analytes. Dyphylline is a derivative of theophylline with - $CH_2CHOHCH_2OH$ radical at N7 (Figure 2.1). Its pKa is 11.6 [3], therefore at the conditions of the CZE method under investigation it migrated with EOF, as was the case with caffeine. Even a high pH, near 12 (CAPS buffer) does not deprotonate dyphylline and it cannot be separated from caffeine this way.

The unique reaction of borate ions with vicinal diols (such as dyphylline) is known [62, 63]. The complexation with borate ions in the background electrolyte (BGE) generates charge on the dyphylline molecules and that is how it emerged after EOF although it possessed no ionisable chemical group [2]. Thus, borate buffer was chosen for separation of the three compounds.

Tables 2.5 and 2.6 show the parameters of the separation while optimising the pH and concentration of borate buffer.

Table 2.5. Parameters of separation for optimising pH of borate buffer, concentration 20 mM. n =3

Buffer pH	R*	N caffeine	N dyphylline	N theophylline
8.5	0.7	75840	106094	130480
8.7	1.4	103599	94048	151168
9.0	2.1	103785	82650	163151

* R – resolution of caffeine and dyphylline peaks. The resolution of dyphylline and theophylline was greater than 10 in all experiments.

Table 2.6. Parameters of separation for optimising concentration of borate buffer, pH 9. n = 3

Buffer concentration	R *	N caffeine	N dyphylline	N theophylline
10 mM	1.2	85565	58164	148528
20 mM	2.1	103785	82650	163151
30 mM	3.3	103661	82222	154636

* R – resolution of caffeine and dyphylline peaks. The resolution of dyphylline and theophylline was greater than 10 in all experiments.

The highest efficiency of analytes and acceptable resolution of caffeine and dyphylline was achieved at pH 9 and concentration 20 mM. It has to be noted that resolution of dyphylline and theophylline was greater than 10 at all times and thus was not under investigation. Further increase in concentration of borate resulted in similar efficiency of peaks and resolution 3.3, which is not required for separation of two analytes. The separation of caffeine, theophylline and dyphylline with 20 mM borate at pH 9 is shown in Figure 2.8.

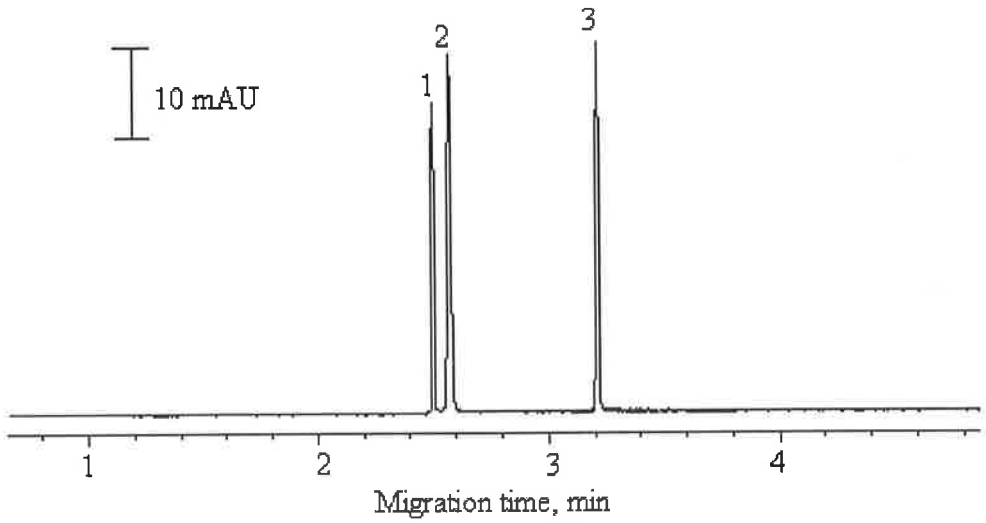


Figure 2.8. Electropherogram of separation of three compounds with 20 mM borate pH 9. Peak identification: 1=caffeine (EOF), 2=dyphylline, 3=theophylline. Concentration of each compound 0.5 mM. Other separation conditions as in Figure 2.2.

2.3.2.1 Capillary length

The full length of the capillary is that from the injection end to the exit end. The effective length is from the point of injection to the point of detection. As UV detection was used in this experiment, effective length was 55.3 cm, while total length of the capillary was 63.3 cm. In the case of detection outside of the capillary (mass spectrometry), both lengths are equivalent. The migration time and mobility depend on the effective length, whereas the electric field is determined by total length [60].

At the capillary length mentioned above, the last component (theophylline) migrated at 3.3 min. When the capillary was shortened by 7 cm, the migration time of theophylline shortened to 2.7 min. The decrease in migration time was accompanied by a decrease in resolution of caffeine and dyphylline, but the small increase in pH up to 9.1 (was 9.0) achieved acceptable resolution. Comparison of the separation at different capillary lengths is shown on Figure 2.9.

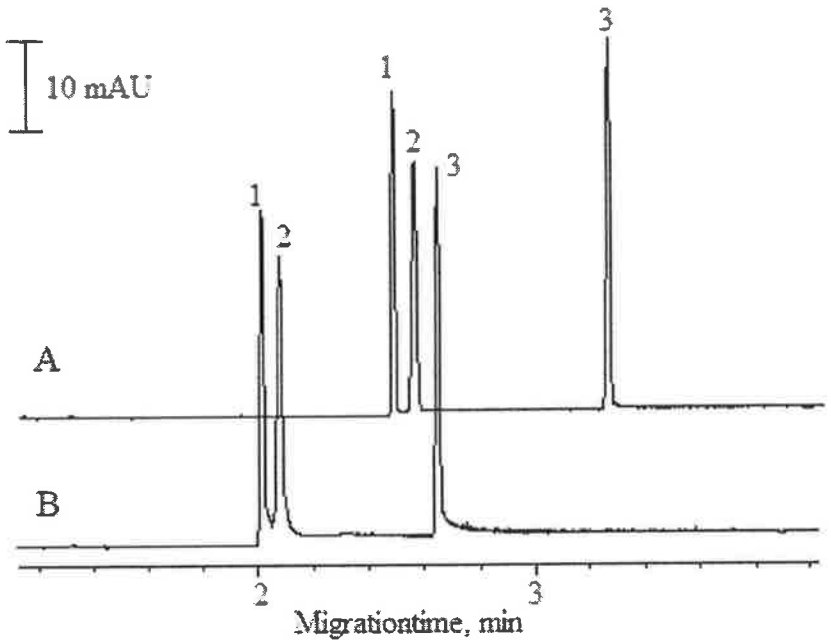


Figure 2.9. Capillary length effect. A – 63.3 cm (55.3 cm), B – 56.3 cm (48.3 cm). Separation conditions: 20 mM borate pH 9.1. Peak identification: 1=caffeine (EOF), 2=dyphylline, 3=theophylline. Concentration of each compound 0.5 mM. Other separation conditions as in Figure 2.2.

Since the total length of the capillary was changed, the electric field has also changed. The linearity of the Ohm's law plot was tested again. The increase in current with voltage was proportional (R^2=0.9967) across the whole interval. The maximum voltage of 30 kV was chosen to achieve the faster separation.

2.3.2.2 Temperature effect

A series of experiments were accomplished in order to investigate the influence of varying temperature on the separation of caffeine, theophylline and dyphylline.

The Agilent CE system is supplied with a thermostat, which allows the temperature of the cassette to be maintained at the 4-60°C. The separation of caffeine, theophylline and dyphylline was performed at 25, 35, and 45, 55°C. From Figure 2.10 it is clear that an increase in the temperature strongly affected the migration parameters of the separation.

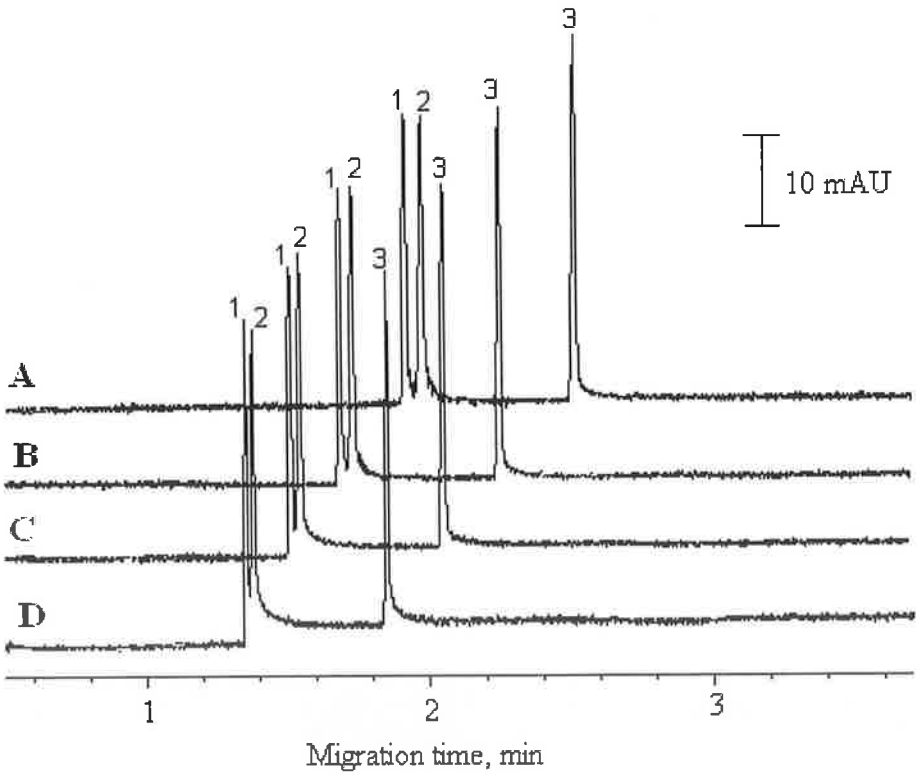


Figure 2.10. Temperature effect. Separation conditions: 20 mM borate pH 9.1. A – 25°C, B - 35°C, C - 45°C, D - 55°C; separation voltage 30 kV; capillary 56.3 cm (48.3 cm - effective length), 50 μm i.d.; hydrodynamic injection 4 seconds; UV detection 200 nm. Peak identification: 1=caffeine (EOF), 2=dyphylline, 3=theophylline. Concentration of each compound 0.5 mM.

Thus, the increase in temperature of the cassette provoked a decrease in resolution of caffeine and dyphylline accompanied by peak broadening. As mentioned above, the resolution of these two compounds is based on complexation of dyphylline with borate ions; as expected, with the temperature increase this complex becomes weaker and, thus, the resolution disimproves. Tailing off of the peaks occurred due to the increase of the diffusion due to overall elevated column temperatures, or radial gradients in temperature causing changes in the pH, mobility, distortion of the flat zone profile, and convection [64].

Increased temperature of the separation resulted in significantly shorter migration time of analytes. Further experiments showed that by increasing the pH of 20 mM borate buffer to 9.4 (previously 9.1) at 55°C, the resolution of caffeine and dyphylline peaks approached 1.5.

Figure 2.11 shows that the current, generated in the capillary at 55°C was nearly twice as great as that generated at 25°C, but the increase in current with voltage remained proportional (R^2=0.9994), therefore the maximum applied voltage 30 kV still could be used for faster separation.

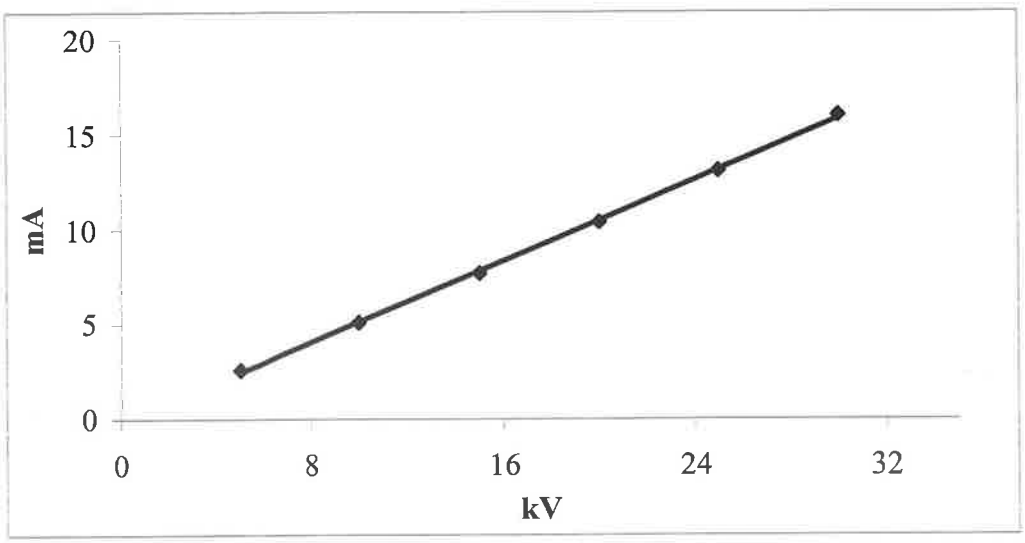


Figure 2.11. Ohm's law plot representing current generated as a function of different applied voltage. Separation conditions: 20 mM borate pH 9.4, capillary length 56.3 cm (48.3 cm to the detector), temperature 55°C.

2.3.2.3 Injection type

CE requires only minute volumes of sample to be loaded into the capillary to maintain high efficiency [60]. Typically the plug length of the sample should be 1-2% of the whole capillary length. Hydrodynamic and electrokinetic injections were investigated in this study. Hydrodynamic injection was performed by applying pressure of 50 mbar for 1, 2, 3, 4, 5, and 6, 10 sec. The parameters of the obtained separations are represented in the Table 2.7.

Table 2.7. Parameters of optimisation of time of hydrodynamic injection (50 mbar). n = 3

Injection time, sec	R *	N caffeine	N dyphylline	N theophylline
1	3.3	386135±28054	120240±3621	447919±21458
2	3.1	293646±32301	116976±13721	421583±38822
3	3.1	259855±54688	123082±5835	371223±53747
4	2.6	166137±3494	99527±1595	236694±5672
5	2.3	100281±11548	77975±5959	152321±18787
6	2.2	97088±12250	75820±8593	146536±38906
10	1.4	33131±682	33107±713	45596±509

* R – resolution of caffeine and dyphylline peaks. The resolution of dyphylline and theophylline was greater than 10 in all experiments.

Electrokinetic injection was accomplished by applying the voltage when the injection end of the capillary was in the sample vial. The results of applying 3, 5, 8, 10, 15 kV for 5 sec are shown in the Table 2.8.

Table 2.8. Parameters of the optimisation of voltage of electrokinetic injection (5 sec). n = 3

Voltage, kV	R *	N caffeine	N dyphylline	N theophylline
3	3.1	281357±35737	119534±8220	422449±43470
5	2.6	153508±5032	101382±1805	329708±14095
8	2.0	79536±872	70672±765	207866±1236
10	1.6	41505±226	42256±405	110880±5698
15	1.1	26255±1065	20888±182	45475±59

* R – resolution of caffeine and dyphylline peaks. The resolution of dyphylline and theophylline was greater than 10 in all experiments.

Form Table 2.7 and 2.8 it can be seen that the increased amount of sample loaded onto the capillary resulted in a decrease in resolution of caffeine and dyphylline and efficiency of all peaks. In case of analysis where the sensitivity of the method is of high importance, the hydrodynamic injection over a longer period of time or electrokinetic injection by a higher voltage is favourable. In this study higher resolution of caffeine and dyphylline and high efficiency of all peaks was taking into the account. The optimum parameters of hydrodynamic injection were 1 sec at 50 mbar and electrokinetic injection – 3 kV for 5 sec. In this case hydrodynamic injection is somewhat more preferable.

2.3.3 Other parameters of separation

Caffeine, theophylline, dyphylline, theobromine and enprofylline were involved at this stage of the study. Theobromine has the same molecular formula as theophylline, but the difference in structure is that one of the $-CH_3$ radicals is at N7 and proton is at N1 (Figure 2.1), pKa 10. Enprofylline is a derivative of theophylline with $-CH_2CH_2CH_3$ at N3 and two protons at N1 and N7 (Figure 2.1), pKa 8.4. At pH 9.3 theobromine was partially negatively charged, which was sufficient for its mobility to be different from all other compounds in the sample and elute the capillary after caffeine and dyphylline.

The difference in pKa of enprofylline and theophylline is 0.4 (Table 2.1) and, therefore, pH 9.4 was not sufficient for their separation even though they were both fully deprotonated. As expected, the long alkyl radical at N3 atom of enprofylline partially compensates the negative charge that appears due to deprotonation of the molecule and that is how enprofylline migrated faster than theophylline in the capillary. A baseline separation of enprofylline and theophylline was achieved with a slight increase in pH of the run buffer or by increased temperature of the separation. The electropherograms of the obtained separations are presented in Figure 2.12. The increase in the temperature was preferential due to the shorter analysis time while the baseline resolution of caffeine – dyphylline and enprophylline – theophylline was achieved (>1.5).

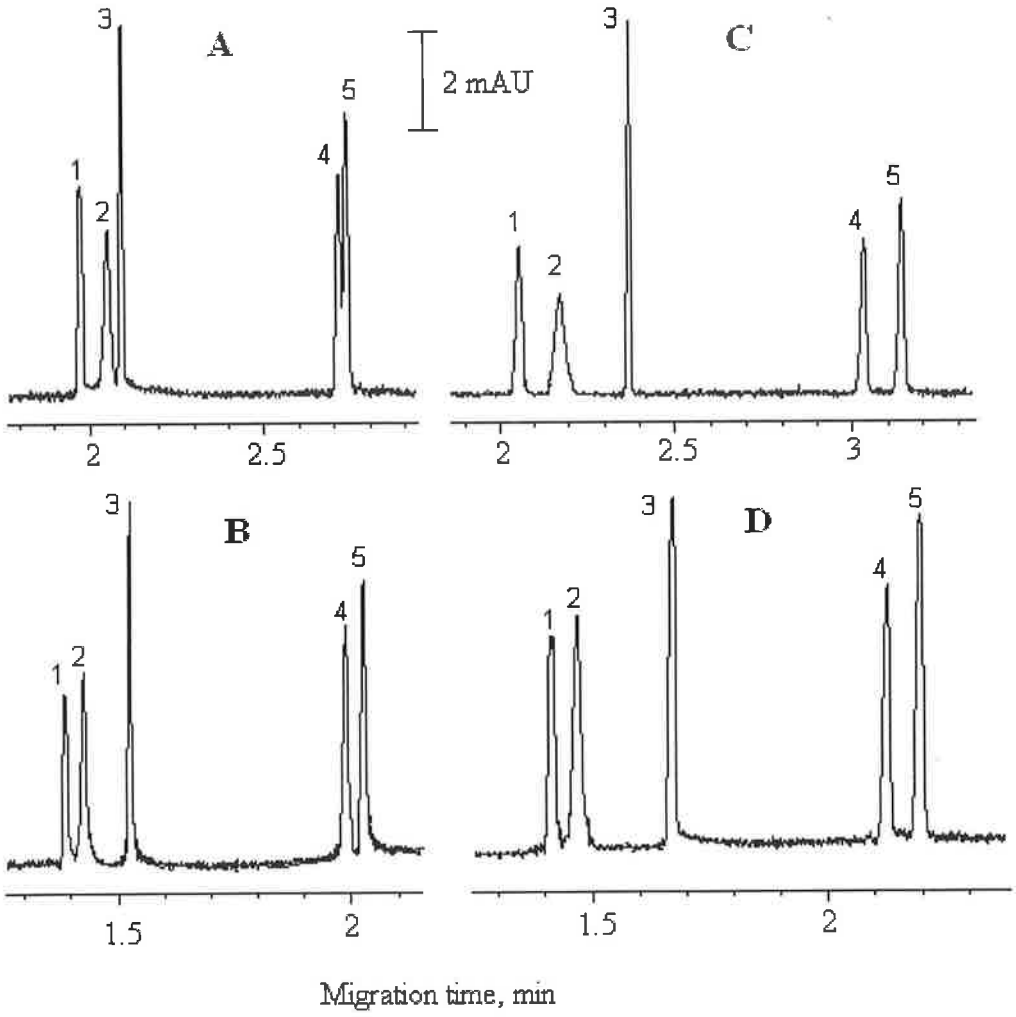


Figure 2.12. Electropherograms of the alkylxanthine separation at different temperature and pH of the run buffer. Separation conditions: 20 mM borate, A – 25°C, pH 9.4, B – 55°C, pH 9.4, C – 25°C, pH 9.6, D – 55°C, pH 9.6; separation voltage 30 kV, capillary 56.3 cm (48.3 cm - effective length), 50 µm i.d., hydrodynamic injection 1 second, UV detection 200 nm. Peak identification: 1=caffeine, 2=dyphylline, 3=theobromine, 4=enprophylline, 5=theophylline. Concentration of each compound 0.05 mM.

2.3.3.1 Rinsing between runs

Many studies have shown lengthy rinse steps in CZE (Table 2.2). This adds to the overall analysis time and is often not reported, thereby providing an ambiguous or false impression of the actual analysis time. A series of experiments were performed in order to investigate the influence of capillary rinsing between runs on the separation. As stated in section 2.2.5, all experiments before this were accomplished with 2 min 0.1 N NaOH, 5 min water, and 5 min buffer rinse. Rinsing time was reduced with all possible combinations of rinses and finally it was found that rinsing could be excluded altogether. Resolution, efficiency and migration time were not affected by this significantly. Some of the results are presented in Table 2.9.

Further experiments were accomplished without rinsing between runs. It allowed a single analysis to be performed in 2 min. And this is the shortest reported time for separation of caffeine, theophylline, dyphylline, enprophylline and theobromine by CZE to the author's knowledge. For 100 injections the migration time demonstrated that buffer depletion had began to occur >1.5% %RSD.

Table 2.9. Integration data for optimising rinsing conditions between runs (n=5). 1 – caffeine, 2=dyphylline, 3=theobromine, 4=enprophylline, 5=theophylline.

№[a]	N1	N2	N3	N4	N5	R1-2[b]	R4-5
1	48907	53547	113432	106452	117494	1.6	1.7
2	67412	70238	147768	149214	162165	1.8	2
3	88326	74792	239434	210579	225465	2	2.3
4	42588	47087	101726	80278	87721	1.5	1.4
5	38345	47801	110259	102368	103683	1.5	1.7

a - Conditions 1 - 1,5 min 0,1 N NaOH+1,5 min water+3 min buff;
Conditions 2 - 1,5 min 0,1 N NaOH+1,5 min water+2 min buff;
Conditions 3 - 1,5 min 0,1 N NaOH+1,5 min water+1 min buff;
Conditions 4 - 1 min 0,1 N NaOH+1 min water+3 min buff;
Conditions 5 - No rinse, injection only;
b - **R2-3** > 5 and **R3-4** > 17 in all cases.

2.3.3.2 Calibration

The calibration graphs were established with the peak-area as ordinate versus the concentration in mM as axis. From the slopes of the curves the method showed greater sensitivity towards theophylline determination and least for dyphylline. The linear regression equations were obtained as follows: for caffeine assay: $y = 17.958x + 0.1636$, $n=5$, $R^2=0.9997$; for theophylline assay: $y = 23.508x + 1.1693$, $n=5$, $R^2=0.9991$; for dyphylline assay; $y = 17.609x + 1.1147$, $n=5$, $R^2=0.9985$; for theobromine assay: $y = 20.402x + 0.0001$, $n=5$, $R^2=0.9991$; for enprophylline: $y = 20.091x + 0.0216$, $n=5$, $R^2=0.9995$. The data indicates good linearity of the method over the studied range 0.01 mM – 8 mM for caffeine and dyphylline, 0.01 mM – 10 mM for theophylline, 0.01 mM – 1 mM for theobromine and 0.001 - 0.8 mM for enprofylline.

2.3.3.3 Precision of the method

In order to measure the precision of the developed method the repeatability of the migration time, peak area and concentration for each analyte were determined. Relative standard deviations of the migration time and peak area of analytes are shown in Table 2.10. Here it can be noted that acceptable %RSD values were achieved. Table 2.11 represents data on repeatability of the method. Here three concentrations are sown (each measured in triplicate) and %RSD for all measurements are below 5%. Results show that in general at higher analyte concentration in each case %RSD values improved with the exception of enprofylline.

Table 2.10. Repeatability parameters of the migration time and peak areas. n = 5

Analyte	t_m (min)	%RSD	Peak area	%RSD
Caffeine	1.28	0.10	3.4	1.67
Dyphylline	1.32	0.06	3.5	1.42
Theobromine	1.42	0.14	4.7	1.24
Enprofylline	1.81	0.22	1.0	3.54
Theophylline	1.85	0.11	4.9	2.06

Table 2.11. Data on repeatability of the method. n = 3

Concentration known (mM)	Concentration found (mM)	RSD (%)	95% Confidence interval
Caffeine			
0.5	0.525 ± 0.015	2.79	0.508 – 0.542
1	1.047 ± 0.032	3.06	1.011 – 1.083
5	5.236 ± 0.106	2.03	5.116 – 5.356
Theophylline			
0.5	0.520 ± 0.016	3.05	0.502 – 0.538
1	1.045 ± 0.017	1.67	1.025 – 1.065
5	5.090 ± 0.056	1.10	5.028 – 5.154
Dyphylline			
0.5	0.524 ± 0.017	3.26	0.504 – 0.543
1	1.048 ± 0.031	2.98	1.012 – 1.083
5	5.236 ± 0.098	1.86	5.126 – 5.347
Theobromine			
0.1	0.103 ± 0.006	4.76	0.097 – 0.108
0.5	0.514 ± 0.021	4.15	0.490 – 0.539
1	1.021 ± 0.006	0.55	1.015 – 1.028
Enprofylline			
0.1	0.107 ± 0.007	2.70	0.103 – 0.109
0.5	0.509 ± 0.006	1.13	0.503 – 0.516
0.8	0.793 ± 0.017	2.20	0.773 – 0.813

2.3.3.4 LOQs and LODs

For this study the limits of detection were calculated on the basis of the baseline noise, where LOD is defined as the concentration of sample that generates a peak of height 3 times the level of the baseline noise. Limits of quantitation were defined as the concentration of sample that generates a peak of height 10 times the level of the baseline noise. The LODs and LOQs are listed in the Table 2.12. Detection for the study varied between 1.8 mg L^{-1} and 2.5 mg L^{-1}. This range is ideally suited for determination of the samples under investigation, as will be discussed later.

Table 2.12. Limits of detection and quantitation.

Compound	LOD, M	LOD, mg L^{-1}	LOQ, mg L^{-1}
Caffeine	$1*10^{-5}$	1.9	3.8
Theophylline	$1*10^{-5}$	1.8	5.4
Dyphylline	$1*10^{-5}$	2.5	10
Theobromine	$1*10^{-5}$	1.8	5.4
Enprofylline	$1*10^{-5}$	1.9	5.7

2.3.4 Application to real samples

The applications of the proposed method in the quality control of theobromine in chocolate samples and theophylline in tablets were studied.

2.3.4.1. Chocolate

Methylxanthine has previously been determined in different types of food and beverages, including coffee, tea, carbonated beverages, some chocolate products, caffeinated water, and chewing gum [5]. The majority of research has focused on the analysis of methylxanthines in coffee, tea and cola beverages [65]. Different variations of HPLC [17, 32, 33, 37, 66] and CE [24, 37, 38, 40, 41] methods were commonly used to determine these aqueous-based beverages and the sample preparation required was minimal. Paper substrate room temperature phosphorescence was reported in the analysis of chocolate, tea and coffee [31]. Noticeably less data exists on the contents of methylxanthines in chocolate food and beverages [65]. Caffeine and theobromine contents have been reported for some chocolate products including commercial hot chocolate [67], bakery products [68], chocolate milk [69], cocoa powder [70] and chocolate cereal [71]. To the author's knowledge there is no information about the CE method being employed in the analysis of methylxanthines in chocolate. And thus this work is the first determination of theobromine in chocolate by CZE.

Due to the global consumption of chocolate and the potential physiological effects of theobromine, both health professionals and consumers desire to know the theobromine contents of food. Levels of theobromine in chocolate products vary and depend on the type of chocolate. For instance, there are higher quantities of theobromine in plain chocolate and in higher quality chocolate; while milk chocolate and lower quality chocolate contains lower levels of theobromine [72].

Sample pretreatment is the most difficult issue in the analysis of any type of food. Since the extraction of theobromine from the chocolate was not the goal of this work, the sample preparation technique was taken directly from [59] and was not optimised. Eight different chocolates from 3 brands and one type of cocoa powder were analysed.

As expected, theobromine was determined in all samples of milk, dark chocolate and cocoa powder, but not in the samples of white chocolate. Figure 2.13 shows electropherograms of the white chocolate "Milkybar", milk chocolate "Bubbly" and dark chocolate "Bournville". It is noticeable from the electropherogram that the concentration of theobromine in dark chocolate was higher than in milk chocolate (as expected from % cocoa in the sample).

The time of a single analysis was less than 2 min. There are three peaks on the electropherogram. The first peak on each electropherogram corresponded to caffeine (spiked) and other components of the sample that migrated with EOF. Since the sample was dissolved in ethanol, the negative peak on the electropherograms was the ethanol peak. The theobromine peak did not interfere with any of them. The calibration of theobromine in ethanol was performed for this analysis. The bracketing standards method was employed in order to reliably quanitate theobromine in chocolate samples. During one day of analysis theobromine standards (n=5) were injected before and after the injections of the chocolate samples to ensure that the obtained concentration of standards was in agreement with the calibration curve. The concentrations of theobromine in all the samples are represented in a Table 2.13. The bar chart in Figure 2.14 represents a comparison between the amount of theobromine and cocoa solids in the chocolate bars.

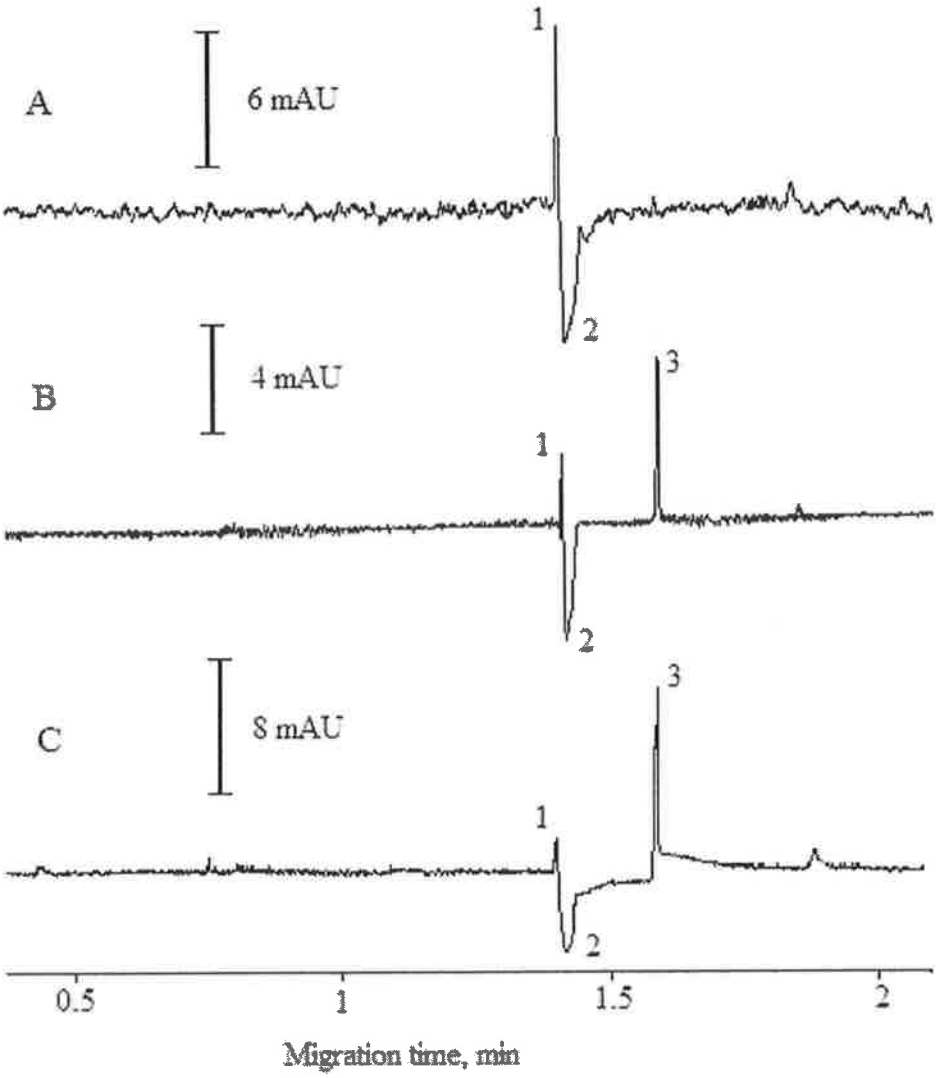


Figure 2.13. Electropherograms of chocolate samples. A – Milkybar, B – Bubbly, C – Bournville. Peak identification: 1=EOF, 2=ethanol, 3=theobromine. Separation conditions as in Figure 2.12 (B).

Table 2.13. Concnetrationof theobrominein the analysed samples.

Name of chocolate	Type of chocolate	Theobromine content, mg g^{-1}
Cadbury "Bubbly"	milk	0.78±0.08
Cadbury "Bournville"	dark	1.46±0.08
Nestle "Milkybar"	white	Not detected
Nestle "Aero All Bubble"	milk	0.81±0.05
Nestle "Aero All Bubble"	milk and mint	0.44±0.07
Cadbury "Buttons"	milk	0.58±0.02
Cadbury "Buttons"	white	Not detected
Terry's "Orange"	milk	0.67±0.11
Cadbury "Cocoa"	cocoa powder	3.91±0.34

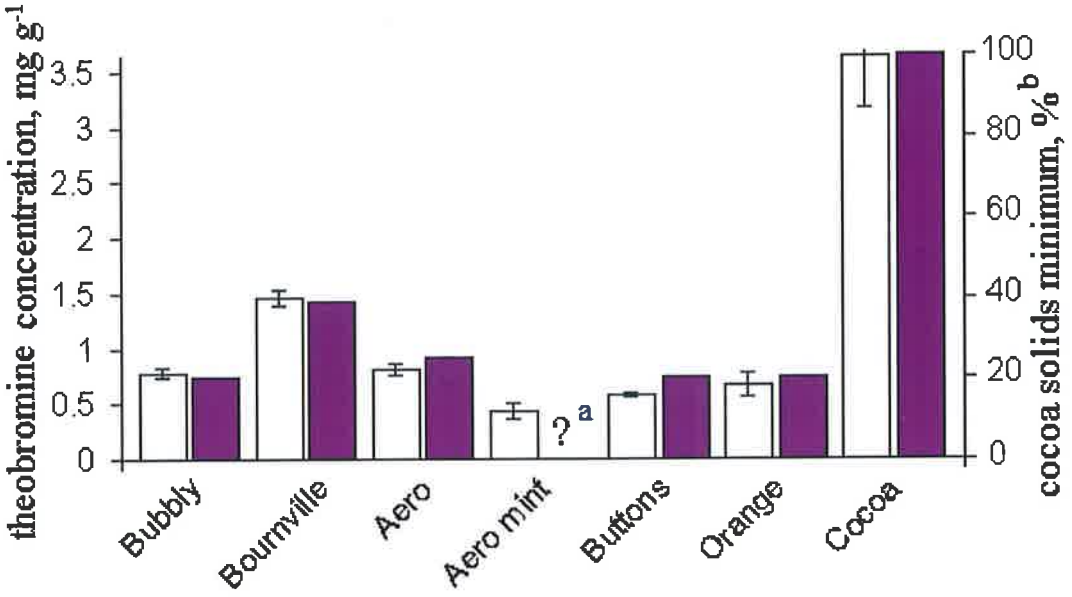


Figure 2.14: The comparison of determined concentration of theobromine and cocoa solids in analysed samples. □ - theobromine concentration, mg g^{-1} , ■ - cocoa solids minimum, %
a – no data, b - information taken from the wrapping.

The highest concentration of theobromine in cocoa powder corresponded to 100% of cocoa solids. A theobromine concentration of 1.46 mg g^{-1} in dark chocolate "Bournville" approached very close to 39% of cocoa solids. All samples of milk chocolate with 20-25% of cocoa solids had some variations in the theobromine concentration. Nestle chocolate "Aero All Bubble" (mint) contained noticeably less theobromine. This is due to the nature of this chocolate, which consists of mint part and milk chocolate outer coating. There is no data on cocoa solids contained on the product, but it is clear that it is only the chocolate part that contains it.

Table 2.14 represents statistical analysis of the data obtained. Relative standard deviations for peak area were less than 5%, while the %RSD of the extraction was over 5%, and especially high in the case of Nestle "Aero All Bubble"(mint) (%RSD 15.02). The reason for this is the heterogeneous nature of the sample. As previously stated, the extraction method was not optimised since it was not the objective of this work.

Table 2.14. Relative standard deviations of CE analysis and extraction. n = 3

Sample	%RSD (area)	%RSD (extraction)
Cadbury	1.72	
"Bournville"	1.79	5.21
	3.32	
Nestle	2.94	
"Aero All Bubble"	2.86	5.81
	0	
Nestle	4.35	
"Aero All Bubble" mint	0	15.02
	3.46	

2.3.4.2 Pharmaceuticals

A tablet sample containing theophylline was analysed using the optimised method. The labeled amount of theophylline was 300 mg. An electropherogram of the theopek sample is shown in Figure 2.15. A single analysis was completed in less than 2 min. Theophylline was the only peak in this separation. Analytical results for content uniformity of theophylline tablet obtained from a commercial source are shown in Table 2.15. Analytical values fell within labeled amount of 94.0-106.0% for theophylline required by the USP 25 [73].

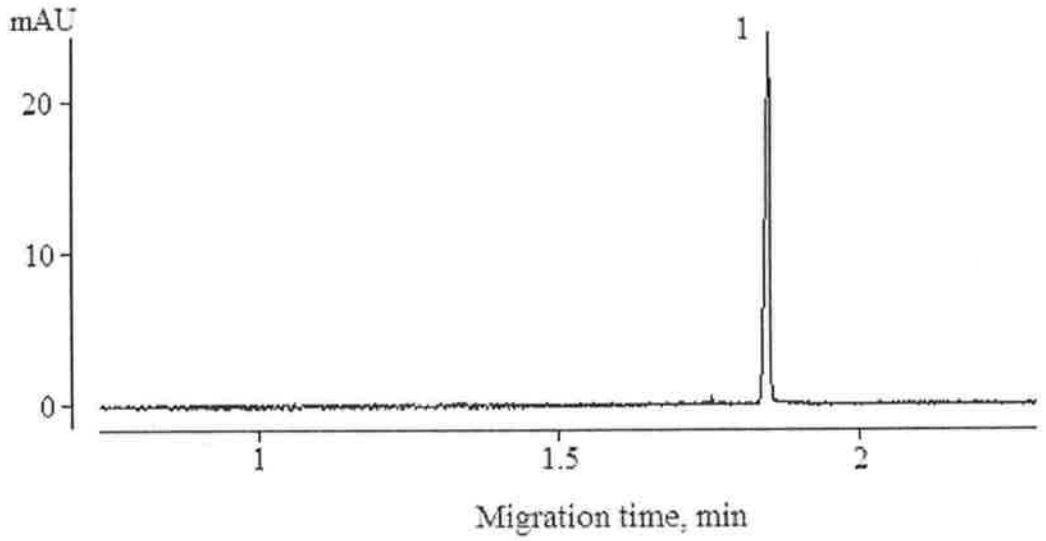


Figure 2.15. Electropherogram of commercial sample. Peak identification: 1=theophylline. Separation conditions as in Figure 2.12 (B).

Table 2.15. Analytical results for content uniformity of theophylline tablet obtained from a commercial source

Tablet[a]	Amount found[b] (mg)	Claimed content (%)
1	292.6 ± 5.2	98
2	304.3 ± 14.9	101
3	291.9 ± 6.3	97
4	303.9 ± 20.6	101
5	297.0 ±11.0	99
6	315.4 ± 13.0	105
7	292.6 ± 2.6	98
8	289.3 ± 5.4	96
9	293.3 ± 9.7	98
10	283.6 ± 9.6	95
Mean (%)		99
S.D.		2.9

[a] Labeled amount of theophylline in each tablet was 300 mg.

[b] Mean ± S.D. (n=3)

2.4 CONCLUSIONS

A rapid CZE method was developed for the determination of alkylxanthines in pharmaceuticals and food samples. The main advantage of this method is a very short (2 min) full time of a single analysis. It is the shortest reported time of a complete analysis of above mentioned components by CZE to the author's knowledge. While 40 sec was reported [57] for analysis of caffeine and theophylline with CE microchip, the devise has to be rinsed for 15 min with the running electrolyte prior to the injection. The same method can be used as for the separation of single compounds as for the mixture of them. The peculiarities of the method are as follows: caffeine cannot be determined if other neutral components are present that migrate with EOF (then MEKC must be employed). If dyphylline is analysed, samples must be dissolved in the borate buffer with concentration and pH close to the run buffer (20 mM borate pH 9.4). It is advisable to rinse the capillary with 1 M HCl, 1 N NaOH, MeOH, distilled water and run buffer once a day (before the analysis). If lower LODs are required, the injection volume may be increased.

This method is a good alternative technique to HPLC and could be useful for quality control in pharmaceutical and food industries. The proposed method has been successfully applied for the essay of theobromine in chocolate samples and theophylline in commercial tablets.

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CHAPTER 3

DETERMINATION OF ASSOCIATION CONSTANTS OF INCLUSION COMPLEXES OF STEROID HORMONES AND CYCLODEXTRINS

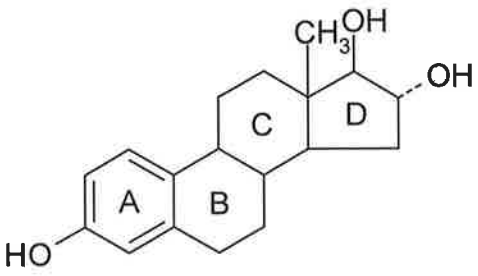
3.1 INTRODUCTION

There is considerable analytical interest in the determination of estrogens. This interest is due to their physiological functions in the body and the potential danger in disruption of the endocrine system. Recently studies linking estrogens to the feminisation of fish, breast and ovarian cancer in women and reproductive health problems in men have generated further interest in these chemicals [1].

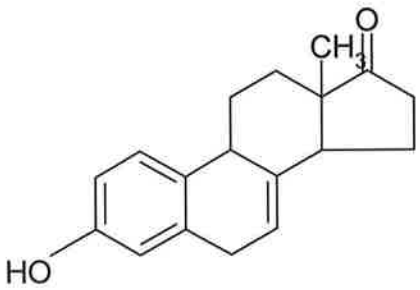
3.1.1 Estrogens

Estrogens belong to the group of hormones called steroids. They are synthesized from cholesterol. Prominent in their structure is a series of carbon rings: typically three six-membered rings conjugated with a five-membered one [2]. Several compounds with a structure similar to natural estrogens have been synthesised for different purposes. Chemical formulas of some natural and synthetic estrogens are shown in Figure 3.1.

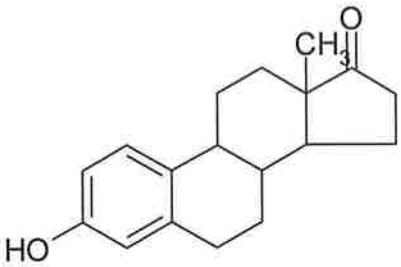
The pKa values of steroids are high (Table 3.1), which means they are more likely to remain in protonated form even at alkaline pH. They display a property of hydrophobic compounds – the magnitudes of solubility are rather low (Table 3.1).



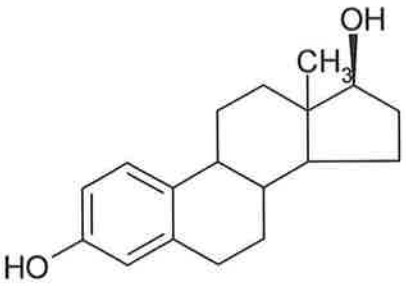
(1) Estriol



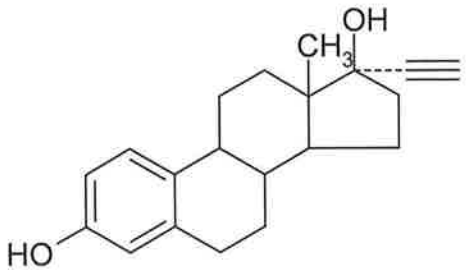
(2) Equilin



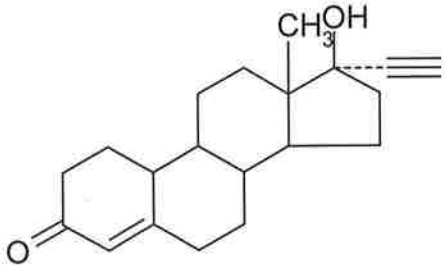
(3) Estrone



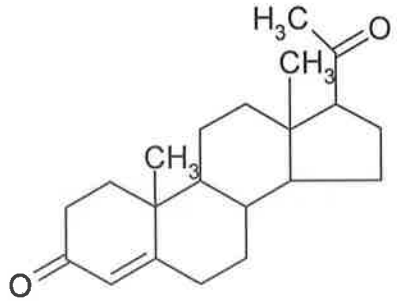
(4) 17β-Estradiol



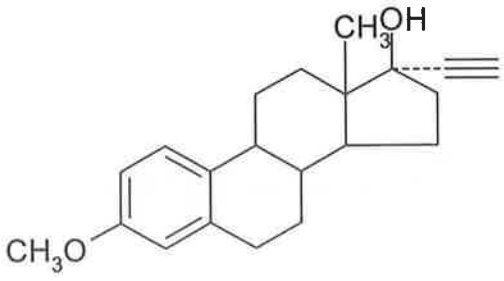
(5) Ethinylestradiol



(6) Norethindrone



(7) Progesterone



(8) Mestranol

Figure 3.1. Structures of steroids.

Table 3.1. Physical characteristics of some steroid hormones.

Analyte	MW, g $mole^{-1}$	pKa	Solubility in water, [3] µg mL^{-1} [8] mg L^{-1}	Log K_{ow} [a] [8]	Dipole moment [9], Debye
Estrone	270.37	10.34 [3], 10.91 [4], 10.26 [5,6], 10.77 [7]	0.8 [3], 13 [8]	3.43	3.35
17α-Estradiol	272.30	10.46 [3], 10.10 [4], 10.30 [5,6]	3.9 [3]	-	2.32
17β-Estradiol	272.30	10.71 [7]	13 [8]	3.94	1.25
Ethinylestradiol	296.40	10.40 [3]	9.7 [3], 4.8 [8]	4.15	-
Estriol	288.39	10.38 [3]	3.2 [3], 13 [8]	2.81	2.30
Equilin	268.35	10.26 [3]	1.4 [3]	-	-

a – octanol-water partition coefficient.

3.1.2 Determination of estrogens

3.1.2.1 Biological assay

The biological potency of estrogens can be determined using *in vivo* or *in vitro* tests. Their activity is expressed relative to that of estradiol, which is the most common endogenous estrogen. Immunoassays, such as radioimmunoassay [10], receptor binding assays [11], DNA binding assays [11] and others, are very important for their ongoing contribution to estrogen analysis. At the same time they contribute to our understanding of estrogen biochemistry and physiology [12]. Despite the wide variety of assays, generally they produce very consistent results, which mean that if a compound is estrogenic in one assay is estrogenic in all others [13]. A chemical that is estrogenic to one living species is estrogenic to all other animals due to the conserved structure of estrogen receptor between species. Currently 0.4 mL or more of plasma is required to test just one estrogen by immunoassay in general [12]. One of the lowest limits of detection 0.48 pg mL^{-1} was reported for estradiol by non-extraction chemiluminescent assay [14].

3.1.2.2 HPLC

Compounds of polar and non-polar nature can be efficiently separated by HPLC through changes in stationary and mobile phases. The octanol-water partition coefficients of estrogens from Table 3.1 show that they are quite hydrophobic. In general, reversed-phase HPLC columns are used in estrogen analysis; the mobile phase consists of acetonitrile and/or methanol in combination with water containing some acid.

All estrogens and their metabolites contain at least one aromatic ring in their structure, which is responsible for their ability to absorb UV light. The UV detection method is widely used in the determination of estrogens [15-17]. Detection based on fluorescence is generally more sensitive than UV absorption [18]. For example, in the

method of Yoon *et al.* [19] for the determination of 17β-estradiol, ethynyl estradiol and bisphenol A by HPLC with fluorescence detection, the LODs for 17β-estradiol and ethynyl estradiol were 1.15 and 0.96 nM, respectively. The detection was performed at an excitation wavelength of 280 nm and an emission wavelength of 310 nm. Electrochemical detection combined with the HPLC separation was also able to provide very sensitive detection of estrogens. This technique was used by Devanesan *et al.* [20] to detect 1 pmole (~300 pg) of estrogen in a sample, which was 1g of tissue of hamster treated with 4-hydroxyestradiol. LODs and LOQs for estrogens in a range of 0.1 – 10 ng mL^{-1} have been reported when using HPLC-ESI-MS-MS [26, 27]. However, liquid-liquid extraction and derivatisation with dansyl chloride had to be employed in a study of Zhang *et al.* [27] in order to be able to detect 17α-ethynyl estradiol in the aquatic environment.

The use of chiral stationary phases can alter the selectivity of HPLC. A β-cyclodextrin was employed in several analyses as a chiral stationary phase to separate structurally related estrogens, natural and synthetic [21-23], or chiral selector can be included in the mobile phase. Several articles by Zarzycki *et al.* [24, 25] are dedicated to the investigation of the effect of β-cyclodextrin and its concentration in the mobile phase for separation of 6 to 8 estrogens. The retention of steroids was strongly influenced by temperature when 12 mM of β-CD was added to the mobile phase.

Unlike GC-MC, a liquid chromatographic technique does not require compounds to be volatile or to have high molecular weight. HPLC-MS has its disadvantages in not being as sensitive as some biological immunoassays but it is available for the analysis of a wide range of compounds and not limited by the availability of specific antiserum [28].

3.1.2.3 Gas chromatography

GC-MS has also been employed extensively for the determination of several estrogens [28-32]. The technique is advantageous in terms of good separation efficiency and selectivity, small sample and reagent demand and is readily coupled to mass spectrometric detection. This provides the sensitivity required for the quantification of environmental and biological levels of estrogens in real samples. They are often present in ng L^{-1} to μg L^{-1} range. Estrogens are not volatile, therefore GC-MS analyses are usually carried out after derivatisation of analytes, which means longer preparation time and increased risk of error.

Hernando *et al.* [29] optimised and compared two GC methods – GC-MS and GC-MS-MS for the analysis of five EDCs in wastewaters (with estrone, 17β-estradiol and ethynyl estradiol among them). For the GC-MS method, compounds were derivatised with N,O-bis(trimethylsilyl)trifluoracetamide. Both methods included solid phase extraction allowing an enrichment factor for wastewater samples of 100-fold. Validation studies obtained comparable results in both cases. LODs for GC-MS and GC-MS-MS were 8.5 and 7.5 ng L^{-1} (estrone), 17.0 and 27.5 ng L^{-1} (17β-estradiol) and 4.0 and 17.5 ng L^{-1} (ethynyl estradiol), respectively.

A combination of different techniques produced promising results in the determination of steroids. R. Gibson *et al.* [16] have developed an analytical method to detect estrogenic compounds in fish bile. Estrogenic metabolites in bile were deconjugated using enzymatic hydrolysis and concentrated by SPE prior to fractionation by reversed-phase HPLC. The estrogenic potency of active HPLC fractions was detected by yeast estrogen receptor transcription screen and analysed by GC-MS after trimethylsilylation. The whole method recovery was reported to be 81±7% (n=7).

3.1.2.4 Capillary electrophoresis

Table 3.1 contains pKa values of several estrogens. It can be seen that they are quite high, ranging from 10.10 to 10.91 and the difference in pKa between each analyte is not very significant, which means that the mechanism of CZE mode cannot be used for their separation. The addition of an organic solvent like methanol can improve the situation somewhat, as studied by Potter *et al.* [33].

In 1995 Chan *et al.* [34] showed successfully developed MEKC method for the determination of 10 estrogens. A 10 mM borate buffer with pH 9.2 containing 100 mM sodium cholate achieved the separation of 9 of 10 estrogens (4-hydroxyestradiol and 4-hydroxyestrone coeluted) in 12 min. UV detection was at 200 nm. The addition of 20% methanol to 10 mM phosphate buffer pH 7.0 containing 50 mM SDS allowed the separation of all 10 compounds in 24 min. Many of the estrogens have very similar structures and some of them are optical isomers, therefore the addition of cyclodextrin is helpful in that type of separation. In the same work Chan *et al.* [34] showed that γ-CD is better than β-CD for the analysis of 10 particular estrogens and allows complete separation in less than 10 min. Although the separation of all analytes was achieved, the reason for choosing the concentration of γ-CD to be 20 mM was not explored, nor was the change in the migration order of analytes with β- and γ-CD.

Ji *et al.* [35] published a method for the separation of urinary estrogens (estrone, estriol and estradiol with internal standard d-equilenin) by MEKC. In this study a cholate was chosen as the surfactant agent and the buffer was a mixture of 5 mM borate and 5 mM phosphate at pH 8.7. With the addition of 20% MeOH to the run buffer the separation of all analytes was achieved in 7 min.

Several recent publications [36-38] showed the advantages of CDs in the run buffer when analysing steroid hormones. Work carried out by Poole [36] studied separation of 10 steroids with α-CD, β-CD and γ-CD in the run buffer. The addition of 20 mM α-CD to the 20 mM sodium borate – sodium phosphate buffer (pH 8) containing 50 mM SDS did not improve resolution of any 10 analytes. While 20 mM β-CD in the run buffer separated 9 peaks out of 10 almost to baseline resolution.

When 20 mM γ-CD was used in place of β-CD significant changes in the migration order of analytes were observed and baseline resolution of all ten estrogens was obtained. It is difficult to judge the migration times of peaks from the figure in the paper but most of the compounds had shorter migration times with γ-CD, while 17α-dihydroequilin and equilin had longer migration time.

Munro and colleagues [37] investigated the enhancement in sensitivity of CE for the analysis of 17β-estradiol, estrone and estriol when charged cyclodextrin-mediated sample stacking was employed. The sulfated β-CD was reported to allow extremely long injections (up to 36% of the capillary) while still providing baseline resolution of the analytes (17 β-estradiol, estrone, estriol). The improvement in sensitivity allows UV detection (200 nm) of estrogens in the part-per-billion range. Different derivatives of cyclodextrin proved to be useful in the separation of estrogens. Deng *et al.* [39] showed that the addition of anionic sulfobutyl ether β-cyclodextrin has advantages over the neutral CD in the separation of five estrogens and two stereoisomers. Specifically, the amount of SBE-β-CD required was markedly less than the neutral CD. A 6-dimethyl- β-CD was employed in the separation of levonorgester, progesterone, testosterone and estradiol by MEKC [38]. Changes in the migration order were reported to have a complicated effect.

Harino *et al.* [40] reported LOD of 0.16-0.30 nM for estrogen in the sample of water that were achieved due to the combination of SPE treatment and sweeping with the MEKC separation method. Katayama *et al.* [41] reported simultaneous determination of a large number of compounds (16 estrogens, dehydroepiandrosterone and their glucuronide and sulphate conjugates) by MEKC within 14 min. A number of articles [42-44] showed the separation of estrogens along with other endocrine disrupting compounds (EDCs). In [44] as many as 19 endocrine EDCs of estrogenic and alkylphenolic nature were separated by MEKC in a single run.

3.1.3 The role of cyclodextrins in the analysis of estrogens

The overview of the existing methods for the determination of steroid hormones reveals that the employment of cyclodextrins in chromatographic [21-25] and, especially, capillary electrophoretic methods [34, 36-39] has the advantages of enhanced selectivity of the separations. The association of steroid hormones with certain types of cyclodextrin was studied by Sadlej-Sosnowska [45, 46] using HPLC and the binding constants of analyte-cyclodextrin equilibrium were determined. Several papers [47-49] have demonstrated that different techniques for the determination of association constants give rather different results, which is likely to be due to the differences in the experimental conditions required by each individual technique.

3.1.4 Theory of CE separation with cyclodextrin

The mechanism of the separation in cyclodextrin modified capillary electrophoresis is revised in detail in Chapter 1.4.3. Cyclodextrins are truncated cone-shaped molecules with a hollow, tapered cavity, which posses a hydrophobic property. It is known that various organic molecules form inclusion complexes with cyclodextrin [50]. Fundamental knowledge of equilibrium and the association constants of analysed compounds and ligand in the system is a powerful factor in the understanding of that system and in discovering ways to evaluate it.

The association of cyclodextrin C and analyte molecules A, and the dissociation of the formed complex AC are controlled by equilibrium for a 1:1 complexation scheme [51]:

$$A + C = AC \qquad \textbf{Equation 3.1}$$

where A, C and AC are the free analyte, cyclodextrin and the complex respectively.

The effective electrophoretic mobility (μ_e) of analyte represents the summary of the complexed (μ_c) and free (μ_f) solute [52]:

$$\mu_e = X_f\mu_f + X_c\mu_c \qquad \textbf{Equation 3.2}$$

where X_f is the molar fraction of free analyte and X_c is the the molar fraction of complexed analyte. Representing the molar fraction through the equilibrium concentration the following is obtained:

$$\mu_e = \frac{[A]}{[A]+[AC]}\mu_f + \frac{[AC]}{[A]+[AC]}\mu_c \qquad \textbf{Equation 3.3}$$

The expression of equilibrium constant for the considered case:

$$K = \frac{[AC]}{[A]_f[C]_f} \qquad \textbf{Equation 3.4}$$

Combined with equation 3.3 the mobility of analyte at any given concentration of the ligand equals [52]:

$$\mu_e = \frac{1}{1+K[C]}\mu_f + \frac{K[C]}{1+K[C]}\mu_c \qquad \textbf{Equation 3.5}$$

The model of that complexation was reviewed by Szejtli [51].

This expression can be rearranged in order to linearise it in a several ways [52]:

(a):

$$\frac{1}{(\mu_c - \mu_f)} = \frac{1}{(\mu_c - \mu_f)K}\frac{1}{[C]} + \frac{1}{(\mu_c - \mu_f)} \qquad \textbf{Equation 3.6}$$

The data can be transformed into a double reciprocal plot of $1/(\mu_e - \mu_f)$ versus $1/[C]$.

From this plot the apparent binding constant K=intercept/slope.

(b):

$$\frac{[C]}{(\mu_e - \mu_f)} = \frac{1}{(\mu_c - \mu_f)}[C] + \frac{1}{(\mu_c - \mu_f)K} \qquad \textbf{Equation 3.7}$$

This equation leads to Y-reciprocal plot of $[C]/(\mu_e - \mu_f)$ versus $[C]$. From there K=slope/intercept.

(c):

$$\frac{(\mu_c - \mu_f)}{[C]} = -K(\mu_e - \mu_f) + K(\mu_c - \mu_f) \qquad \textbf{Equation 3.8}$$

This leads to the X-reciprocal plot of $(\mu_e - \mu_f)/[C]$ versus $(\mu_e - \mu_f)$, where K = -slope.

3.1.5 Aim of this work

To the authors' knowledge the association constants for steroids and cyclodextrins have not previously been determined from capillary electrophoresis separation data. The aim of this work was to fill this gap, as this data has a high importance in the understanding of the electrophoretic behavior of analytes in the presence of cyclodextrins in the run buffer. The objectives involved the examination of the mechanism of inclusion of cyclodextrins and steroids. Different types of natural CDs and their derivatives were investigated in that regard. This work also demonstrated the potential of capillary electrophoresis as a fundamental research tool.

3.2 EXPERIMENTAL

3.2.1 Equipment

CE separations were performed using the system described in Chapter 2. The fused silica capillaries (Composite Metal Services ltd., The Chase, Hallow, Worcs. WR2 6LD, UK) were 54 cm in length (46 cm to the detector) with an internal diameter 50 μm.

3.2.2 Reagents

All analyte compounds investigated in the separations had purity of 99% and were used without further purification. Estriol, estrone, 17β-estradiol, ethinylestradiol, equilin, norethindrone, mestranol, progesterone, α-cyclodextrin, 2HP-α-CD, β-cyclodextrin, γ-cyclodextrin, 2HP-β-CD (degree of substitution ~ 4-10), 2HP-γ-CD (molecular substitution ~ 0.6), sodium phosphate (dibasic), HCl, NaOH, methanol (HPLC grade), SDS were purchased from Sigma-Aldrich, Dublin, Ireland. Buffers were prepared using distilled water and adjusted to the desired pH using 1 M and 0.1 M NaOH.

3.2.3 Standards

Stock solutions of estriol, estrone, ethinylestradiol, 17β-estradiol, equilin, norethindrone, mestranol and progesterone were prepared in methanol of concentration 1 mM. Stock solutions were stored in darkness and evaporation of methanol from the flasks was prevented.

3.2.4 Separation conditions

The electrophoretic buffer was optimised in advance and consisted of 20 mM borate pH 11.5 and 10 mM SDS. β-CD, γ-CD, 2HP-β-CD, 2HP-γ-CD were added to the buffer separately in a range of five concentrations from 1 mM to 10 mM. Separations were carried out at 30 kV electrophoretic voltage and at a temperature of 20°C. Injections were carried out hydrodynamically at 50 mbar for 2 sec. UV detection was at 214 nm and 254 nm. The concentration of each analyte in a mixture was 0.125 mM in methanol. Conditioning of the CE capillary between runs was performed with 0.1 M NaOH for 2 min and run buffer for 2 min. All separations were repeated at least three times.

3.3 RESULTS AND DISCUSSION

3.3.1 Initial conditions

The theory of the formation of inclusion complexes was reported to be valid only for cases of 1:1 stoichiometry [51]. A number of steroid hormones, including four of the target analytes in this work (estriol, 17β-estradiol, estrone and ethinylestradiol), have been investigated by N. Sadlej-Sosnowska [45, 46]. The conclusion was that 1:1 stoichiometry applies to the complexes of studied steroids with β-CD and γ-CD. Due to the high structural similarity of steroids it was assumed in this paper that inclusion of equilin, mestranol, norethindrone and progesterone follows the same pattern.

Throughout the series of experiments, the run buffer composition was held constant (except for cyclodextrin type and concentration which were varied). Thus the analytes were involved in two equilibria: the analyte-micelle and analyte-cyclodextrin. The presence of surfactant (SDS) and operating pH of 11.5 requires explanation. Compounds 1-5 (as in Figure 3.1) were in their deprotonated form at this pH because their pKa value lies between 10.26 and 10.71 [3, 5]. The structures of mestranol, norethindrone and progesterone pointed to even higher pKa values (pKa=13.1 for mestranol [53]) and thus they were neutral at the conditions of separation. Such close pKa magnitudes and molecular weights of steroids did not allow their separation in a simple mode of capillary zone electrophoresis. All target analytes were quite hydrophobic. The calculated $\log K_{ow}$ values are 2.81, 3.22, 3.43, 3.94, 4.12, 4.68, 2.99 and 3.67, for estriol, equilin, estrone, 17β-estradiol, ethinylestradiol, mestranol, norethindrone, and progesterone respectively [54]. The latter provoked their interaction with the micelles in the run buffer as well as cyclodextrins when added. The interaction between molecules of surfactant and cyclodextrins also takes place. In the work of Lin *et al.* [55] it was found that the complexes formed between β-CD and SDS monomers exist predominantly in the form of a 1:1 stoichiometry. The variations of the electrophoretic mobility of probe

molecules as a function of surfactant concentration suggested that as a consequence of a strong inclusion complexation between β-CD and SDS, the encapsulation of β-CD with probe molecules is greatly diminished. Even though the interaction of the cyclodextrins and analytes was to a less degree due to the surfactant, the presence of the micelles in the run buffer was necessary in order to observe the difference in the mobility of analytes while adding neutral cyclodextrins.

The top electropherogram in Figure 3.2(A) represented the separation of target estrogens at primary conditions, *e.g.* without cyclodextrin. It could be seen that analytes reached the detection window after the electro osmotic flow; association with negatively charged micelles was the reason for their migration towards the anode, which was against the EOF in this separation mode.

According to Rekharsky and Inoue [50] any differences in the free analyte's interaction with the buffer prior to inclusion complexation did not contribute measurably to the complexation thermodynamics. Since the concentration of SDS was held constant at every separation, the true effect of cyclodextrin was seen while adding it at different concentrations. The separation with 1 mM of each cyclodextrin is shown in Figure 3.2 (B-E). The change in migration time of analytes clearly demonstrated the occurrence of analyte-cyclodextrin interaction. The mobility changes were more significant in the case of γ-CD and 2HP-γ-CD than those of β-CD and 2HP-β-CD. Analyte mobility was increased due to the interaction with neutral cyclodextrins. Attraction to the anode was lower.

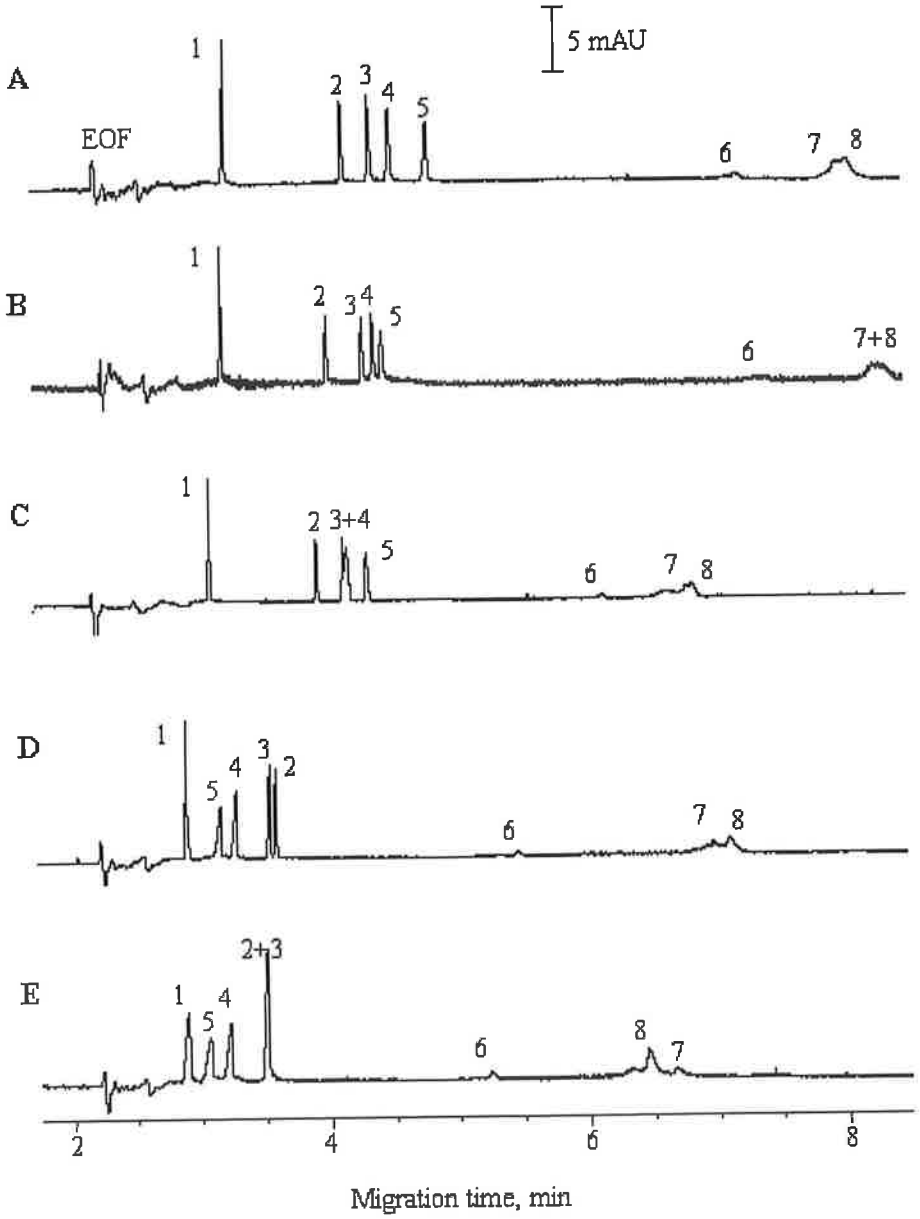


Figure 3.2: Electropherogram of separation of 8 steroid hormones. Separation conditions: 20 mM phosphate buffer pH 11.5 and 10 mM SDS, A – no CD added, B - 1 mM β-CD, C – 1 mM 2HP-β-CD, D – 1 mM γ-CD, E – 1 mM 2HP-γ-CD; separation voltage 30 kV; capillary 54 cm (46 cm is effective length), 50 μm i.d.; 20°C; hydrodynamic injection 2 seconds; UV detection 214 nm. Concentration of each analyte is 0.125 mM in methanol. Peak identification 1 – estriol, 2 – equilin, 3 - estrone, 4 - 17β-estradiol, 5 – ethinylestradiol, 6 – norethindrone, 7 – progesterone, 8 - mestranol.

Association constants were calculated from the mobility values of estrogens, which were determined at 1 mM, 3 mM, 5 mM, 7 mM and 10 mM of each cyclodextrin in the run buffer. The effect of the concentration of cyclodextrins in the separation buffer on the mobility of analytes is presented in Figure 3.3 – 3.7. The calculated values of the constants determined by three linearisation methods are given in Tables 3.2 – 3.5 along with R^2 of each plot and %RSD. The association constants for a single compound obtained using the plotting methods show quite different values. Regardless of that fact, the same relative order of the constants within the same plotting method was observed for each compound. The exception to this case is shown in Table 3.2. For 17β-estradiol and estriol the relative values for the double reciprocal fit data differed from Y-reciprocal and X-reciprocal fit. It was shown previously by Rundlett and Armstrong [56] that at low ligand concentrations (in this case cyclodextrin) the double reciprocal fit masks deviations from linearity. The data obtained in this work indeed proved this to be the case as R^2 values for the double reciprocal fit for each complexation shows good linearity. The negative association constant values however, pointed to the lack of inclusion of the analytes (progesterone and mestranol) with the CD. This leads to the conclusion that the Y- and X - fits are more reliable in assessing analyte (steroid) – ligand interaction.

3.3.2 α-CD

In this study an attempt was made to investigate the nature of the inclusion of steroids with α-CD. This study was performed using estriol, estrone, 17β-estradiol and ethinylestradiol. It can be seen in Figure 3.2 that the modification of the effective mobility value of the analytes in this study occurred quite gradually over the investigated concentration range of the cyclodextrin. The size of α-CD cavity (~5 Å) is not large enough for the inclusion complex to be formed, while the diameter of only one benzene ring is 5 Å. Thus, the formation of the inclusion complex of each steroid with α-CD was not possible. An attempt was made to arrange this data according to the 3.6 – 3.8. The values of R^2 for linearisation trendlines were very poor and apparent binding constants had negative values (results are not shown). This was expected since no inclusion complexes occurred.

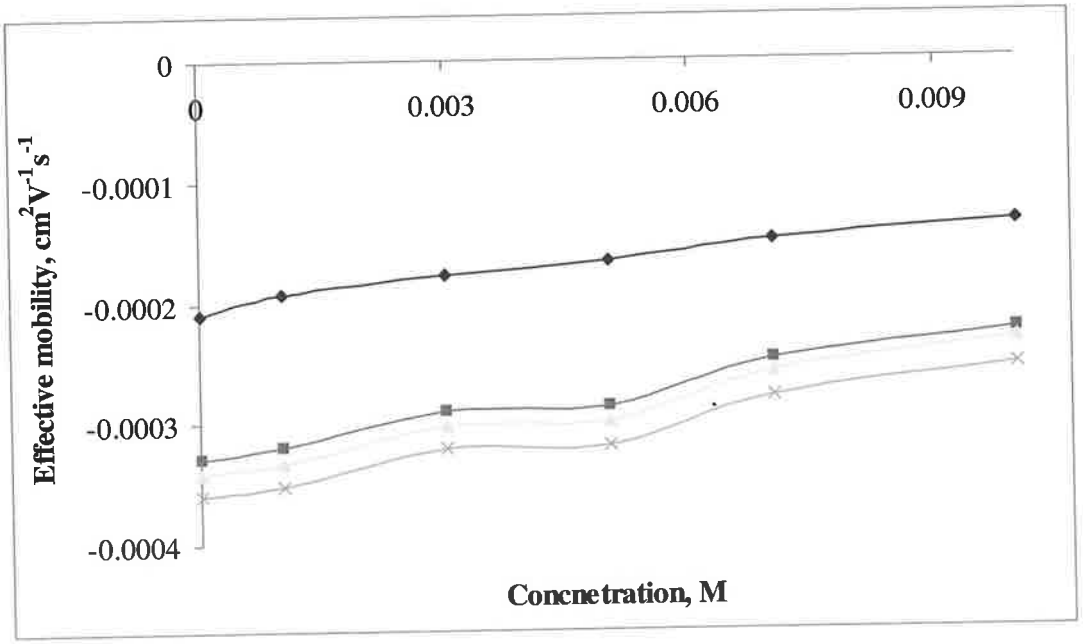


Figure 3.3: Effect of the concentration of α-CD on the electrophoretic mobility of steroids. Separation conditions as in Figure 3.2. Analyte identification:
Estriol, **17β-Estradiol,**
Estrone, **Ethinylestradiol.**

3.3.3 γ-CD

From this study it was found that the highest magnitudes of association constants belong to the inclusion of target steroids in γ-CD (Table 3.2). The diameter of the inside cavity of γ-CD was 10Å, which is more sterically suitable for the 4-ring conventional structure of analytes than β-CD [57] with the diameter 8Å. Large association constants of 331 M^{-1} (Y-reciprocal fit) for equilin and up to 1770 M^{-1} (X-reciprocal fit) for ethinylestradiol were observed.

The double reciprocal fit and the Y-reciprocal fit were found to have R^2 values in the range 0.9797-0.9989. The relative standard deviations were observed to be less than 3.5% for the association of compounds 1-5. The latter demonstrated that the analyte inclusion process took place with the CD and its stoichiometry was 1:1. It should be noted that the R^2 value for the X-reciprocal fit for those compounds lay in a lower range (R^2 = 0.8969-0.9665) of linearity. The effect of varying the CD concentration on the mobility was most pronounced using the X-reciprocal fit compared with other linearity approaches. The latter is due to the plot parameters of $(\mu_a - \mu_f)/[C]$ versus $(\mu_a - \mu_f)$. The concentration range chosen for the plots also affects the R^2 values obtained.

Table 3.2. The value of association constants of target steroids and γ-CD, R^2 for each linearisation method, %RSD (n=3) and literature values of constants.

	K (M^{-1})	%RSD	R^2	K (M^{-1}) lit.[46]
Estriol				3200±370
Double reciprocal fit	1282	0.95	0.9585	
Y-reciprocal fit	798	2.75	0.9936	
X-reciprocal fit	1088	1.32	0.8981	
Equilin				
Double reciprocal fit	477	3.44	0.9812	
Y-reciprocal fit	331	1.15	0.9821	
X-reciprocal fit	387	2.28	0.8969	
Estrone				2550±230
Double reciprocal fit	642	2.33	0.9797	
Y-reciprocal fit	461	0.83	0.9901	
X-reciprocal fit	545	1.64	0.9162	
17β-Estradiol				7100±400
Double reciprocal fit	1231	1.61	0.9844	
Y-reciprocal fit	953	1.73	0.9978	
X-reciprocal fit	1137	1.40	0.9583	
Ethinylestradiol				10600±960
Double reciprocal fit	1873	1.57	0.9842	
Y-reciprocal fit	1506	2.22	0.9989	
X-reciprocal fit	1770	1.51	0.9665	
Norethindrone				
Double reciprocal fit	91	4.64	0.9980	
Y-reciprocal fit	91	2.58	0.8669	
X-reciprocal fit	75	3.31	0.7375	
Progesterone				
Double reciprocal fit	-44	13.56	0.9984	
Y-reciprocal fit	-42	4.92	0.6164	
X-reciprocal fit	-52	2.44	0.7235	
Mestranol				
Double reciprocal fit	-62	12.12	0.9984	
Y-reciprocal fit	-44	7.29	0.5591	
X-reciprocal fit	-56	4.20	0.6997	

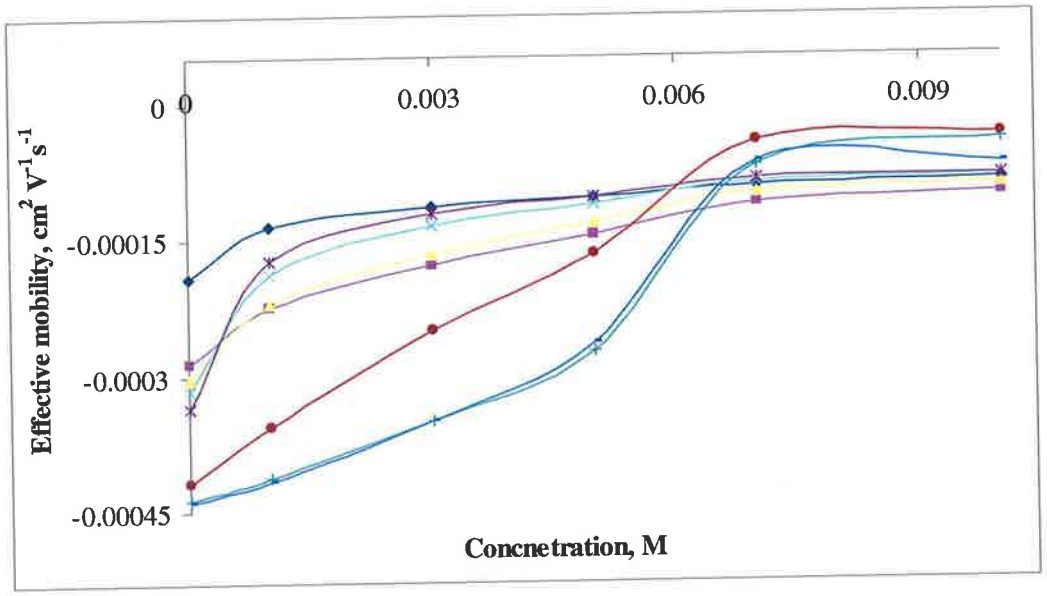


Figure 3.4: Effect of the concentration of γ-CD on the electrophoretic mobility of steroids. Separation conditions as in Figure 3.2. Analyte identification:

- **Estriol,**
- **Equlin,**
- **Estrone,**
- **17β-Estradiol,**
- **Ethinylestradiol,**
- **Norethindrone,**
- **Progesterone,**
- **Mestranol.**

It can be noted that the mobility plots of analytes in Figure 3.4 corresponded to the association constants of analytes. For compounds 1-5 the decrease in mobility occurred quite sharply; at the concentration of 1-3 mM of cyclodextrin the mobilities have reached their maximum. The magnitudes of the association constants for these compounds represent sufficiently large values (Table 3.2). Norethindrone, progesterone and mestranol had very small or non interaction with the cyclodextrin. The change in mobility of these analytes was gradual over the investigated concentration range of γ-CD and the maximum was at 7 mM.

Negative values of constants for progesterone and mestranol, along with poor linearity and precision, pointed to the fact that these steroids did not interact with γ-CD or the nature of interaction was something other than inclusion. Inclusion of

norethindrone was characterised by the association constant 75 M^{-1} (X-reciprocal fit) with %RSD less than 5% and linearity 0.73.

Rekharsky and Inoue [50] attributed the following contributions to the stabilisation of cyclodextrin complexes from a thermodynamic point of view: a) penetration of the hydrophobic part of the guest molecule into thecyclodextrin cavity, b) dehydration of the organic guest, c) hydrogen-bonding interaction, d) the release of the water molecules originally included in the cyclodextrin cavity to bulk water [58-60] and e) the conformational changes or strain release of the cyclodextrin molecule upon complexation [61-63].

According to the factors listed above the possible reasons for strong inclusion of compounds 1-5 and weak or absence of inclusion for compounds 6-8 should be explained by referring to the differences in structural characteristics of the complexes, i.e. structures of steroids incorporated into a cyclodextrin type, which was kept constant. According to the literature hydrophilic groups, such as hydrohxyl, amino and carboxyl [64], remain exposed to the bulk solvent even after inclusion of the hydrophobic moiety [50]. An exception to this general rule is the aromatic hydroxyl group, which can penetrate deeply into the cyclodextrin cavity where it hydrogen-bonds to one of cyclodextrin's peripheral hydroxyl groups [65]. Ring A of compounds 1-5 is identical; it was aromatic with a hydroxyl group on it. The difference was in the ring D (and nonsaturated bond in ring B of equilin) where hydrophilic hydroxyl and ketone groups and hydrophobic ethilyn group attached. According to the literature estriol, equilin, estrone, 17β-estradiol and ethinylestradiol penetrated γ-CD and form a hydrogen bond with the hydroxyl group of CD. The structural difference of ring D and $\log K_{ow}$ values explained the different K values obtained. Norethindrone and progesterone did not contain any aromatic ring with a hydroxyl group on it and the aromatic hydroxyl group of mestranol was methylated. Ring D of the latter compounds contained hydrophilic and hydrophobic groups. However, neither constituent of ring A nor ring D appeared to be the reason for the inclusion as the calculated association constants were negative. A small degree of interaction was observed in the case of norethindrone but as to which one of the above mentioned

contributions or their combination was responsible for the association constant of 75 M^{-1} (X-reciprocal fit) was not clear.

Table 3.2 contains some literature association constant values for several steroids, obtained from HPLC data. To the authors knowledge association constants for other investigated steroids and other cylodextrin types are not available. It was reported previously [47-51] that equilibrium constants determined by different experimental methods for the same reaction can significantly deviate from each other. The HPLC constants were much greater than the data obtained in this work but notably the relative order of the association constants was the same. The mechanism of the separation method influences the magnitude of the association constant values. In CE, the binding constant values are determined mainly by the charge/mass ratio. In HPLC the distribution of analyte between the mobile and stationary phases play an important role. The effect of SDS on the association constants for CD in CE is discussed later.

3.3.4 β-CD

From this investigation a significant decrease in the magnitude of the association constants was found for the interaction of the selected steroids with β-CD (Table 3.3). Figure 3.5 shows that at lower concentrations of β-CD the change in mobility of steroids was rather small, particularly for compounds 6-8, in which case no change in the mobility could be noticed at 1 mM β-CD in the buffer. Increasing further the concentration of β-CD caused a sharper change in mobility for compounds 6-8.

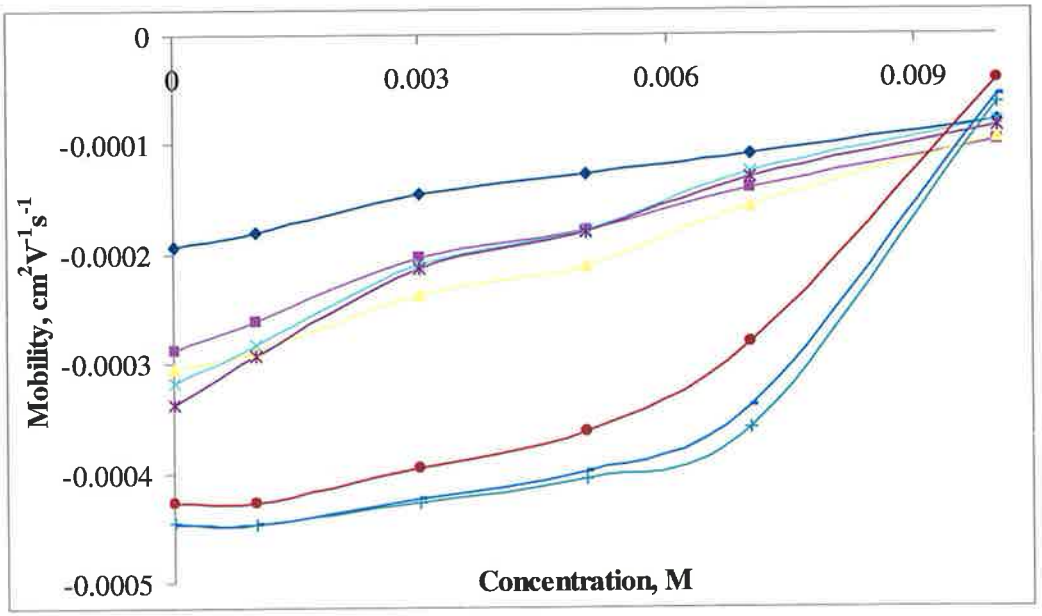


Figure 3.5: Effect of the concentration of β-CD on the electrophoretic mobility of steroids. Separation conditions as in Figure 3.2. Analyte identification as in Figure 3.4.

Association constants values as low as 56 M^{-1} (X-reciprocal fit) for estriol and 120 M^{-1} (Y-reciprocal fit) for ethinylestradiol were found. The association constants were accompanied by a slightly increased deviation from linearity (R^2) for estriol, equilin and 17β-estradiol and a large decrease in linearity for estrone and norethindrone. Relative standard deviation of the obtained K was quite high for estriol (over 30%). The small diameter of β-CD (8Å [56]) did not allow penetration of steroids to the same degree as the cavity of γ-CD. It is likely that the inclusion of only the most hydrophobic part of the molecules took place. The rest of the structure remained in the bulk solvent. Ethinylestradiol had the highest association constant among β-CD complexes followed by 17β-estradiol, equilin and estriol. It is interesting to note that the last three compounds had the same K values calculated by the three linearisation methods within experimental error. Partial interaction of the same structural fragment of the three molecules took place. Estrone and norethindrone did not undergo any interaction with β-CD, which was supported by negative constants. It has to be noted that in case of γ-CD equilin exhibited less interaction than estrone. This was not true for inclusion into β-CD. Because there is no other difference in the steroid structures it is likely that the double bond of the B-ring of equilin induced a different interaction with the CD.

Table 3.3. The value of association constants of target steroids and β-CD, R^2 for each linearisation method, %RSD (n=3) and literature values of constants.

	K (M^{-1})	%RSD	R^2	K (M^{-1}) lit.[46]
Estriol				4700±400
Double reciprocal fit	43	71.23	0.9976	
Y-reciprocal fit	57	35.86	0.8621	
X-reciprocal fit	56	39.09	0.7621	
Equilin				
Double reciprocal fit	57	17.82	0.9973	
Y-reciprocal fit	68	8.81	0.9213	
X-reciprocal fit	97	9.52	0.8376	
Estrone				3100±600
Double reciprocal fit	-43	20.67	0.9942	
Y-reciprocal fit	-17	24.34	0.2645	
X-reciprocal fit	-16	21.54	0.2577	
17β-Estradiol				6830±680
Double reciprocal fit	75	10.61	0.9981	
Y-reciprocal fit	79	5.43	0.9384	
X-reciprocal fit	78	6.50	0.8801	
Ethinylestradiol				7600±770
Double reciprocal fit	126	6.30	0.9982	
Y-reciprocal fit	120	2.33	0.9675	
X-reciprocal fit	123	2.36	0.9307	
Norethindrone				
Double reciprocal fit	-181	12.33	0.9592	
Y-reciprocal fit	-129	13.53	0.5087	
X-reciprocal fit	-90	3.56	0.9513	
Progesterone				
Double reciprocal fit	-250	2.95	0.9103	
Y-reciprocal fit	-196	23.86	0.2786	
X-reciprocal fit	-96	2.11	0.9682	
Mestranol				
Double reciprocal fit	-201	13.50	0.9416	
Y-reciprocal fit	-150	19.30	0.4113	
X-reciprocal fit	-93	2.90	0.9687	

3.3.5 2HP-γ-CD and 2HP-β-CD

Modification of the structure of the cyclodextrin has many beneficial effects, contributing to its solubility or affinity for analytes. In this study 2 hydroxypropyl functionalised γ-CD and β-CD were investigated. It was found that a general decrease in association constants could be noted for the inclusion of target steroids into 2HP-γ-CD (Table 3.4). It was reported that hydroxypropyl substituents elongate the actual CD cavity when attached through primary or secondary hydroxyl groups of the glucose ring [51]. Molecular substitution of the employed 2HP-γ-CD was 0.6 but it was not controlled and thus the actual position of hydroxypropyl groups was unknown. The comparison of the association constants for 2HP-γ-CD with parent γ-CD would suggest that this substitution caused some structural difficulties for steroid molecules to penetrate the cavity and/or the hydrogen bonds were not able to form between analytes and substituted hydroxyls on the CD.

The plots of the mobility of analytes against the concentration change of 2HP-γ-CD and 2HP-β-CD are shown in Figures 3.6 and 3.7. They correspond to a trend of the mobility change as for natural γ-CD and β-CD, respectively. The compounds that had a high degree of inclusion have shown a sharper change in mobility at lower concentration of CD in the separation buffer. The slow reduction in mobility was correlated to negative constant values of progesterone and mestranol.

The data in Table 3.5 shows the association constants of steroids with 2HP-β-CD. A slight deviation from linearity of the plots could be noticed here, which was similar to the linearisation plots for natural β-CD. There was noticeably improved linearity for estrone accompanied by increased association constants. Even though the %RSD of the calculated constants was high, a general increase in the constant value was noted with the functionalised CD compared to the natural β-CD. Hydroxypropyl groups are oriented so that penetration of the part of analytes was quite similar, thereby not distinguishing types clearly. Due to the fact that there is only a slight difference between constant values for several compounds (Table 3.5), it is evident that the HP-substituted β-CD (degree of substitution ≈ 4-10) does not recognise the structural variations of analytes to the same degree as γ-CD for instance.

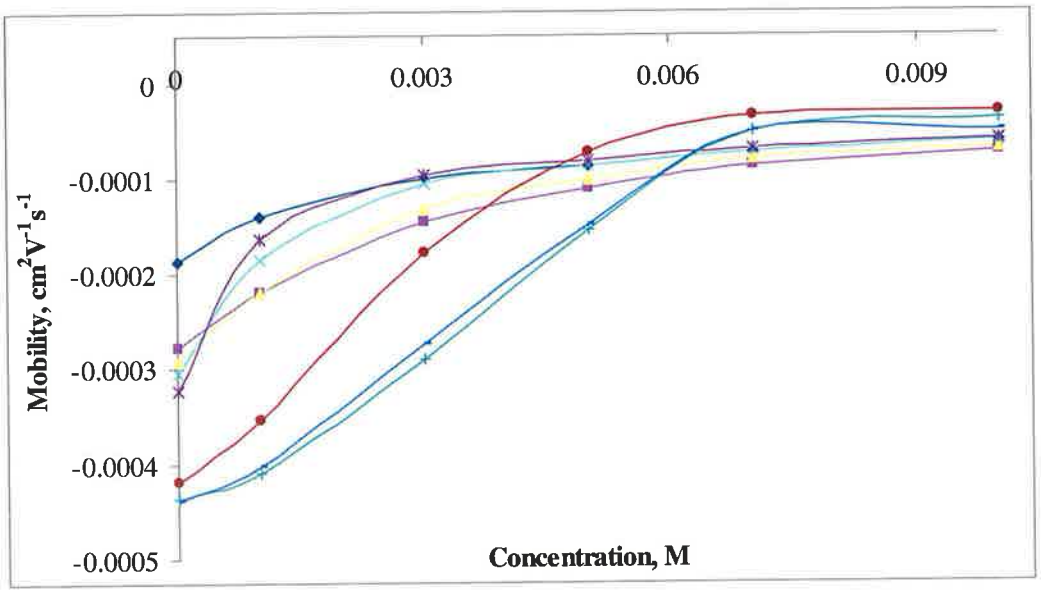


Figure 3.6: Effect of the concentration of 2HP-γ-CD on the electrophoretic mobility of steroids. Separation conditions as in Figure 3.2. Compound identification as in Figure 3.4.

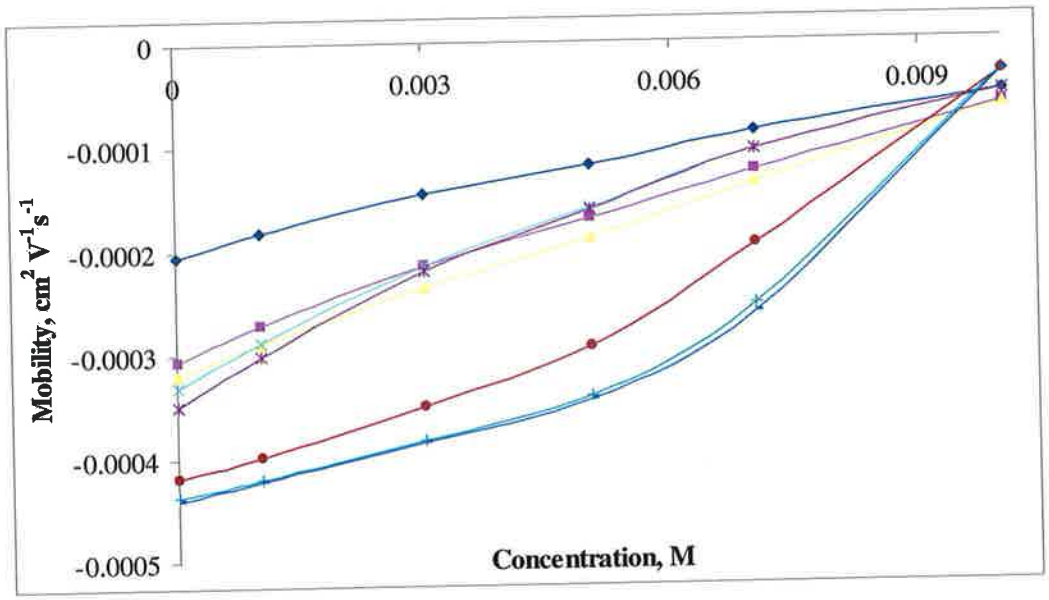


Figure 3.7: Effect of the concentration of 2HP-β-CD on the electrophoretic mobility of steroids. Separation conditions as in Figure 3.2. Compound identification as in Figure 3.4.

Table 3.4. The value of association constants of target steroids and 2HP-γ-CD, R^2 for each linearisation method, %RSD (n=3).

	K (M^{-1})	%RSD	R^2
Estriol			
Double reciprocal fit	592	4.58	0.9971
Y-reciprocal fit	505	9.32	0.9961
X-reciprocal fit	565	5.77	0.9800
Equilin			
Double reciprocal fit	288	4.71	0.9994
Y-reciprocal fit	318	7.31	0.9978
X-reciprocal fit	297	4.20	0.9907
Estrone			
Double reciprocal fit	372	3.41	0.9982
Y-reciprocal fit	432	7.12	0.9977
X-reciprocal fit	389	3.26	0.9824
17β-Estradiol			
Double reciprocal fit	911	2.97	0.9988
Y-reciprocal fit	937	6.25	0.9997
X-reciprocal fit	918	3.09	0.9954
Ethinylestradiol			
Double reciprocal fit	1642	7.96	0.9984
Y-reciprocal fit	1429	6.85	0.9997
X-reciprocal fit	1630	3.32	0.9954
Norethindrone			
Double reciprocal fit	48	10.26	0.9889
Y-reciprocal fit	154	5.27	0.8341
X-reciprocal fit	86	4.41	0.4344
Progesterone			
Double reciprocal fit	-81	6.49	0.9894
Y-reciprocal fit	-23	20.97	0.1156
X-reciprocal fit	-36	8.83	0.2584
Mestranol			
Double reciprocal fit	-49	13.05	0.9916
Y-reciprocal fit	11	53.63	0.0419
X-reciprocal fit	-10	42.06	0.2584

Table 3.5. The value of association constants of target steroids and 2HP-β-CD, R^2 for each linearisation method, %RSD (n=3).

	K (M^{-1})	%RSD	R^2
Estriol			
Double reciprocal fit	114	20.42	0.9908
Y-reciprocal fit	74	11.88	0.9404
X-reciprocal fit	81	14.32	0.8926
Equilin			
Double reciprocal fit	96	31.79	0.9940
Y-reciprocal fit	63	21.31	0.9369
X-reciprocal fit	69	24.29	0.8881
Estrone			
Double reciprocal fit	57	58.59	0.9941
Y-reciprocal fit	31	45.43	0.8616
X-reciprocal fit	33	48.71	0.8206
17β-Estradiol			
Double reciprocal fit	102	30.55	0.9971
Y-reciprocal fit	84	17.82	0.9875
X-reciprocal fit	88	21.39	0.9689
Ethinylestradiol			
Double reciprocal fit	105	28.78	0.9969
Y-reciprocal fit	84	17.16	0.9922
X-reciprocal fit	88	20.54	0.9775
Norethindrone			
Double reciprocal fit	-35	102.46	0.9970
Y-reciprocal fit	-47	25.48	0.7930
X-reciprocal fit	-48	18.03	0.8680
Progesterone			
Double reciprocal fit	-17	278.04	0.9925
Y-reciprocal fit	-53	23.22	0.6962
X-reciprocal fit	-59	12.96	0.8611
Mestranol			
Double reciprocal fit	-10	466.88	0.9919
Y-reciprocal fit	-50	23.73	0.6558
X-reciprocal fit	-58	12.59	0.8472

3.3.6 The effect of SDS

While it is clear from the association constants determined that the concentration and type of CD is important in selecting particular steroids, a study of the effect of SDS was carried out to investigate the impact of SDS on the analyte interaction with the CD. The effect of SDS concentration in the run buffer was investigated using 2HP-γ-CD. The value of association constants of target steroids and 2HP-γ-CD with two run buffers containing 10 mM SDS and 20 mM SDS are presented in Table 3.4 and 3.6, respectively. The steroid-micelle interaction is strong due to high hydrophobicity of analytes ($\log K_{ow}$ values of each analytes have been mentioned above). It was found that by increasing the concentration of SDS, this leads to the equilibrium being moved toward increased micelle interaction.

This fact provoked a decrease in inclusion of steroids into the cyclodextrin cavity when the concentration of SDS was 20 mM. The values of the association constants at 20 mM SDS (Table 3.6) were 2 to 3 times less than at 10 mM SDS (Table 3.4). It has to be noted that K value for estriol was affected significantly less than the rest of the constants. The value for estriol was even higher than the association constant for 17β-estradiol (Y and X fits). Although estriol is the least hydrophobic compound of all target analytes it strongly included into the 2HP-γ-CD cavity. These results demonstrated that in this case, the inclusion is due to the strong hydrogen bonding of the vicinal hydroxyl groups on the D ring with hydroxyls of the cyclodextrin. As expected, the increase in the concentration of SDS in the run buffer did not affect the association constants of the compounds 6-8, which remain negative.

Table 3.6. The value of association constants of target steroids and 2HP-γ-CD with 20 mM SDS in the run buffer, R^2 for each linearisation method, %RSD (n=3).

	K (M^{-1})	%RSD	R^2
Estriol			
Double reciprocal fit	395	6.10	0.9996
Y-reciprocal fit	410	4.64	0.9996
X-reciprocal fit	403	5.41	0.9968
Equilin			
Double reciprocal fit	97	28.16	0.9994
Y-reciprocal fit	102	14.06	0.9861
X-reciprocal fit	99	16.70	0.9694
Estrone			
Double reciprocal fit	134	20.08	0.9995
Y-reciprocal fit	137	10.23	0.9938
X-reciprocal fit	134	12.60	0.9842
17β-Estradiol			
Double reciprocal fit	405	3.57	0.9993
Y-reciprocal fit	388	2.34	0.9995
X-reciprocal fit	396	2.83	0.9957
Ethinylestradiol			
Double reciprocal fit	638	6.78	0.9994
Y-reciprocal fit	610	4.34	0.9999
X-reciprocal fit	628	5.64	0.9973
Norethindrone			
Double reciprocal fit	-12	88.51	0.9992
Y-reciprocal fit	8	99.32	0.1742
X-reciprocal fit	4	182.67	0.0880
Progesterone			
Double reciprocal fit	-39	39.32	0.9999
Y-reciprocal fit	-40	21.13	0.9571
X-reciprocal fit	-38	17.89	0.9749
Mestranol			
Double reciprocal fit	-49	46.51	0.9997
Y-reciprocal fit	-42	30.99	0.9126
X-reciprocal fit	-41	28.74	0.9316

3.4 CONCLUSIONS

This chapter documents a study using capillary electrophoresis to determine the association constants of inclusion complexes of steroid hormones and cyclodextrins. The mechanism of inclusion of steroids by cyclodextrins was investigated. The most powerful agent for the inclusion of the target analytes was found to be γ-CD followed by 2HP- γ-CD. High values of association constants were accompanied by good linearity of the plots. The constants values obtained for individual analytes correlate with their structural characteristics contributing to their inclusion. Interaction with the cavity of β-CD was rather weak while 2HP-β-CD failed to recognise the difference in structure between estriol, equilin, ethinylestradiol, 17β-estradiol and estrone. It was found that the association constant values decreased when the concentration of micelles in the run buffer was increased.

In general the estimation of association constants of steroid compounds with cyclodextrins was a straightforward process due to the high efficiency and simple performance of capillary electrophoresis when combined with the three plotting methods used.

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CHAPTER 4

DETERMINATION OF MONTELUKAST SODIUM AND SIGNIFICANT IMPURITIES

4.1 INTRODUCTION

Montelukast sodium (MK) is currently being produced at Merck & Co as an active ingredient for treatment of bronchial asthma. To monitor the manufacturing process an HPLC method is being employed. This method requires up to 35 min for each run, consumes large volume of organic solvents and uses expensive HPLC columns. In this work a CE method is proposed as an alternative to HPLC. Significantly shorter analysis times, inexpensive equipment along with reproducible and very efficient separations are incontestable advantages of the CE method.

4.1.1 Montelukast

Montelukast sodium (free acid of Singulair™), also known as MK-0476 [1-(((1(R)-(3-(2-(7-chloro-2-quinolinyl)-(E)-ethenyl)phenyl)(3-2-(1-hydroxy-1-methylethyl) phenyl) propyl) thio) methyl) cyclopropane) acetic acid sodium salt], was developed by Merck & Co [1] as a therapeutic agent for the treatment of bronchial asthma. The chemical structure of the compound is presented in Figure 4.1.

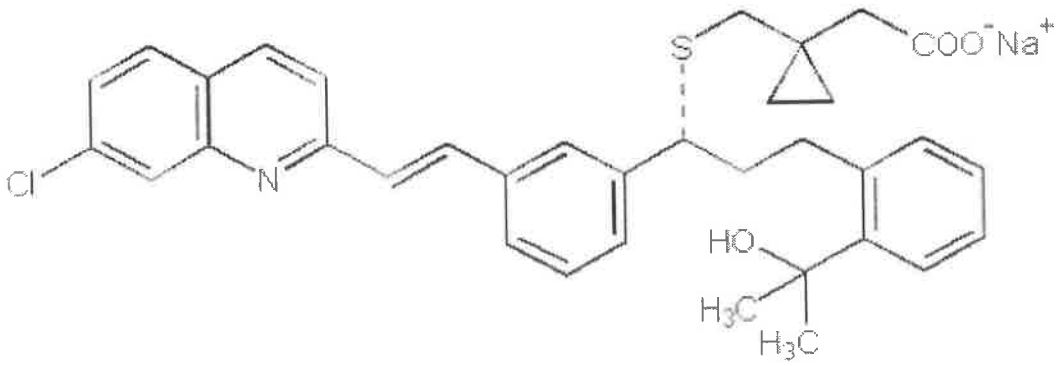

Figure 4.1. Chemical structure of montelukast sodium.

The only available data about montelukast sodium was received from Merck & Co. It is summarised in Table 4.1.

Table 4.1. Information on montelukast sodium.

Information	Value
MW	608.2 g $mole^{-1}$
pH	Between 9.4 and 10.2
Solubility in water at 25°C	Greater than 100 mg mL^{-1}
Soluble in other solvents	Ethanol, methanol
Melting point	135.5°C
Partitioning coefficient Log K_{ow}	2.3

Montelukast sodium is very sensitive to exposure to light or moisture. Light exposure is reported to cause isomerisation (formation of the *cis*-isomer). Exposure to oxygen causes formation of sulfoxide impurities at elevated levels. In case of prolonged exposure to the atmosphere the compound picks up moisture and degrades. According to the literature provided by Merck & Co, Montelukast degrades at 60°C.

4.1.2 Montelukast in the treatment of asthma

Montelukast sodium is a potent and selective leukotriene D-4 (cysLT-1) receptor antagonist [2, 3]. The role of the cysteinyl leukotrienes (leukotrienes C-4, D-4 and E-4) in asthma has been established [4]. Leukotrienes are produced and released from proinflammatory cells, including eosinophils and mast cells, and are at least 1000 times more potent than histamine or methacholine as bronchoconstrictors in healthy and asthmatic subjects. Leukotrienes mediate many of the pathophysiologic processes associated with asthma, including microvascular leakage, bronchoconstriction, and eosinophil recruitment into the airways. Agents that interrupt the action of the leukotrienes (5-lipoxygenase inhibitors and leukotriene receptor antagonists) have improved measures of chronic asthma in clinical studies, thus providing evidence for the role of cysteinyl leukotrienes in this disease [5].

Montelukast has been shown to substantially block airway leukotriene receptors in patients with asthma for as long as 24 hours after oral dosing [6]. Administered once daily, it reduced symptoms of chronic asthma in adults and children [7, 8] and attenuated exercise-induced bronchoconstriction [9, 10].

4.1.3 Determination of Montelukast

Relatively few methods have been reported for the determination of montelukast in the open literature. The majority of the methods have been dedicated to the analysis of montelukast in biological fluids and only a few methods were developed for pharmaceutical formulations. All methods are discussed below.

Amin *et al.* [11] developed an HPLC assay for the determination of montelukast in human plasma involving protein precipitation and fluorescence detection. With this method, chromatography was performed on a C_{18} column with a mobile phase of acetonitrile-ammonium phosphate (pH 3.5; 50 mM) (62:38, v/v). The assay was linear in the range of 28.9 – 2890 ng mL^{-1} of montelukast. Montelukast and the internal standard (very similar in structure to montelukast) were separated in 5 min, which is reasonable and would allow the analysis of a number of samples in a clinical laboratory. The advantage of sensitive fluorescence detection was low LOD 5 ng mL^{-1}, which would be useful for analysis of traces of montelukast in plasma.

Liu *et al.* [12] outlined a method for analysis of montelukast enantiomers in human plasma. Samples of plasma undergo extraction on the Chromospher 5 Biomatrix extraction column, with subsequent switching to an analytical stereoselective column of an HPLC instrument. The assay included protein precipitation and fluorescence detection. Separation was performed on an α-acid glycoprotein chiral column with a mobile phase of acetonitrile-ammonium acetate (pH 5.8; 10 mM) (25/75, v/v). Separation of two enantiomers and an internal standard required up to 35 min. The linearity range for enantiomers was narrower than for single montelukast and was between 28.9 and 386 ng mL^{-1} of free acid of montelukast and its S-enantiomer. The research group studied several healthy volunteers who received a dosage of montelukast daily and concluded that there is no apparent bioinversion of montelukast to its S-enantiomer in humans.

In a study by Ochiai *et al.* [13] a sample pre-treatment step was developed for a direct injection of plasma sample onto the HPLC system with C_{18} column. A six-port switching valve was employed to transfer the sample from pre-column to the analytical column. A mobile phase of acetonitrile-acetate buffer (pH 3.5, 25 mM) (80:20, v/v) was used to elute montelukast from the analytical column. The

temperature of the analytical column was maintained at 40°C. A linearity range of 1 – 500 ng mL^{-1} was achieved. Although the limit of detection was not determined, it could be sufficient as fluorescence detection was employed. The time required for processing samples using this method was shortened due to on-column pre-treatment but the actual separation time was quite long – just under 25 min.

Al-Rawithi *et al.* [14] described a human plasma assay for the analysis of montelukast. Authors claimed that a simple extraction of the plasma is less time consuming than column switching. That is why extraction was used before the drug was measured by HPLC with a C-8 4-μm particle size radial compression cartridge at 40°C and identified with fluorescence detector with the excitation and emission wavelengths set at 350 and 400 nm, respectively. The mobile phase was delivered at 1.0 mL min^{-1}. This mobile phase consisted of 200 mL of 0.025 M sodium acetate, pH adjusted to 4.0 with acetic acid, to 800 mL of acetonitrile, with 50 mL triethylamine. With a run time of only 10 min per sample, this assay had an overall recovery of >97% and improved detection limit of 1 ng mL^{-1}.

The most recent human plasma assay for the analysis of montelukast sodium was published by Kitchen and colleagues [15]. The novelity of the method was in the use of a semi-automated 96-well protein precipitation method. The analysis was performed by HPLC with fluorescence detection. The method offered significant time savings over the manual methods and reached limit of quantitation of 3 ng mL^{-1}.

Radhakrishna *et al.* [16] published the only up to date HPLC method for the simultaneous determination of montelukast and loratadine in pharmaceutical formulations and separation of impurities of montelukast using high-low chromatography technique. HPLC separation was achieved with a Symmetry C18 column and acetonitrile-sodium phosphate buffer (pH 3.7; 0.025 M) (20:80, v/v) as eluent at a flow rate of 1.0 mL min^{-1}. UV detection was performed at 225 nm. The separation of impurities of montelukast was performed in approximately 35 min per single run, which would be quite a time consuming procedure in the busy environment of the pharmaceutical industry. As an alternative, second derivative spectrophotometry was reported to be successful for the simultaneous determination of montelukast and loratadine in pharmaceutical formulations. The second-order derivative spectrophotometry was applied due to overlap of the UV

spectra of loratadine and montelukast in the area from 200 nm to 310 nm. The zero-crossing technique was applied at 276.1 nm, but for montelukast, peak amplitude at 359.7 nm (tangent method) was used.

Pressurised liquid extraction technology for the extraction of montelukast sodium from oral chewable tablets was introduced. In the study by Hoang *et al.* [17] samples were extracted using two cycles of water for 2 min with a cell temperature of 40°C and a pressure of $1*10^4$ kPa, to disintegrate the tablet, followed by three cycles of methanol for 3 min at 70°C and $1*10^4$ kPa, to solubilise montelukast sodium. The method demonstrated an improvement in extraction efficiency of 98.2% of label claim compared to 97.6% obtained using a validated mechanical extraction method, the % RSD was 1.3% (n=10) compare to 0.9% RSD from mechanical method. Quantitative analysis was performed using an HPLC system.

A high performance thin layer chromatographic method for the determination of montelukast sodium in bulk drug and pharmaceutical preparations was reported by Sane *et al.* [18]. Chromatography was performed on silica gel $60F_{254}$ HPTLC plates, the mobile phase used for elution was toluene-ethyl acetate-glacial acetic acid, 6.0 + 3.4 + 0.1 (v/v). Plates were evaluated by densitometry at 344 nm. The linearity of the method was established in the range 0.8 – 10 μg mL^{-1}. Coefficient of variation was less than 2%. Each analysis required approximately 45 min, which is even longer than the reported [16] HPLC method.

A case of chiral separation of montelukast and its S-enantiomer by capillary electrophoresis has been reported by Hoang *et al.* on the Tenth International Symposium on Pharmaceutical and Biomedical Analysis [19]. However the results of the above were not published in the peer-reviewed literature.

Duran Meras *et al.* [20] studied the complexation of montelukast and heptakis-(1, 6-di-*O*-methyl)-β-cyclodextrin using fluorimetry. It has been proven that fluorescence of montelukast was significantly intensified in the presence of cyclodextrins. A 1:1 stoichometry was established and the association constant of the inclusion complex was 959 M^{-1}. The pKa values of montelukast were also

estimated in two different ethanol:water media, 70:30(v/v) and 10:90 (v/v) and were pKa = 2.9 ± 0.1, pKa_1 = 2.0 ± 0.1and pKa_2 = 6.5 ± 0.1, respectively.

As reported at present mainly HPLC and HPTLC methods have been developed for this purpose. All of them are quite time consuming methods – the separation time of HPLC method is just under 35 min in reported work of Radhakrishna *et al.* [16], 45 min is required for an HPTLC analysis [18]. Obviously long analysis time in HPLC requires larger quantities of mobile phase, waste of which can add to the cost of analysis.

Even though a capillary electrophoresis method has not been developed yet, this technique proved to be very advantageous in pharmaceutical separations. Many ways to vary parameters of the run buffer lead to the possibility of adapting each composition for the specific case whether charged or neutral compounds, structurally related or optical isomers have to be separated. The high efficiency of the separation, shorter analysis time, small volumes of samples and buffers required – these are the reasons why this method should be investigated in the determination of montelukast sodium and its related impurities.

4.1.4 Aim of this work

The aim of this work was to determine montelukast sodium and significant impurities. A fast analysis time and reproducible separation were among the objectives of the research in order to develop a capillary electrophoresis method comparable to the conventional methods.

4.2 EXPERIMENTAL

4.2.1 HPLC instrumentation

HPLC separations were performed on a Hewlett Packard series 1050 system with a Model 78953C variable wavelength detector. The instrument was operated using Agilent ChemStation Software version A.09.03 (Agilent Technologies, Palo Alto, CA, USA). An Alltech BRAVA BDS column (25 cm x 4.6 mm, particle size 5 μm) was used. The mobile phase flow was maintained at 1 mL min^{-1}. Separations were performed at 20°C. UV detector was at 225 nm unless otherwise stated.

4.2.2 CE instrumentation

CE separations were performed using the system described in Chapter 2. The fused silica capillaries (Composite Metal Services ltd., The Chase, Hallow, Worcs. WR2 6LD) were 64 cm in length (56 cm to the detector) with an internal diameter of 50 μm unless otherwise stated.

4.2.3 Reagents

All compounds investigated in the separations had purity 99% and were used without further purification. Montelukast sodium was kindly supplied by Merck, Sharp & Dohme Irl Ltd. (Ballydine, Kilsheelan, Co. Tipperary, Ireland). NaH_2PO_4, HCl, NaOH, sodium dodecyl sulphate, (2-hydroxypropyl)-α-cyclodextrin (MS ~ 0.6), (2-hydroxypropyl)-β-cyclodextrin (DS ~ 4-10), (2-hydroxypropyl)-γ-cyclodextrin (MS ~ 0.6), γ-cyclodextrin, KBr were purchased from Sigma-Aldrich, Dublin, Ireland. Methanol (HPLC grade) and acetonitrile (HPLC grade) were from Lab-Scan, Dublin Ireland. Boric acid was from Riedel-de Haën, Seelze, Germany. Buffers were prepared using distilled water and adjusted using 1 M and 0.1 M NaOH and 0.1 M HCl.

4.2.4 Standards

Stock solutions of 1 mM or 10 mM montelukast sodium were prepared in methanol for CE experiments. For the HPLC experiment 8.2 mM stock solution of montelukast sodium was prepared in water:ACN=20:80 (v/v). All were kept in amber flasks. Samples of montelukast for analysis were exposed to the light and air (oxygen) for 4 days unless otherwise stated.

4.2.5 Procedure

All procedures with montelukast sodium were performed in a dark room. All buffers were filtered through a 0.2 μm filter before use. Separations were carried out at 30 kV electrophoretic voltage and temperature 20°C. Injections were hydrodynamic 50 mbar for 2 sec. Conditioning between runs was performed with 0.1 M NaOH for 1 min, followed by MeOH for 1 min and run buffer for 2 min. All separations were repeated at least three times.

4.3 RESULTS AND DISCUSSION

4.3.1 HPLC

In order to be able to observe impurities it is necessary to inject large sample concentration. While this chapter's main focus was the development of a CE method, it was first desirable to detect the impurities in the HPLC separation and compare the results with the literature. In the work of Radhakrishna *et al.* [16] the impurities of montelukast sodium were determined by HPLC method. A high-low chromatography technique was employed in this case.

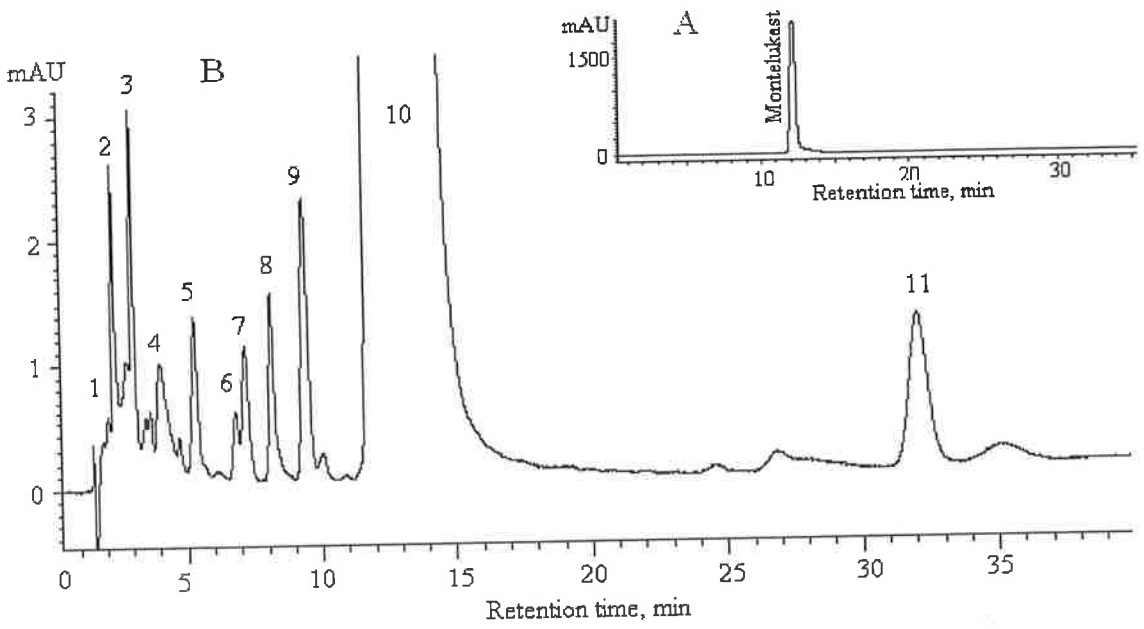

Figure 4.2. A - high-low chromatogram of montelukast. B – expanded high-low chromatogram of montelukast. Chromatographic conditions: Alltech BRAVA BDS C_{18} column, 250*4.6 mm, 5 µm column; mobile phase 0.025 M NaH_2PO_4 pH 3.7:ACN = 20:80 (v/v), flow rate 1 mL min^{-1} ; injection 10 µL; detection at 200 nm. Peak identification: 1-9, 11 – not identified, 10 – montelukast. Concentration of montelukast 1.64 mM.

The aim of the method was to analyse the sample containing a high concentration of montelukast and improve sensitivity toward low-level impurities. The weight percentage of each impurity present in the sample was calculated by comparing its response to the response of diluted montelukast (0.5%), prepared from concentrated sample (5 mg mL^{-1}). Three unknown impurities and *cis*-isomer were separated from the main component in less than 35 min. Since this was the only available information about impurities of montelukast sodium, it was taken into account for future investigation.

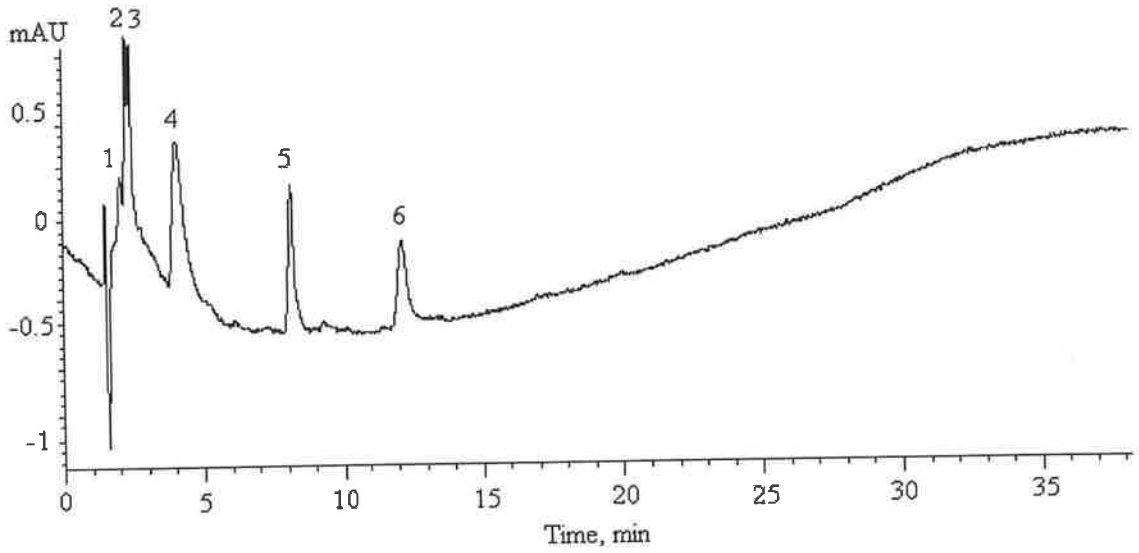

Figure 4.3. Chromatogram of the injection of water:ACN = 20:80 (v/v). Chromatographic conditions as in Figure 4.2. Peaks 1-6 not identified.

The method reported by Radhakrishna *et al.* [16] was applied to the sample of montelukast sodium under investigation. The resultant chromatogram is shown in Figure 4.2. The elution time of montelukast (peak 10 on the chromatogram) was in accordance with the chromatogram in the work [16]. The number of obtained peaks exceeded the number of peaks in work of Radhakrishna *et al.* [16]. Four peaks in this separation belonged to the impurities of the solvent, in which a fresh sample of montelukast was prepared. Figure 4.3 shows a chromatogram obtained after the injection of water:ACN = 20:80 (v/v). Peaks 1, 2, 4 and 5 with the elution times 2.06 min, 2.27 min, 4.03 min and 8.14 min, respectively, corresponded to peaks 1 (2.07 min), 2 (2.28 min), 4 (4.03 min) and 8 (8.13) in Figure 4.2. It has to be noted that the elution time of peak 6 in the injection of the solvent (12.15 min) was identical to the montelukast peak (12.15 min) in Figure 4.3. This leads to the view that the method of Radhakrishna *et al.* [16] was not completely suitable for the analysed system, unless the area of that peak was ignored due to its small value.

Peak 9 on the chromatogram of the montelukast was proposed to be a *cis*-isomer of montelukast as it corresponded to the identified peak in work of Radhakrishna and colleagues [16]. Peaks 5 and 11 also agreed with the elution times of two impurities in the literature [16]. One of the peaks in the sample of Radhakrishna *et al.* [16] did not correspond to any of the peaks in Figure 4.2 where several other peaks were present. This and other obtained peaks could be due to the specifity of the analysed sample of montelukast sodium.

The chromatographic analysis of the sample under the investigation showed at least 6 peaks of different intensity (peaks 3, 5, 6, 7, 9 and 11), which could repesent the potential impurities, although a number of less significant peaks were present on the chromatogram.

4.3.2 CZE

The common drawback of the CE-UV analysis is poor sensitivity. This could strongly affect the aim of this study to develop a separation of montelukast and impurities, as the impurities in montelukast sodium are present at elevated levels. It was thought that intentional exposure of the montelukast sodium sample to the light and air (oxygen) would cause an increase in the concentration of the potential impurities, which would be desirable especially for the method development.

Several modes of CE were tested in order to separate impurities of montelukast sodium. Figures 4.5-4.7 show electropherograms in CZE, MEKC and CD-MEKC modes. Free zone elctrophoresis (Figure 4.5) was found to be unsuitable for such a challenging problem and only a single peak was obtained in this separation. Montelukast and its impurities possess a negative charge at the conditions of the separation (pH 9.2) and comigrate against EOF with comparable velocities, which points to their high similarity in structure. The pH of the experiment was then gradually increased to 10.2. A change in mobility was observed but no separation of the compounds was observed.

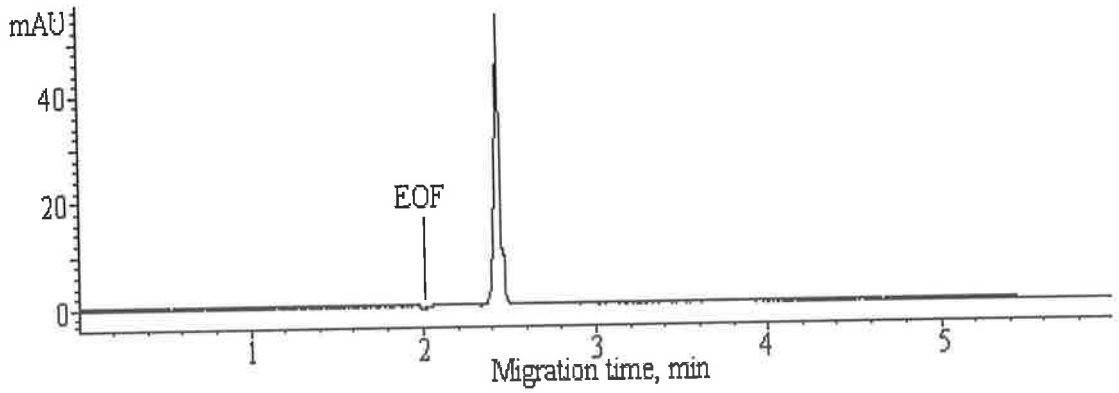

Figure 4.5. Electropherogram of CZE separation of MK. Separation conditions: 20 mM borate buffer pH 9.2, 30 kV, 20°C, hydrodynamic injection 2 sec., detection at 225 nm, capillary 56cm (48cm), 50 µm i.d. 1mM MK in methanol exposed.

4.3.3 MEKC

Montelukast sodium is quite a hydrophobic compound as its $\log K_{ow}$ is 2.3 (Table 4.1).The resolving power of MEKC [21] provides separation capability for compounds of hydrophobic nature. The hydrophobic properties of montelukast would induce its incorporation into the micelles. Figure 4.6 shows the electropherogram of MEKC separation of exposed sample of monteluklast sodium. A micelle-forming additive SDS, was introduced to the run buffer in concentration from 2.5 mM to 50 mM. Additional peaks were observed in the eletropherograms. It was found that at lower concentration of SDS they eluted the capillary with better resolution than at higher SDS concentration. The optimum was found to be at 6 mM SDS, which is lower than the critical micelle concentration of SDS, 8.2mM (this magnitude is lower in a buffer solution than in water) [22]. Thus the mechanism of actual MEKC separation should be questioned in this separation.

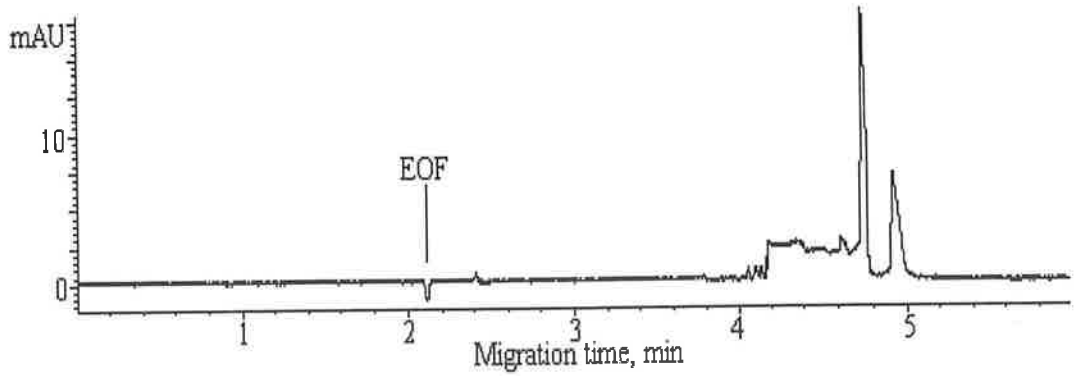

Figure 4.6. Electropherogram of MEKC separation of MK. Separation cond.: 20 mM borate buffer pH 9.2 6 mM SDS, 30 kV, 20°C, hydrodynamic injection 2 sec., detec. at 225 nm, capillary 56cm (48cm), 50 μm i.d. 1mM MK in methanol exposed.

4.3.4 CD-MEKC

4.3.4.1 The type of cyclodextrin

It is known that one of the main impurities in montelukast sodium is its *cis*-isomer. The addition of cyclodextrin to the run buffer should have a positive effect on this separation and help to resolve such closely structured compounds. A range of cyclodextrins was investigated in this study. The electropherograms illustrating the separations are shown in Figure 4.7. A concentration of 5 mM of each cyclodextrin was added to the run buffer containing 20 mM borate at pH 9.2, with 6 mM SDS. This composition was optimised in advance. It can be seen that the separation with 2HP-α-CD or 2HP-β-CD was not successful, as it resulted in coelution of the analyte and impurities. The addition of 2HP-γ-CD and γ-CD improved the separation and several additional peaks could be noted in Figure 4.7 (C, D). These results pointed to the fact that the size of the cavity of the employed cyclodextrins played the key role in the separation. The cavity of 2HP-α-CD and 2HP-β-CD was not large enough for the molecule of montelukast or its *cis*-isomer to penetrate the cyclodextrin and form the inclusion complex. The diameter of the cavity of γ-CD is reported to be 10Å [23]. This is likely to have allowed the formation of the inclusion complex of the molecule of analyte and cyclodextrin to occur.

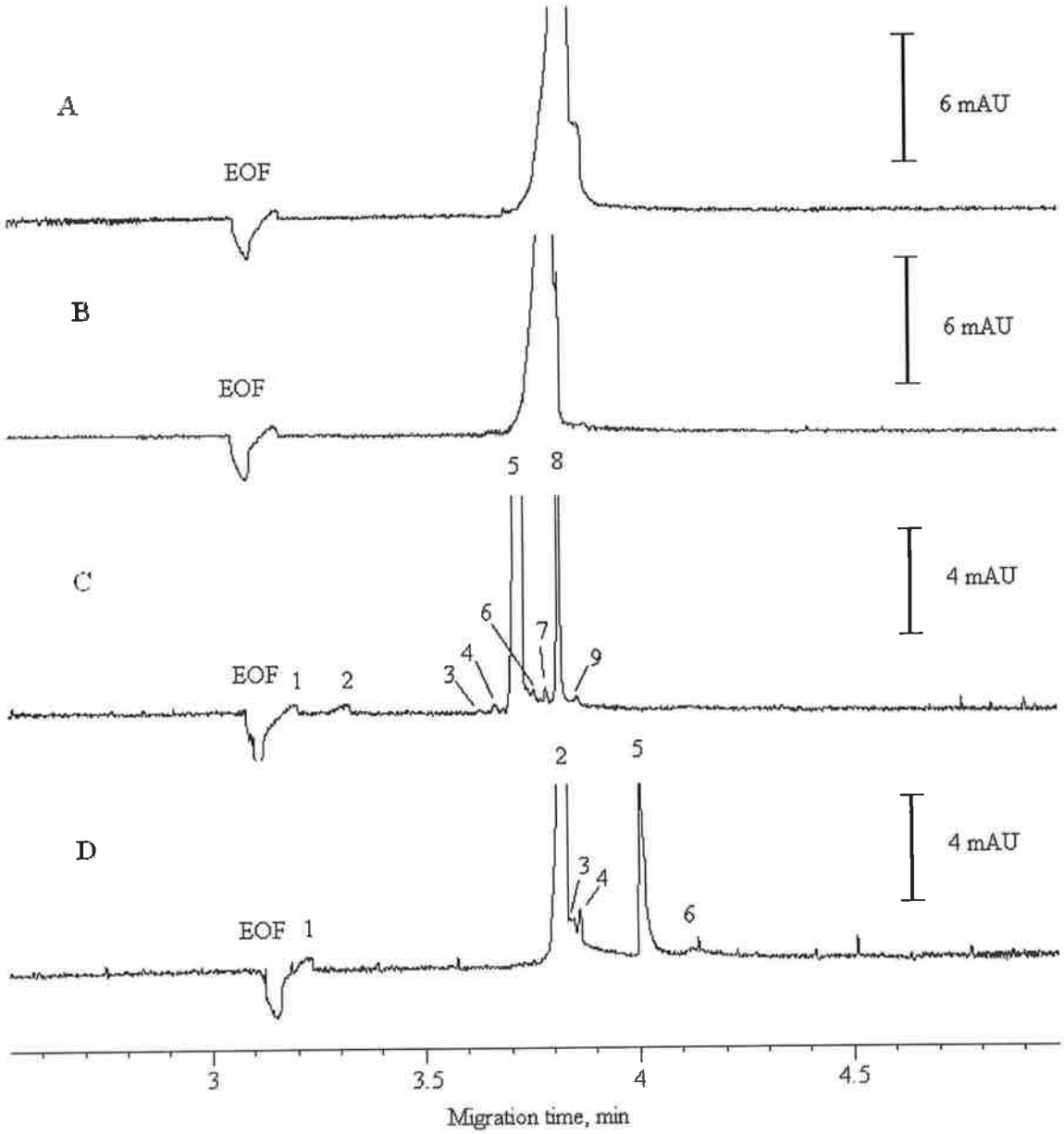

Figure 4.7. Electropherograms of CD-MEKC separation of MK. Separation cond.: 20 mM borate buffer pH 9.2, 6 mM SDS, 5 mM of A - 2HP-α-CD, B - 2HP-β-CD, C - 2HP-γ-CD, D - γ-CD, 30 kV, 20°C, hydrod. inj. 2 sec., detec. at 225 nm, capillary 64 cm (56 cm), 50 μm i.d. 1mM MK in methanol exposed.

Interestingly, Meras *et al.* [20] studied the complexation of montelukast and heptakis-(1, 6-di-*O*-methyl) subsitutes of β-cyclodextrin using fluorimetry. Their investigation showed that a complex between montelukast and cyclodextrin was formed. The association constant of the inclusion complex was found to be 959 M^{-1}. The cyclodextrin investigated in this work and in work [20] are substitutes of β-CD and thus the cavity of both cyclodextrins should be similar in size (7 glucose unite in the ring).

There can be several reasons for the difference in the behaviour of cyclodextrins. Firstly, the difference in the nature of functional groups of β-cyclodextrins and the degree of cyclodextrin substitution can make significant inpact on the results [24]. Secondly, the concentration of cyclodextrin can have an effect. In this study 5 mM 2HP-γ-CD was used with 1 mM montelukast (before the sample was exposed). In the work of Meras *et al.* [20] it was demonstrated that greater than 1000 times CD – montelukast molar concentration ratio did show inclusion. Cyclodextrin was employed in the concentration 6.9 mM while the investigated level of montelukast was 1.8 μM [20]. Such a low concentration of montelukast could not be investigated in this work as the objective was to study the impurities. Thirdly, the solvent employed in the study of Meras *et al.* [20] consisted of ethanol, acetate buffer and water. At such conditions montelukast is in its protonated form; according to the results of this paper pKa of montelukast is 2.9. For the capillary electrophoretic analysis montelukast has negative charge as borate buffer pH 9.2 was used. The hydrophobic inclusion is less likely to occur while the guest molecule was charged.

One major peak and one peak of a lower intensity can be seen in the electropherogram in Figure 4.7 with γ-CD and 2HP-γ-CD. One of the peaks is montelukast. The second peak is likely its *cis*-isomer as the sample was exposed to the light and air for 4 days.

There were other minor peaks separated with the investigated conditions. At the concentration of 5 mM the number of peaks separated with 2HP-γ-CD was higher than with γ-CD. Also, tailing of peaks could be noted in the separation with γ-CD, indicating that the mobility of the analytes is lower that the mobility of the buffer [25]. Peaks 1 and 2 on the electropherogram of the separation with 2HP-γ-CD are due to buffer constituents. Their presence was observed on the electropherogram of the blank injection. The number of the peaks (6) matching the impurities formed after the exposure of the montelukast to the light and air corresponded to the number of impurities obtained in the separation by HPLC method (Figure 4.2).

4.3.4.2 Identification of montelukast

In order to identify the peak due to montelukast in the separation with 2HP-γ-CD the effective mobility of a fresh montelukast sample was compared to the effective mobility of two main peaks in the separation of the sample exposed to daylight and air over a period of time. The results are presented in Table 4.2.

Table 4.2. Effective mobility of montelukast and *cis*-isomer in the fresh samples and exposed in time. Separation cond.: 20 mM borate buffer pH 9.2, 6 mM SDS, 5 mM 2HP-γ-CD, n=3.

	Peak 5*		**Peak 8***	
Sample MK 1 mM	**Effective mobility, $cm^2 V^{-1} s^{-1}$**	**%RSD**	**Effective mobility, $cm^2 V^{-1} s^{-1}$**	**%RSD**
Fresh	-	-	$-11.89\ 10^{-5}$	0.54
Exposed, 1 day	$-9.95\ 10^{-5}$	0.30	$-11.25\ 10^{-5}$	0.52
Exposed, 2days	$-10.46\ 10^{-5}$	0.72	$-11.83\ 10^{-5}$	0.69
Exposed, 3 days	$-10.53\ 10^{-5}$	0.32	$-11.82\ 10^{-5}$	0.28
Exposed, 4 days	$-10.54\ 10^{-5}$	0.40	$-11.77\ 10^{-5}$	0.57
Exposed, 15 days	$-10.23\ 10^{-5}$	0.39	$-11.34\ 10^{-5}$	0.28

* the numeration of the peaks correspond to the Figure 4.7(C).

The negative values of the mobility of montelukast and its *cis*-isomer mean that the molecules migrated against EOF. It can be seen that the mobility of peak 8 in the separation with 2HP-γ-CD (Figure 4.7(C)) corresponded to the effective mobility of montelukast in the fresh sample. The effective mobility of montelukast was consistent while the sample was exposed to the light and air for up to 15 days. It has to be noted that the area of montelukast peak 8 in Figure 4.7(C) is smaller than area of peak 5, which is supposedly its *cis*-isomer. This sample was exposed to the daylight and air for approximately 4 days, when most of the montelukast was converted to the isomer.

Table 4.3. Effective mobility of montelukast and *cis*-isomer in the fresh samples and exposed in time. Separation cond.: 20 mM borate buffer pH 9.2, 6 mM SDS, 5 mM γ-CD, n=3.

	Peak 2*		**Peak 5***	
Sample MK 1 mM	**Effective mobility, $cm^2 V^{-1} s^{-1}$**	**%RSD**	**Effective mobility, $cm^2 V^{-1} s^{-1}$**	**%RSD**
Fresh	-	-	$-15.04\ 10^{-5}$	0.24
Exposed, 4 days	$-13.68\ 10^{-5}$	0.67	$-15.28\ 10^{-5}$	0.24

* the numeration of the peaks correspond to the Figure 4.7(D).

A similar study was performed to identify the montelukast peak in the separation with γ-CD. The effective mobility is shown in Table 4.3. Thus the mobility of peak 5 in Figure 4.7(D) corresponds to the mobility of the montelukast in a fresh sample. Even though the study on the inclusion complexation of γ-CD and 2HP-γ-CD and montelukast was not performed, the obtained data on the effective mobility can represent this information to some extent. The inclusion of analyte molecule in 2HP-γ-CD occurred to a greater degree than in γ-CD, as the effective mobility had a less negative magnitude. The molecules of cyclodextrin did not posses their own mobility at the conditions of the experiment as they were neutral and migrated along the capillary with EOF. Any interaction of the analyte molecule, which possesses negative mobility, with a neutral species would result in the change of the analyte mobility to the less negative magnitude as the general mobility of the complex. The stronger interaction would lead to a greater change in the mobility. The effective mobility of montelukast in the case of the separation with 2HP-γ-CD was -11.89 10^{-5} cm^2 V^{-1} s^{-1}, which is higher than the mobility of montelukastat in the case of separation with γ-CD -15.04 10^{-5} cm^2 V^{-1} s^{-1}.

4.3.4.3 Concentration of cyclodextrins

Both cyclodextrins (γ-CD and 2HP-γ-CD) had shown an improvement in the separation of montelukast and its impurities, therefore it was decided to investigate the influence of the concentration of each cyclodextrin on the separation. The range of concentration 3 – 10 mM was evaluated for γ-CD and 3 – 20 mM for 2HP-γ-CD. Tables 4.4 and 4.5 represent the data obtained. Since the aim of this research was to separate the montelukast from its impurities, only the peak of montelukast was considered when choosing the parameters of separation. In the table 4.4 it could be seen that resolution of montelukast peak was very good (>2) in the full range of the investigated concentration of γ-CD. The efficiency of the peak was over 200000 theoretical plates. The migration time was very precise as %RSD was between 0.23 and 1.07. The actual magnitude of the migration time of montelukast did not change significantly. Thus, the concentration of γ-CD that allowed more efficient separation of montelukast could be chosen for further investigation.

Table 4.4. Effect of the concentration of γ-CD on the parameters of the separation. Separation cond.: 20 mM borate buffer pH 9.2, 6 mM SDS, n=3.

Conc. γ-CD	t_m (min)	%RSD (t_m)	N	R 1*	R 2**
3 mM	3.96	1.07	204505±10952	>2	>2
5 mM	4.02	0.52	355314±48184	>2	>2
7.5 mM	3.90	0.73	231074±37720	>2	>2
10 mM	3.91	0.23	314830±17729	>2	>2

* resolution of peaks 5 and 4 in Figure 4.7(D).
** resolution of peaks 5 and 6 in Figure 4.7(D).

The determination of montelukast with 2HP-γ-CD in the run buffer was even more efficient and the general trend of the efficiency was to increase with increased concentration of cyclodextrin. Only at 20 mM 2HP-γ-CD the efficiency was observed to decrease. The results are presented in Table 4.5. The %RSD of the migration time was under 2%, while the actual magnitude of the migration time increased with increased concentration of 2HP-γ-CD in the run buffer. The resolution of montelukast peak has shown to improve with higher concentration of cyclodextrin. For the resolution with the peak 7 (as in Figure 4.7(C)) a maximum was observed at the concentration 10 mM of 2HP-γ-CD, after which the resolution decreased again. A 10 mM concentration could be chosen for further investigation of the separation with 2HP-γ-CD.

Table 4.5. Effect of the concentration of 2HP-γ-CD on the parameters of the separation. Separation cond.: 20 mM borate buffer pH 9.2, 6 mM SDS, n=3.

Conc. 2HP-γ-CD	t_m (min)	%RSD (t_m)	N	R 1*	R 2**
3 mM	-	-	-	0	0
5 mM	3.92	0.52	380091±35660	1.31	1.46
7.5 mM	4.09	1.71	438768±17994	1.53	2.49
10 mM	4.11	1.65	476570±93654	1.76	2.65
20 mM	4.30	0.72	223008±24456	1.55	2.85

* resolution of peaks 8 and 7 in Figure 4.7(C).
** resolution of peaks 8 and 9 in Figure 4.7(C).

4.3.4.4 The effect of organic solvent

The addition of an organic solvent such as acetonitrile or methanol to the run buffer often increases the efficiency and improves the resolution of separation [26, 27]. A 10% MeOH concentration was added to the run buffer comprising 20 mM borate pH 9.2, 6 mM SDS, 10 mM 2HP-γ-CD. The comparison of the obtained electropherogram and the electropherogram without organic additive is shown in Figure 4.8. The addition of methanol to the run buffer resulted in a significant decrease in EOF, which led to longer time of analysis. The poor efficiency and resolution and diffusion of the leading edge of the peaks could be noted in this separation. The latter is due to the higher mobility of the solute zone in comparison to the mobility of the buffer [25]. It was decided not to employ methanol in further separation of the impurities of montelukast by CD-MEKC.

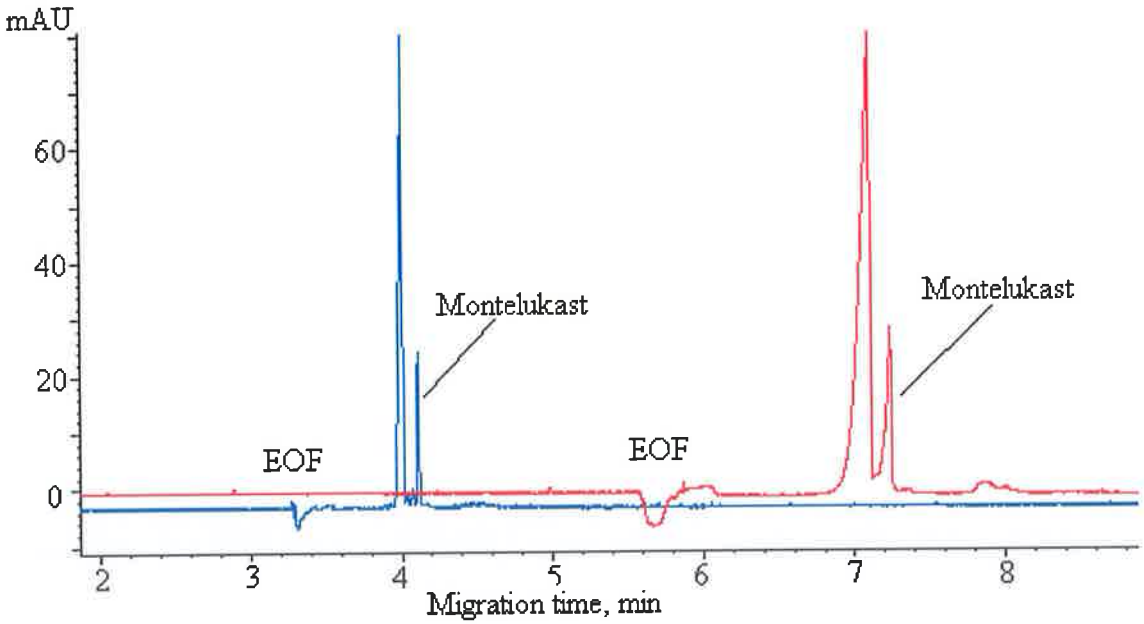

Figure 4.8. Electropherograms of CD-MEKC separation of MK with and without MeOH in the run buffer. Separation cond.: 20 mM borate buffer pH 9.2, 6 mM SDS, 10 mM 2HP-γ-CD, ---- – with 10% MeOH, ---- – without MeOH, 30 kV, 20°C, hydrod. inj. 2 sec., detec. at 225 nm, capillary 64cm (56cm), 50 μm i.d. 1mM MK in methanol exposed.

4.3.4.5 Concentration of SDS

In order to optimise the concentration of the surfactant in the CD-MEKC system the range of 3 – 15 mM SDS was studied. The parameters of the obtained results are presented in Table 4.6. The resolution of montelukast with peaks 7 and 9 (as in Figure 4.7(C)) was not affected significantly by the changes in the concentration of SDS; only at higher concentration (15 mM) was the resolution found to be less than 1. Good reproducibility of the migration time (%RSD < 2) could be observed. A definite increase in the efficiency of the montelukast peak followed the increase of the SDS concentration up to 10 mM SDS where the maximum efficiency was over 900000 theoretical plates. The latter conditions were chosen as optimum in this study.

Table 4.6. Effect of SDS concentration on the parameters of the separation. Separation cond.: 20 mM borate buffer pH 9.2, 10 mM 2HP-γ-CD, n=3.

Conc. SDS	t_m (min)	%RSD (t_m)	N	R 1*	R 2**
3 mM	3.99	0.45	309427±12258	1.76	2.21
6 mM	4.11	1.65	476570±93654	1.76	2.65
10 mM	4.03	0.14	936859±34080	1.72	2.52
15 mM	-	-	-	<1	<1

* resolution of peaks 8 and 7 in Figure 4.7(C).
** resolution of peaks 8 and 9 in Figure 4.7(C).

4.3.4.6 Purity of montelukast peak

The purity of the montelukast peak was established by the peak-area ratio method for fresh standard and the sample of montelukast exposed to the light and air for 4 days at different wavelengths. The results are demonstrated in Table 4.7 and Table 4.8. The developed method has yielded consistent purity of montelukast peak with cyclodextrins, γ-CD and 2HP-γ-CD, in the run buffer.

Table 4.7. Purity of montelukast peak established by the peak-area ratio method of standard solutions and exposed sample at various wavelengths. Separation cond.: 20 mM borate buffer pH 9.2, 10 mM SDS, 10 mM 2HP-γ-CD.

Wavelength ratio λ_1/λ_2	Peak area ratio Fresh MK*	Peak area ratio MK exposed*
214/200	0.71	0.71
225/214	0.79	0.78
254/225	0.71	0.70

* Mean from three determinations with %RSD <5%.

Table 4.8. Purity of montelukast peak established by the peak-area ratio method of standard solutions and exposed sample at various wavelengths. Separation cond.: 20 mM borate buffer pH 9.2, 6 mM SDS, 5 mM γ-CD.

Wavelength ratio λ_1/λ_2	Peak area ratio Fresh MK*	Peak area ratio MK exposed*
214/200	0.71	0.72
225/214	0.81	0.80
254/225	0.74	0.71

* Mean from three determinations with %RSD < 5%.

4.3.4.7 Validation of the method

The method development has shown that both cyclodextrins γ-CD and 2HP-γ-CD are suitable for the separation of montelukast and its impurities. For method validation the buffer containing 2HP-γ-CD was chosen, as the cost of this compound was significantly less than the natural γ-CD. The parameters of the optimised method are summarised in Table 4.9.

Table 4.9. The parameters of the optimised method for the determination of montelukast and its impurities.

Buffer	20 mM borate pH 9.2
Buffer additives	10 mM SDS
	10 mM 2HP-γ-CD
Temperature of separation	20°C
Separation voltage	30 kV
Injection conditions	50 mbar, 2 sec
Detection	225 nm
Capillary	64 cm (56 cm), 50 μm i.d.
Preconditioning	1 min 0.1 M NaOH
	1 min MeOH
	2 min run buffer

The calibration graph was established with the peak-area as ordinate versus the concentration in mM as axis. The obtained linear regression equation was $y = 166.71x - 6.2014$, $R^2=0.9941$, $n=3$. The data indicates good linearity of the method over the studied concentration of montelukast 0.01 mM – 1 mM. Relative standard deviations of the migration time and peak area of 1 mM fresh sample of montelukast sodium were 0.53% and 4.50%, respectively.

The developed method only allows the determination of montelukast from its impurity in the samples that have been exposed to the light or air, which increases the concentration of the impurity in the sample. No impurity could be detected at their elevated levels in the fresh stock of montelukast. Further improvements of the detection limits are necessary in order to be able to employ the developed method for the purity test of montelukast sodium.

4.3.4.8 Temporal study of the degradation of montelukast

The developed method was employed in the investigation of the degradation of montelukast sodium under exposure to air and light. One series of 1 mM solutions of montelukast sodium were exposed to the light and air for 1, 2, 6 and 8 days. Before capillary electrophoresis analysis the solvent level of each sample was adjusted to obtain the same concentration. The second series of 1 mM solutions of montelukast were kept in air tight vials and exposed to light only, for 1, 2, 6, and 8 days. It has to be mentioned that the light exposure was to indoor light for 24 hours a day. The concentration of montelukast was obtained through the calibration curve. The results are presented in Figure 4.9. It can be seen that the most significant decrease in the concentration of montelukast occurs during the first two days of the sample being exposed to light and air. The concentration of montelukast in the sample that was exposed only to light decreased faster and to around 0.3 mM after 2 days. The concentration of the sample exposed to both light and air approached 0.5 mM after 2 days.

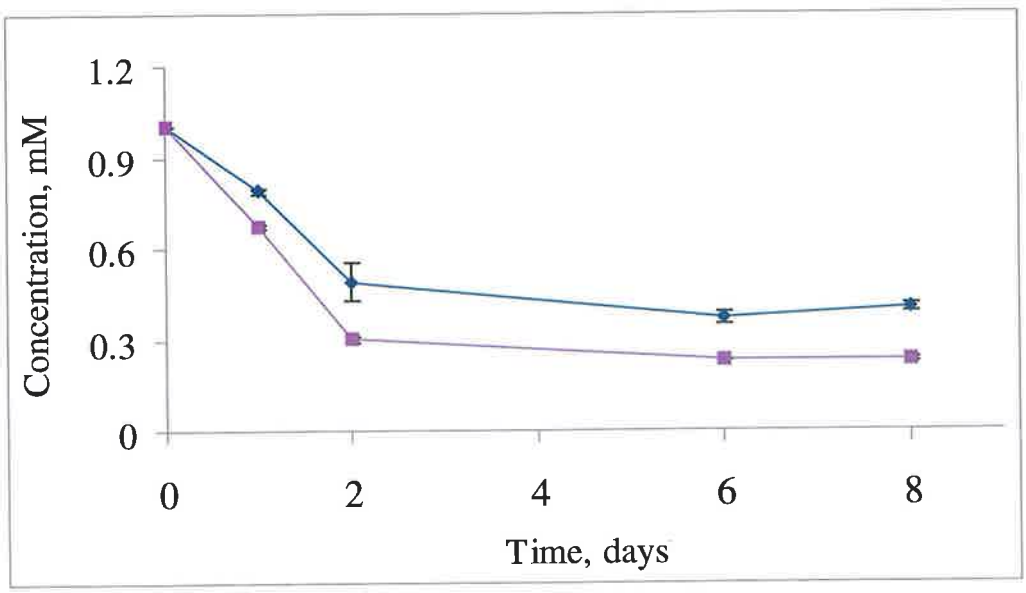

Figure 4.9. Effect of light and air on the concentration of montelukast. Separation cond.: 20 mM borate buffer pH 9.2, 10 mM SDS, 10 mM 2HP-γ-CD, 30 kV, 20°C, hydrod. inj. 2 sec., detec. at 225 nm, capillary 64cm (56cm), 50 µm i.d. 1mM MK in methanol exposed. —♦— - exposure to light and air, —■— - exposure to light.

Figure 4.10 shows the effect of light and air on the peak area of *cis*-isomer of montelukast. It gradually increased during all period of exposure. Noticeably, the peak area of *cis*-isomer from the sample, which was in contact only with light, became more intense than from the sample where both light and air were involved. These results from Figure 4.9 and 4.10 pointed to the fact that degradation of montelukast and the formation of *cis*-isomer were to a higher degree when there was no contact with the air (oxygen) in the exposed sample. It was thought that the presence of oxygen induced some other processes in the sample, which slowed down the formation of *cis*-isomer. This rather unusual kinetics of the degradation of montelukast and the formation of *cis*-isomer in presence of light and light and air can suggest that most of the montelukast is transformed into its *cis*-isomer under "the light conditions". The presence of oxygen (from the air) appears to slow down the degradation of montelukast and the formation of *cis*-isomer, possibly due to the acceleration of an alternative degradation mechanism and alternative degradation products, which were not detected in current study.

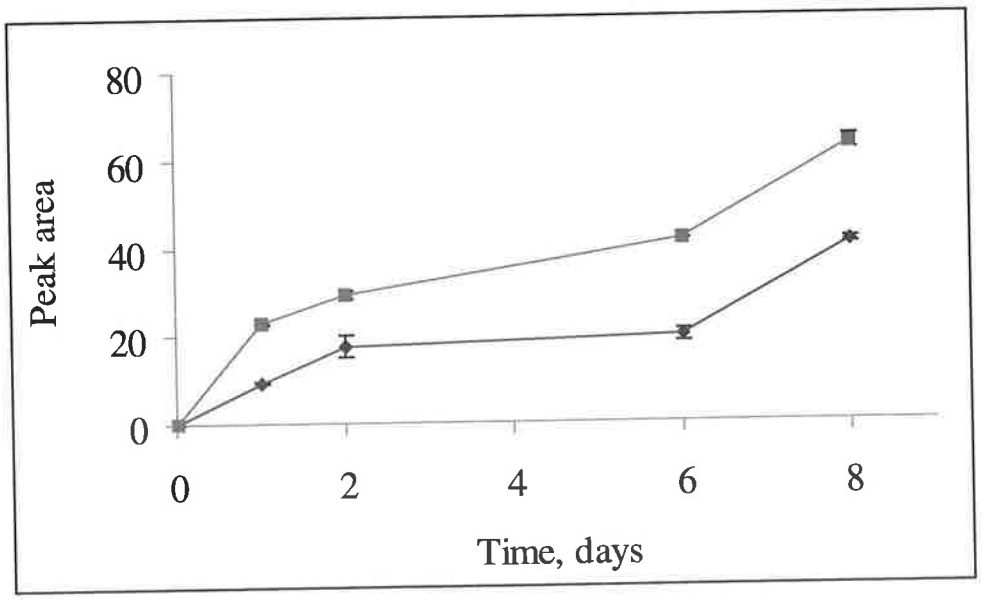

Figure 4.10. Effect of the light and air on the peak area of *cis*-isomer of montelukast. Separation cond.: 20 mM borate buffer pH 9.2, 10 mM SDS, 10 mM 2HP-γ-CD, 30 kV, 20°C, hydrod. inj. 2 sec., detec. at 225 nm, capillary 64cm (56cm), 50 μm i.d. 1mM MK in methanol exposed. —♦— - exposure to light and air, —■— - exposure to light.

The developed method for the determination of montelukast and its impurities allowed to obtained valuable information about the stability of montelukast and different rates of the conversion to its *cis*-isomer. This data has never been obtained before by capillary electrophoresis or other analytical techniques.

4.3.5 Comparison of HPLC and CE methods

The comparison with the HPLC method published in literature [16] of the developed capillary electrophoresis method for the determination of montelukast and its impurity is presented in Table 4.10. It can be seen that one CE analysis required only 9 min, 4 of which were for conditioning the capillary prior the analysis. The HPLC analysis took over 30 min. The montelukast peak had an acceptable resolution from its impurity, even though that its resolution was less than in HPLC method. Extremely high efficiency of capillary electrophoresis separation could be noted in Table 4.10. The repeatability of the migration time was very good for both methods.

Table 4.10. Comparison of the parameters of the HPLC and CE methods for the separation of the impurity in montelukast sodium.

	HPLC	**CE**
t_r/t_m of MK	12.15 min	4.15 min
Complete time of 1 analysis	33 min	9 min
Resolution of MK peak	9.4 and 12.2	1.72 and 2.72
Efficiency of MK peak	3544±10	936859±34080
Repeatability (%RSD of t_r/t_m)	0.23%	0.53%

4.4 CONCLUSIONS

The aim of this work was to develop the capillary electrophoresis method for the separation of the related impurities in montelukast sodium. The completed study has not been achieved at this point as the available instrumentation did not allow sufficient detection sensitivity of the impurity at the elevated levels as in a fresh stock of montelukast. However, even at the present stage of developing the method, CE has showed good potential and many advantages over HPLC method for the determination of montelukast sodium. The advantages of the CE method for the determination of montelukast sodium to date are:

1. The full analysis time is less than 9 min, which is significantly less than the 35 min of HPLC method [16];
2. A high efficiency of the montelukast sodium peak over 900000 theoretical plates was recorded with the achieved method;
3. The impurities were not identified but their quantities were in accordance with HPLC results obtained with the method [16];
4. Further experiments are required to improve the sensitivity of the method and to identify the peaks of the impurities. The employment of the mass spectral detection system could possibly achieve these goals.

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CHAPTER 5

SIMULTANEOUS SEPARATION OF ANTHRACYCLINES AND TAXANES BY CAPILLARY ELECTROPHOERSIS

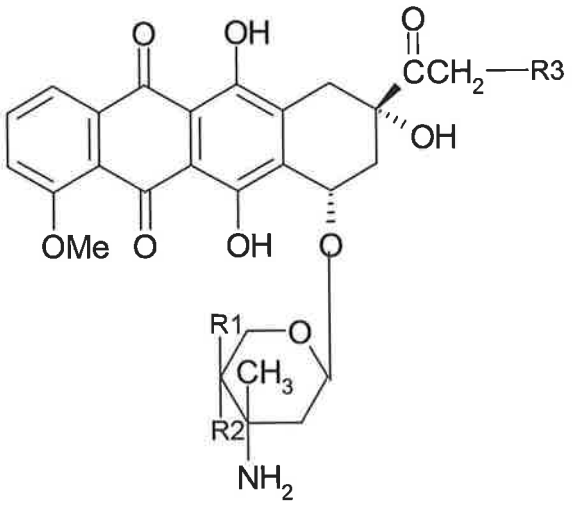
5.1 INTRODUCTION

Cancer causes 7 million deaths every year or 12.5% of deaths worldwide [1]. There are over 200 different types of cancer, and each has a specific name, treatment and chance of being cured [2]. When a cancer cell begins to grow it multiplies out of control, taking over the organ. An intensive growth of cancer cells leads to formation of a tumor. This tumor can cause serious health problems by blocking internal ducts, or by pressing against other organs, preventing their normal function.

Treatment consists of surgery (removal of affected tissue by an operation), chemotherapy (treatment using drugs) or radiotherapy (using radiation) [2]. Chemotherapy may be given before surgery (neo-adjuvant therapy) to shrink a tumour so that it is easier to remove from the body, after surgery (adjuvant therapy) to make sure that any cancerous cells left in the body after removal of a tumour are killed (this makes it less likely that the cancer will come back) and in advanced cancer to remove cancerous cells that have spread through the body, or to slow the progress of the disease [3]. Most chemotherapy drugs work by affecting the DNA in cancer cells. Alkylating drugs, such as cyclophosphamide, stick to one of the DNA strands and prevent them dividing. Antimetabolites, such as methotrexate, stop cells working by preventing them making and repairing DNA. Vinca alkaloids, such as vindesine, disrupt the mechanism that enables one cell to split into two new cells. Cytotoxic antibiotics, such as doxorubicin, stick to DNA causing it to become tangled and preventing the cell from dividing. Other drugs include taxanes and platinum compounds like carboplatin. They have various modes of action [3].

5.1.1 Anthracyclines

Doxorubicin (DOX), daunorubicin (DAU) and epirubicin (EPI) belong to the anthracycline antibiotic family. They are isolated from a pigment-producing *Streptomyces* [4]. Chemical structures are shown in Figure 5.1. A planar, hydrophobic tetracycline ring of anthracyclines is linked to a daunosamine sugar through a glucosidic linkage. Anthracyclines are among the most widely used anticancer agents. In the past 40 years over 200 naturally occurring anthracyclines have been identified and numerous derivatives have been synthesised in order to minimise their side effects and multi-drug resistance [5, 6]. The mechanism of their action is uncertain and complex. Doxorubicin intercalates between adjacent DNA base pairs, which interferes with DNA strand separation and inhibits DNA topoisomerase II, DNA and RNA polymerises. This also inhibits DNA replication and transcription, which eventually induces DNA fragmentation [7]. A cumulative dose-related cardiotoxicity is one of the most severe toxic effects of anthracyclines [8]. Controversially, doxorubicin-mediated free radical formation and lipid peroxidation, which produce direct membrane damage, are more directly responsible for its cardiotoxic effect than antitumor effect [8-10]. Epirubicin is a 4'-epimer of doxorubicin due to the difference of spatial orientation of the hydroxyl group at the C-4' daunosamine ring. The latter does not change the therapeutic efficacy of epirubicin but reduces its toxicity. DOX and EPI are prescribed to patients with solid tumors such as breast, ovarian cancer, lung cancer, lymph system cancer or stomach cancer [11]. DAU is utilised in treatment of leukaemia [12, 13].



	R1	R2	R3
Daunorubicin	H	OH	CH_3
Doxorubicin	H	OH	OH
Epirubicin	OH	H	OH

Figure 5.1. Chemical structure of anthracyclines. Daunorubicin, doxorubicin and epirubicin.

5.1.2 Taxanes

Paclitaxel (Taxol®) is a powerful chemotherapeutic drug. It was isolated from the bark of the Pacific yew tree *Taxus brevifolia*. The chemical structure is shown in Figure 5.2. The chemical name for paclitaxel is 5β,20-Epoxy-1,2α,4,7β,10β,13α -hexahydroxytax- 11-en-9-one 4, 10-diacetate 2-benzoate 13-ester with (2R,3S)-N-benzoyl-3-phenylisoserine. Paclitaxel shows activity against a wide range of tumor types including breast [15, 16], ovaries [17], lung [18] and brain [19] tumors.

Docetaxel (Taxotere®) is an analogue of paclitaxel and is obtained by semisynthesis from 10-deacetyl-baccatin III, which is extracted from the needles of the European Yew Tree *Taxus baccata* [20, 21]. The chemical structure is shown in Figure 5.2. The chemical name for docetaxel is (2R, 3S)-N-carboxy-3-phenylisoserine, N-*tert*-butyl ester, 13-ester with 5β-20-epoxy-1,2α, 4,7β,10b,13α-hexahydroxytax-11-en-9-one 4-acetate 2-benzoate.

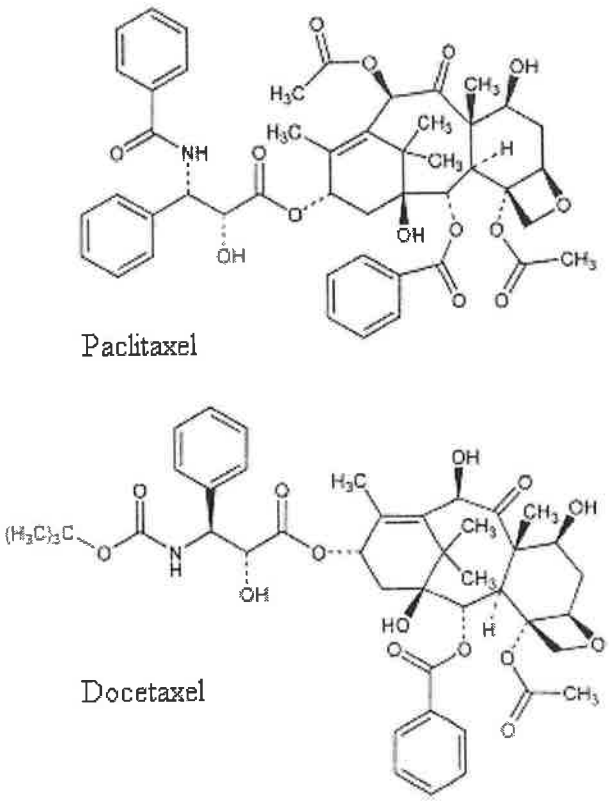


Figure 5.2. Chemical structures of paclitaxel and docetaxel [14].

The cytotoxic properties of taxanes are due to their ability to inhibit microtubule depolymerisation and to promote tubulin assembly [22, 23]. This restrains the normal dynamic reorganization of the microtubule network, which is essential for vital interphase and mitotic cellular functions. This leads to the production of microtubule bundles without normal function and to the stabilisation of microtubules, which results in the inhibition of mitosis in cells.

The binding of taxanes to microtubules does not alter the number of protofilaments in the bound microtubules, a feature that differs from most spindle poisons currently in clinical use.

Docetaxel was first given to cancer patients in 1990 and has proven to be generally more active than paclitaxel [24-26]. Paclitaxel is formulated using Cremophor EL due to its poor solubility in water. This excipient is quite toxic and responsible for strong hypersensitivity reactions [27, 28]. Docetaxel is solubilised in a less toxic polysorbate 80 formulation; nevertheless, the hypersensitivity reactions in patients have occurred at high rates [29]. All patients should be premedicated with oral corticosteroids such as dexamethasone in order to prevent fluid retention. Myelosuppression, skin toxicity and peripheral neurotoxicity are among other side effects [21].

Paclitaxel displays nonlinear pharmacokinetics [30]. The relationship between dose and peak plasma concentration does not correspond to a linear equation [31]. The monitoring of plasma concentration should be of high priority as to prevent side effects of overdosing.

5.1.3 Combination therapy of anthracyclines and taxanes

Combination therapy is a method of treating disease through the simultaneous use of a variety of drugs to eliminate or control the biochemical cause of the disease [32]. It is used in the treatment of conditions such as tuberculosis, leprosy, cancer, malaria, and HIV/AIDS [33]. The first attempt to combine two or more chemotherapeutic drugs was in the 1950s to manage metastatic breast cancer [34]. Taxanes and anthracyclines have emerged as the most active agents for treating breast cancer. The rationale for combining docetaxel with an anthracycline includes high clinical activity of each individual agent, lack of complete clinical cross resistance, and non-overlapping toxicity profiles [34].

The combination therapy of anthracyclines and taxanes has shown survival benefits. The disease-free survival after 4 or 5 years is in a range 65%-75% and an overall survival rate of 77%-87 was reported in several studies [35-38]. An overall reduction of 71-81% was found in patients with metastatic breast cancer after doses of 50–75 mg/m^2 docetaxel in combination with 40–50 mg/m^2 doxorubicin administered every 3 weeks as first-line chemotherapy [39]. Paclitaxel–doxorubicin combination gave similar results [40].

Paclitaxel significantly increases the peak concentration of DOX when injected first in combination therapy [41]. The distribution and elimination of DOX is non-linear and dose-dependent [42]. It was found that paclitaxel followed by DOX was more toxic than the opposite sequence [43] and could sometimes involve a risk of congestive heart failure. The combination of docetaxel and epirubicin could represent a safer alternative. Side effects like fluid retention and cardiotoxicity were not seen in a clinical trial with 75 mg/m^2 docetaxel plus 90 mg/m^2 epirubicin, whereas neutrophenia was the dose-limiting toxicity [44]. The administration of EPI does not affect the pharmacokinetics of paclitaxel even at high concentrations (90 mg/m^2 paclitaxel plus 200 mg/m^2 EPI), while the level of EPI was reduced as the paclitaxel dose increased [45]. The time interval at which paclitaxel is injected has significant impact on the metabolism of EPI [46].

5.1.4 Methods for identification of anthracyclines and taxanes

It has to be noted that all the methods of identification reported in the literature are developed either for anthracyclines or taxanes only, no single method for simultaneous determination of the drugs of interest has been published up to date. Ceruti *et al.* [46] investigated the pharmacokinetic interactions of epirubicin and docetaxel in metastatic breast cancer. Over period of 24 hours after treatment the levels of drugs in plasma can vary from 4500 ng mL^{-1} to 20 ng mL^{-1}. Thus, very sensitive methods are required for the determination of target compounds during therapy. The most sensitive methods have been reported using mass spectral detection 0.5 ng mL^{-1} [47-50] and immunoassay, 0.3 ng ml^{-1} [51]. Both anthracyclines and taxanes have chromophores due to the extensive ring structure and thus UV detection is used with chromatographic and capillary electrophoretic methods for their determination [52-54]. Anthracyclines contain both electroactive and native fluorescent quinone moiety and thus electrochemical [55, 56], fluorescence and laser-induced fluorescence detection systems have been used [7, 57-74].

5.1.4.1 Sample preparation

In order to exclude interference from biofluids (protein binding), it is necessary to carry out a sample pretreatment step prior to analysis. A liquid-liquid phase extraction using *tert.*-butylmethyl ether was used for paclitaxel sample manipulations in human plasma [53]. Methanol was used by Fahmy *et al.* [75] for protein precipitation prior to sample preparation of DOX in combination with 5-FU. The percentage recovery was found to be between 99.4 and 100.4%. Acetonitrile deproteinisation of serum samples for the determination of different anthracyclines is widely employed [61, 62]. Hempel *et al.* [65] employed liquid-liquid extraction with the mixture of sodium phosphate buffer at pH 7.4 and chlorophorm (1:10) when analysing plasma with low concentration of DOX (up to 50 μg mL^{-1}). The recovery was 72.8% accompanied by 7.86% RSD, which means that a significant amount of drug was co-extracted with proteins.

A 92±2% extraction efficiency of docetaxel was achieved by loading plasma onto the C_2 SPE microcolumn followed by RP HPLC separation [76]. C_8 SPE cartridges have also been used for sample pretreatment of anthracyclines and taxanes analysis [47]. C_{18} and C_2 cartridges were used in the analysis of EPI and docetaxel, respectively, in a combinational therapy [46]. On-line column switching from a clean-up column to the analytical column eliminates the need for sample clean-up from plasma. Mader *et al.* [77] used C_4 clean up column in combination with C_8 analytical column for the determination of paclitaxel in human plasma. The mean overall recovery was 98.8%. Alvarez-Cedon *et al.* [78] developed a single solvent deproteinising step (using methanol-$ZnSO_4$) with a high recovery of doxorubicin (close to 100%) while Murdter *et al.* [79] successfully used $AgNO_3$ for this purpose.

5.1.4.2 Chromatographic methods

The majority of analytical techniques for anthracyclines described in the literature use reversed-phase HPLC in combination with fluorescence detector. If the method is developed for a single anthracycline only minor adjustments are needed to analyse related compounds and most methods employ one of the anthracyclines as an internal standard in the analysis of others.

DOX and DAU (I.S.) were separated [7] on a C_{18} column using a complicated gradient elution of ACN and 1-heptanesulfonic acid. With the excitation and mission of fluorescence detection set at 480 nm and 550 nm, respectively, LOD for DOX was 1 ng mL^{-1}. The method is precise with inter-day and intra-day RSD within 0.5% and 6.7%. Picomolar limits of detection for the determination of DOX and its metabolites in biological fluids were reported by Israel *et al.* [80] when using fluorescence as a detection technique. The plasma concentration (<38 ng mL^{-1}) of EPI and its metabolites was analysed by HPLC with fluorescence detector [81]. The method has been shown to be applicable to clinical plasma sample taken during a 96-h infusion of epirubicin in a patient with multiple myeloma. Only small volumes (<200 μL) were required to be treated by precipitation, the elution time of EPI was around 10 min. For human serum containing 25 ng/ml of epirubicin, the inter- and intra-day variation was <10%.

The metabolites epirubicinol and 7-deoxydoxorubicinolone, but not 7-deoxydoxorubicinone, were also detected and measured.

Badea *et al.* [82] developed a method for RP HPLC separation of 7 anthracyclines including EPI, DOX and idarubicin (IDA) hydrochloride on C_8 column with the mobile phase containing SDS. The separation was performed in 10 min. Using UV detection the achieved LODs for DAU, DOX and EPI were 42, 110 and 128 ng mL^{-1}, respectively. Paclitaxel in human plasma was separated on C8 analytical column (after switching from a clean-up column) with ammonium acetate buffer - ACN mobile phase using UV detection [74]. The overall analysis time was rather long (30 min). LOD in plasma was 11 ng ml^{-1}. The mean accuracy of the method was 1.4%.

Docetaxel and epirubicin were analysed by two different methods in work of Ceruti *et al.* [46], where the pharmacokinetic interactions of these drugs were investigated in combinational therapy of metastatic breast cancer. In this work the HPLC-UV method for the determination of docetaxel was developed by [39] and employed ACN : water (60:40, v/v) mobile phase. The analysis of EPI required 75% phosphate buffer and 25% ACN. UV detection was at 229 nm and 232 nm for EPI and docetaxel, respectively. The methods had shown to be quite sensitive with LODs of 20 ng mL^{-1} for docetaxel and 30 ng mL^{-1} for epirubicin. The procedure resulted in a very long total analysis time as it involved 6 min separation time for docetaxel, 15 min for EPI and the time required for a separate sample pretreatment of each drug prior to the analysis.

Microbore HPLC offers rapid column equilibration and enhanced sensitivity and resolution, and applicability to HPLC-MS methodologies. Zao and Dash [59] carried out their analysis on a 50x1-mm C_{18} Luna column with a flow rate of 0.1 mL min^{-1} and a mobile phase of ACN-1% acetic acid (80:20). Fluorescence detection (λ_{Ex} = 505 nm, λ_{Em} = 550 nm) achieved the detection limits of 1 ng mL^{-1}, which are very similar to those on a normal column.

5.1.4.3 Capillary electrophoresis

Although a significant number of the routine analysis of anthracyclines and taxanes are still performed by chromatographic techniques [7, 39, 47, 59, 77, 82], there have been numerous attempts to challenge the resolving power of capillary electrophoresis in the separation of these compounds [54, 56, 61-63, 67, 83, 84]. It has to be mentioned once again that all methods developed to date do not allow simultaneous determination of anthracyclines and taxanes. Table 5.1 shows several of the most representative CE methods.

Both reported methods for the separation of taxanes [83, 84] used buffers at alkaline pH and involved MEKC mechanism of separation, although a significant amount of organic phase (ACN) 30-35% was required. The time of the run, at which the paclitaxel and docetaxel could be separated were 18 and 12 min [83, 84]. The high concentration of SDS 100 mM in the first method was the reason for a longer run time even though only two compounds were analysed. Shao *et. al.* [84] achieved the separation of paclitaxel and 13 related taxanes in only 12 min with 40 mM SDS in the separation buffer. A 10 mM urea was added to the latter system but the authors suggested that it did not affect the separation significantly.

Most of the CE methods for the determination of anthracyclines employed free zone electrophoresis with 30% to 70% of organic additive, such as MeOH or ACN [54, 61-63]. Hu and colleagues [56] used only high concentration phosphate buffer at pH 7.8 for the determination of a single compound DAU. The amperometric detection which was employed in this method was able to achieve LOD of 0.8 μM in urine, while neither sample preparation nor preconcentration were involved, apart from filtering. LIF was widely employed as a detection technique [61-63, 67], the benefit of which was a very low LODs (Table 5.1). Gavenda *et al.* [54] used reversed polarity of separation at low pH 2.5 for DOX and DAU. Employing a sweeping technique to preconcentrate the analytes and a traditional UV detector (234 nm) it was possible to quantify 10 nM of analytes in plasma. A single cell analysis of DOX, doxorubicinol (DOXol) and other metabolites was investigated by Andersson *et al.* [67] using MEKC mode of separation and LIF detector. A five min separation time and LOD of 61±13 zmol were the achievements of the method.

MEKC was also employed to reveal differences in intracellular metabolism between liposomal and free doxorubicin treatment of human leukaemia cells [74]. Liposome-encapsulated form is where anthracycline is in a small vesicle composed of 2:1 molar ratio of distearoyl phosphatidylcholine and cholesterol [85]. It was reported to be less cardiotoxic then corresponding anthracycline, DAU or DOX [86]. An organic modified CZE method was developed for the determination of free and liposome-associated DAU and daunorubicinol (DAUol) in plasma [71].

A pressurised capillary electrochromatography method for the determination of DOX was developed by Nagaraj and Karnes [87]. A 100 μm i.d. fused silica capillary was packed with 3 μm C-18 particles and separation was carried out at 25 kV using 70% ACN : 30% phosphate (10 mM, pH 4.8) as mobile phase. Doxorubicin was derivatised in order to use visible diode laser induced fluorescence. The obtained results showed a very attractive LOD (1.7 ng mL^{-1}).

5.1.5 Observations

The analysis of anthracyclines and taxanes is of a high importance for cancer therapy, as evidenced by the numerous developed analytical methods for the determination of these compounds. Neither chromatographic nor capillary electrophoretic methods developed to date offer the possibility of simultaneous analysis of anthracyclines and taxanes, which are administered together in combinational therapy of metastatic breast cancer. The literature review shows that the methods employing CE can offer low LOD, which are similar to those in HPLC, and shorter times of analysis.

Table 5.1. Some representative methods for CE separation of anthracyclines and taxanes.

Analytes	Time, min	Buffer & pH	Additives	Capillary length x ID	Voltage	Injection	Temperature	Detector	Sample prep. / preconc.	LOD	Ref.
Paclitaxel, Docetaxel	18	25 mM Tris-phosph pH 8.5	100 mM SDS, 35% ACN	67/60 cm x 50 µm *	418 V/cm	0.5 psi 12 sec	Not reported	UV	Yes/yes (10 times)	20 ng mL^{1} Plasma, 50 mg mL^{1} urine	[83]
Paclitaxel & 13 taxanes	12	25 mM Trizma pH 9	40 mM SDS, 30% ACN, 10 mM urea	72/50 cm x 50 µm	25 kV	5 in. Hg 1 sec	30 °C	UV	Not reported	Not reported	[84]
DOX, DAU	14	10 mM phosph pH 2.5	40% MeOH	75/45 cm x 50 µm	-30 kV	30 kV 3-5 min **	25°C	UV	Yes/no	10 nM plasma	[54]
DOX, EPI, DAU	8	100 mM phosph pH 4.2	70% ACN	70/65 cm x 75 µm	20 kV	12 kV 5 sec	Not reported	LIF	Yes/yes	35, 70, 50 pg mL^{-1}	[61]

Table 5.1. Continue

Analytes	Time, min	Buffer & pH	Additives	Capillary length x ID	Voltage	Injection	Temperature	Detector	Sample prep. / preconc.	LOD	Ref.
DOX, DOXol, DAU, IDA , IDAol	5	100 mM phosph pH 5	60 µM spermin, 70% ACN	40(ef) cm x 50 µm	25 kV	10 kV 7 sec	Not reported	LIF	Yes/yes (2 times)	<2 µg L^{-1} (LOQ)	[63]
DOX, DAU, IDA	10	100 mM borate pH 9.5	30% ACN	57/50 cm x 75 µm	15 kV	3.45 kPa 5-15 sec	25°C	LIF	Yes/no	<0.9 ng mL^{-1} (LOQ)	[62]
DOX, DOXol & metabolites	5	10 mM borate pH 9.3	10 mM SDS	35 cm x 50 µm	14.12 kV	3.53 kV 5 sec	Not reported	LIF	Yes/no	61±13 zmol ***	[67]
DAU	6	150 mM phosph pH 7.8	non	35 cm x 25 µm	8 kV	8 kV 10 sec	Not reported	Amperometry	No/no	0.8 µmol L^{-1} urine	[56]

*Extended light pass was employed, ** Sweeping was employed, *** Single cell analysis.

5.1.6 Aim of this work

The above review has shown that anthracyclines and taxanes have been prescribed to cancer patients in combination and such therapy has proven to increase survival rates. Only one anthracycline and one compound of the taxane type are administered at the same time. Although these drugs are taken by patients simultaneously, their control and determination in biological fluids is still performed separately. Hence, it is worthwhile for high sample throughput to examine the feasibility of analysing these compounds by a single method. Anthracyclines and taxanes are quite different compounds by their nature, which makes the development of a method for their simultaneous determination very challenging.

The fundamental aim of this work was to investigate the capability of the capillary electrophoretic technique for simultaneous determination of taxanes and anthracyclines.

5.2 EXPERIMENTAL

5.2.1 CE instrumentation

CE separations were performed using the system described in Chapter 2. The fused silica capillaries (Composite Metal Services ltd., The Chase, Hallow, Worcs. WR2 6LD) were 56 cm in length (48 cm to the detector) with an internal diameter of 50 μm.

5.2.2 Reagents

All analyte compounds investigated in the separations had purity of at least 99% and were used without further purification. Daunorubicin, doxorubicin hydrochloride, docetaxel, paclitaxel, equilin, naproxane, 2, 4-dichlorophenoxyacetic acid, Tris ([hydroxymethyl] aminomethane hydrochloride and Trizma base), NaH_2PO_4, HCl, NaOH, sodium dodecyl sulphate, human plasma and bovine plasma were purchased from Sigma-Aldrich, Dublin, Ireland. *n*-octane and *n*-heptane were from BDH Laboratory Supplies, Poole, England. *n*-butyl alcohol was from May & Baker ltd., Dagenham, England. Methanol (HPLC grade), acetonitrile (HPLC grade) and ethyl acetate were from Lab-Scan, Dublin Ireland. Boric acid and phosphoric acid were from Riedel-de Haën, Seelze, Germany. Buffers were prepared using distilled water and adjusted using 1 M and 0.1 M NaOH, 0.1 M HCl or diluted phosphoric acid.

5.2.3 Standards

Stock solutions of docetaxel 2.5 mg mL^{-1} in methanol and paclitaxel 2.5 mg mL^{-1} in methanol were kept in a freezer, while 2 mg mL^{-1} daunorubicin hydrochloride in methanol and 2 mg mL^{-1} doxorubicin hydrochloride in 0.9% solution of NaCl were stored in a refrigerator. Stocks of 2 mg mL^{-1} equilinl, 3.46 mg mL^{-1} 2, 4-dichlorophenoxyacetic acid and 2 mg mL^{-1} naproxen were made up in methanol and stored in a refrigerator.

5.2.4 Plasma

5.2.4.1 Direct plasma injection

Distilled water was added to the dry human plasma in the amount suggested by the manufacturer. Obtained plasma was diluted with the separation buffer in ratios 20: 80, 50 : 50, 80 :20 and 100 : 0 (v/v) to obtain 0.5 mL solution in total. Each of these samples was spiked with 0.04 mL DAU, 0.04 mL docetaxel and 0.003 mL naproxen (IS) from their stocks.

5.2.4.2 Plasma pretreatment

Distilled water was added to the dry bovine plasma in the amount suggested by the manufacturer. The procedure for plasma pre-treatment was taken from the work of Polson *et al.* [88] with some modifications. A 0.2 mL aliquot of plasma was mixed with 0.005 mL DAU (45.5 μg mL^{-1}), 0.005 mL docetaxel (56.8 μg mL^{-1}) and 0.01 mL equilin (IS) 2 mg mL^{-1}(90.9 μg mL^{-1}). This mixture was vortexed for 10 sec. Acetonitrile was added in amount of 0.6 mL; this was vortexed for 20 sec and then left for 20 min ensuring protection from light. The mixture was placed in a centrifuge at 3000 rpm for 10 min. A 0.7 mL of supernatant was transferred into an eppendorf and evaporated under nitrogen until dryness. The residue was reconstituted in 0.05 mL of separation buffer of choice before the analysis. In order to inject such a small quantity of sample, small eppendorfs (with lids cut off) were inserted into the standard CE vials. The offset of the capillary was set to 13 mm (3 mm in all other separations).

5.2.5 Procedure

All buffers were filtered through a 0.2 μm filter before use. Taxanes and anthracyclines were detected at 230 nm. Separations were carried out at 30 kV electrophoretic voltage and temperature 20°C, unless otherwise stated. Injections

were hydrodynamic 50 mbar for 6 or 12 sec for CZE and MEKC experiments, 50 mbar for 2 sec for octane MEEKC separations and -50 mbar for 2 sec for high-speed MEEKC, unless otherwise stated. CZE, MEKC and octane MEEKC separations included conditioning between runs with 2 min MeOH, followed by 0.1 M NaOH for 3 min and run buffer for 3 min. Conditioning for high-speed MEEKC included 1 min with 0.1 M NaOH, followed by MeOH for 1 min and run buffer for 1 min. Samples were diluted in the run buffer, unless otherwise stated. All separations were repeated at least three times.

5.3 RESULTS AND DISCUSSION

5.3.1 Separation of anthracyclines and taxanes by CZE

For the purpose of this study doxorubicin and docetaxel were used as test analytes.

As it can be seen in Table 5.1 a lot of the methods developed for the separation of anthracyclines have employed organically modified free zone electrophoresis. The separation buffers have been employed in the low pH (below 5) and high pH (8-9) range in order to ensure full or partial ionisation of analytes. The presence of organic solvent in the separation buffer was necessary in order to increase solubility of hydrophobic docetaxel in the aqueous electrolyte. It was decided to use this strategy for the simultaneous separation of anthracycline and taxane. The addition of two organic solvents methanol and acetonitrile at different concentrations to a 20 mM borate buffer pH 9 was investigated. The electropherograms of the obtained separations are presented in Figure 5.3 and 5.4. Methanol in the concentration 30% or 70% didn't have enough resolving power for the separation of two analytes, they coeluted with the EOF.

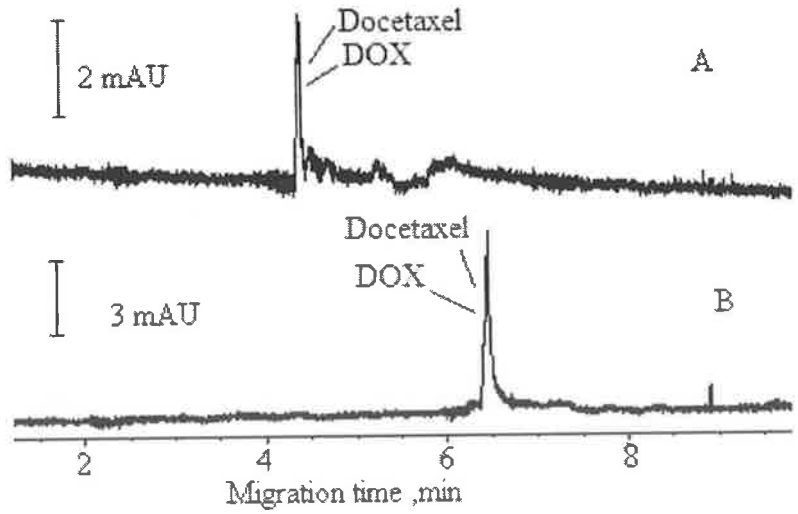


Figure 5.3. Electropherograms of CZE separation of docetaxel and DOX. Separation cond.: 20 mM borate buffer pH 9, A – with 30% MeOH, B – with 70% MeOH, 30 kV, 20°C, hydrod. inj. 2 sec., detec. at 230 nm, capillary 56cm (48cm), 50 μm i.d. Sample: DOX 54.1 μg mL^{-1} and docetaxel 67.6 μg mL^{-1} in the run buffer.

Resolution of more than 1.5 was obtained when acetonitrile was added to the run buffer. It can be seen that the increased amount of organic solvent resulted in an decrease in EOF and that is why the migration times of the analytes with 70% MeOH (Figure 5.3 B) or ACN (Figure 5.4 B, C) in the buffer were longer. A higher amount of acetonitrile added to the separation buffer was beneficial for the separation as the efficiency of both analyte peaks increased. The data is shown in Table 5.2. At the conditions of the separation (pH 9) doxorubicin possessed some negative charge as the phenolic hydroxyl group was deprotonated. This was the reason why the molecules of DOX showed negative mobility and migrated after EOF and docetaxel.

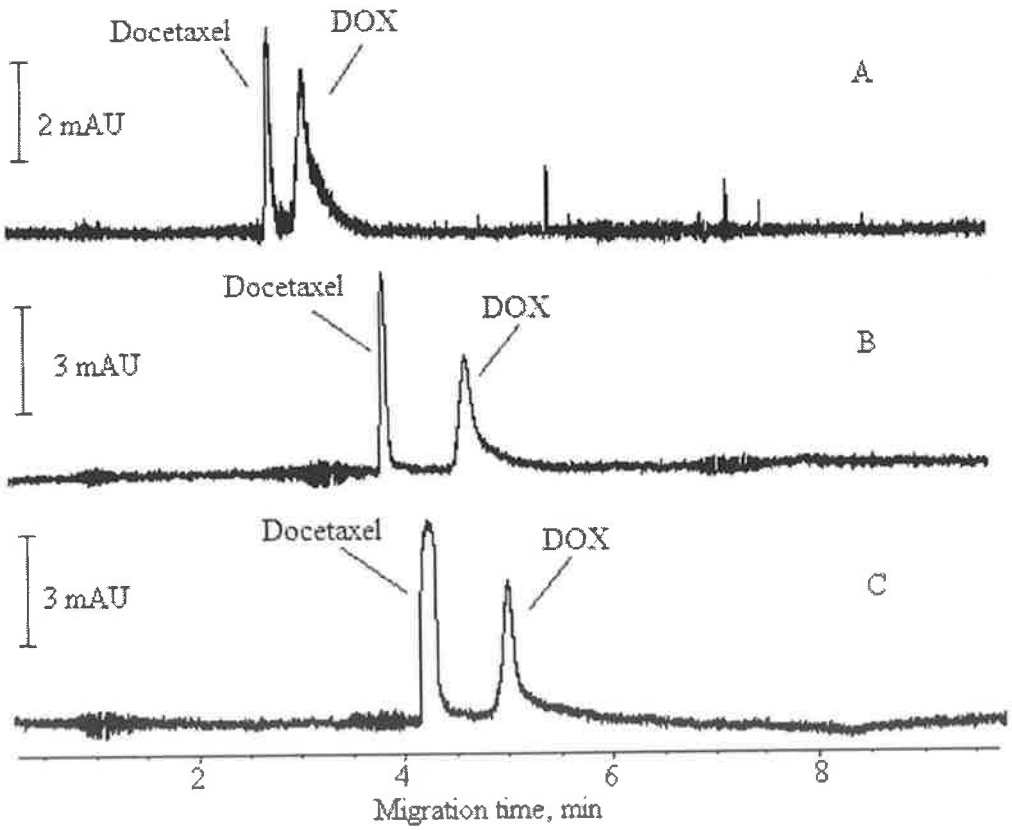


Figure 5.4. Electropherograms of CZE separation of docetaxel and DOX. Separation cond.: 20 mM borate buffer pH 9, A – with 30% ACN, B, C – with 70% ACN, 30 kV, 20°C, hydrod. inj. 6 sec., detec. at 230 nm, capillary 56cm (48cm), 50 μm i.d. Sample: DOX 54.1 μg mL^{-1} and docetaxel 67.6 μg mL^{-1} in the run buffer (A and B) / ACN (C).

The electropherogram in Figure 5.4 C and data in Table 5.2 showed that the composition of the injected sample had an impact on the separation. The efficiency of docetaxel injected in 100% ACN was significantly less than that in the run buffer. On the other hand, the efficiency of doxorubicin was higher in the solvent. The repeatability of the migration time was good as the %RSD was less than 3% (Table 5.2).

Table 5.2. Effect of the ACN concentration and the composition of injection of the parameters of separation. n = 3

Conditions	**Docetaxel**		**Doxorubicin**	
	N	**%RSD (t_m)**	**N**	**%RSD (t_m)**
30% ACN, sample in the run buffer	11376±1576	0.21	1167±608	0.23
70% ACN, sample in the run buffer	18838±1732	2.83	2376±513	2.47
70% ACN, sample in ACN	3827±288	0.76	3973±567	2.04

5.3.2 Separation of anthracyclines and taxanes by MEKC

The methods reported in the literature for determination of taxanes employed micellar electrokinetic chromatography separations [83, 84]. Taxanes tend to have similar, large partition coefficients in the micelle phase due to their small aqueous solubility [84]. Addition of an organic solvent to the separation buffer increased the solubility of these compounds in the electrolyte. A significantly higher concentration of organic solvent is reported to induce changes in size and structure of the micelles [89-90]. Vindevogel and Sandra [91] deduced that SDS micelles might be stable at high concentrations of ACN in the aqueous buffer due to the absence of the precipitation of SDS monomers, which are insoluble in ACN. Shao and Locke [84] concluded that it is still not clear in precisely what form surfactant molecules exist in the presence of organic solvent, but separations achieved in these systems are not possible in the absence of the organic additive.

One of the methods for the separation of doxorubicin and its metabolites by Anderson *et al.* [67] employed MEKC with 10 mM SDS, but no organic phase was required to achieve this separation. The methods for the separation of taxanes [82, 83] and anthracyclines [67] used buffers at alkaline pH. Since MEKC was previously reported to be successful in the independent determinations of taxanes and anthracyclines, it was decided it would be worthwhile to test its resolving power for the simultaneous separation of taxane and anthracycline.

For the purpose of this study doxorubicin and docetaxel were used as test analytes. An alkaline buffer 25 mM Tris-phosphate at pH 9 was employed in the MEKC separation as it created a strong EOF for the negatively charged micelles to be swept towards the detector. As a zwitterionic buffer Tris generated minimal current. The buffer contained 70% MeOH.

5.3.2.1 The effect of SDS on the separation of analytes

Figure 5.5 shows the effect of different concentration of SDS in the run buffer. Analytes were diluted in the run buffer. The concentration of SDS was varied from 10 mM to 100 mM. The migration times of analytes were noted to increase. In this separation doxorubicin migrated along the capillary first and before EOF, followed by docetaxel. The migration of the analytes in such a manner suggests that it did not interact with micelles in the run buffer and also possessed some positive charge as its mobility was higher than that of EOF. The mobility of docetaxel was lower at high content of SDS as the hydrophobic interaction with micelles was enhanced. The molecules of docetaxel were dragged by micelles to the oposite direction of EOF. Docetaxel and doxorubicin were resolved at the concentration of SDS above 50 mM. At this concentration the peak of docetaxel migrated extremely close to the EOF. A 100 mM SDS resulted in sufficient resolution of both peaks with each other and the EOF.

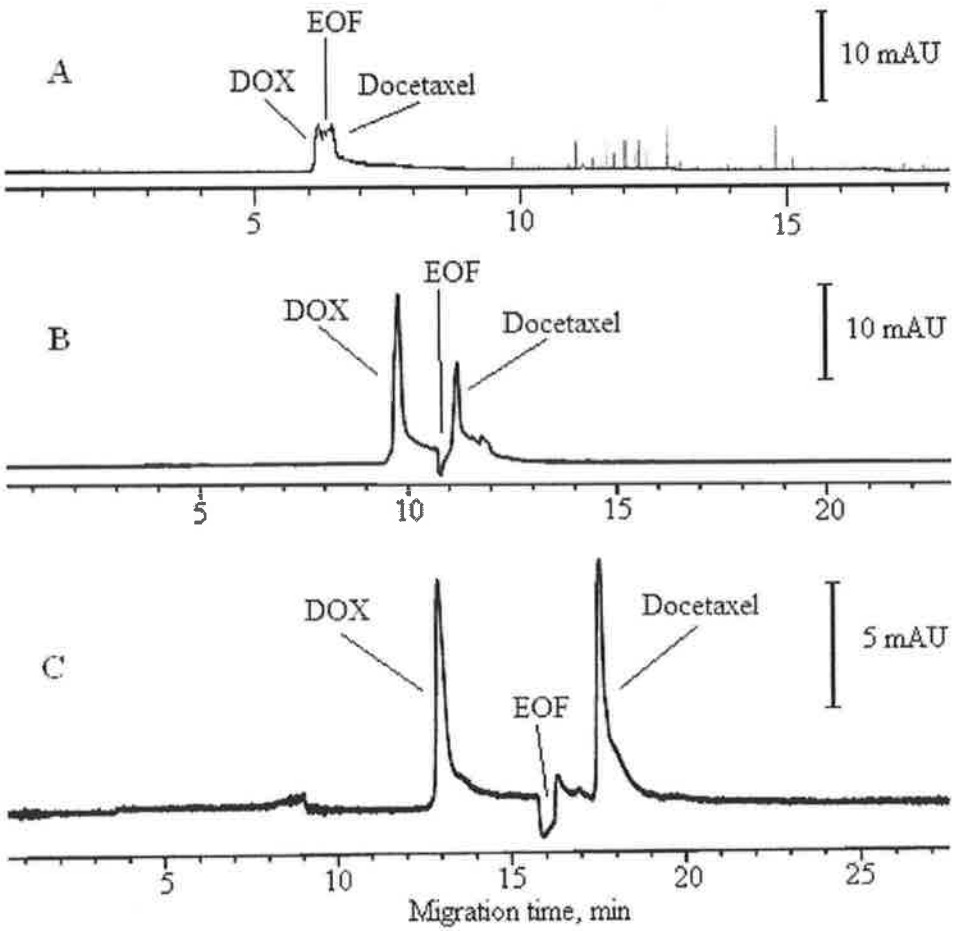


Figure 5.5. Electropherograms of MEKC separation of docetaxel and DOX. Separation cond.: 25 mM Tris-phosphate buffer pH 9, 70% MeOH, A – 10% SDS, B – 50% SDS, C - 100% SDS, 30 kV, 20°C, hydrod. inj. 12 sec., detec. at 230 nm, capillary 56cm (48cm), 50 µm i.d. Sample: DOX 0.121 mg mL^{-1} and docetaxel 0.109 mg mL^{-1} in the run buffer.

5.3.2.2 Effect of sample matrix

Unacceptably poor resolution of docetaxel and doxorubicin was found when using the optimum SDS concentration (100 mM) if the sample dissolved in methanol was injected, as shown in Figure 5.6. The explanation for this is a mass transfer factor [93]. The molecules of analyte are transferred into the micelles from the injection solution at a slow rate, which is not the case for the injection of already micellized solutions. However, this was only relative to the molecules of docetaxel. From a previous experiment it was found that doxorubicin did not interact with the micelle. The injection of the sample in methanol confirmed this as the peak of DOX was very sharp compared to docetaxel, which means the peak shape was independent of the injection media.

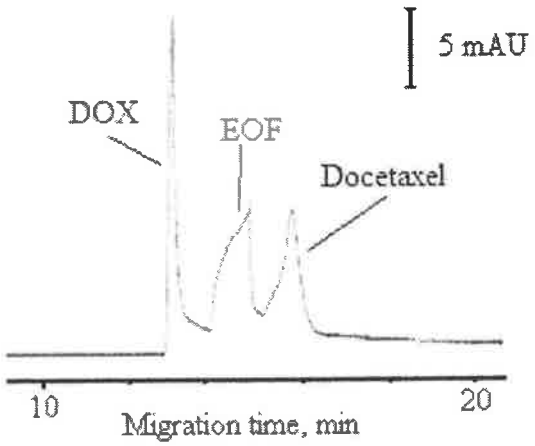


Figure 5.6. Electropherograms of MEKC separation of docetaxel and DOX. Separation cond.: 25 mM Tris-phosphate buffer pH 9, 70% MeOH, 100% SDS, other separation cond. as in Figure 5.5. Sample: DOX 0.128 mg mL^{-1} and docetaxel 0.113 mg mL^{-1} in MeOH.

5.3.2.3 Effect of the pH

The effect of the pH of the run buffer on the mobility of the analyte's peaks is shown in Figure 5.7. The increase of pH from 7.5 to 8.6 resulted in a decrease in the mobility of DOX; at the pH 8.6 – 9.3 the mobility was almost constant with a slight minimum at pH 9.0. At the pH below its pKa (8.34) [94], doxorubicin was only partially deprotonated and had high effective mobility. When the pH was in the region of the pKa value, the molecules of DOX had high negative charge and their mobility toward cathode was slower. The effective mobility of docetaxel was not significantly affected by the variation of the pH of the separation buffer. This is because it was migrating with the micelles along the capillary. The mobility of the micelles was affected by the pH through the change in EOF, which was higher at high pH.

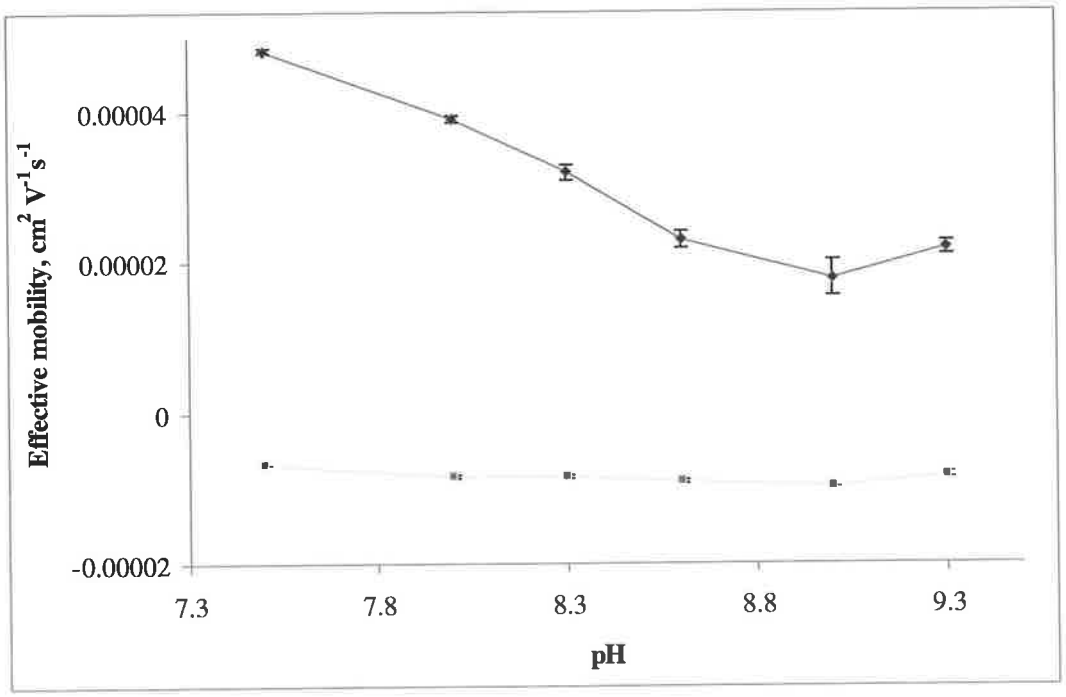


Figure 5.7. Effect of the pH on the effective mobilities of analytes. Separation cond.: 25 mM Tris-phosphate buffer, 70% MeOH, 100% SDS, other conditions as in Figure 5.5. —■— DOX, docetaxel.

5.3.3 Separation of anthracyclines and taxanes by MEEKC

Microemulsion electrokinetic chromatography is known to be a powerful technique in the separation of neutral and charged solutes. The principles of MEEKC have been discussed previously in Chapter 1.4.4 MEEKC allows the separation in a similar fashion as MEKC with the advantage of easier penetration and partitioning of analytes into microemiltion droplets. This is because the structure of the microemulsion is less rigid than that of a micelle [95]. While MEEKC has been known to have a great separation capability for highly hydrophobic compounds [96-100], it also was demonstrated to be a reliable separation tool for hydrophilic analytes [101, 102]. According to the above mentioned reports, MEEKC could potentially be suitable for the separation of a mixture of highly hydrophobic taxanes and hydrophilic anthracyclines.

5.3.3.1 Effect of separation voltage

In this study a typical composition required for the formation of oil droplets 0.81% w/w octane, 3.31% w/w SDS and 6.61% w/w butan-1-ol [103-105] was employed in a combination with a 20 mM phosphate buffer pH 2.5 to suppress EOF. At these conditions both anthracyclines and taxanes posses some positive charges due to the presence of protonated amino groups. At low pH it is necessary to reverse the voltage to attract the negatively charged droplets towards the detector [97]. Figure 5.8 shows the effect of applied voltage on the separation of docetaxel and doxorubicin. The velocities of analytes were found to increase when the voltage was increased from -15 kV to -30 kV resulting in shorter migration times and better resolution. It has to be noted that the acceptable resolution wasn't achieved even with the maximum voltage. Voltage increase was found to induce the enhanced current in the capillary, which was –38 uA, -85 uA and -125 uA for -15 kV, -25 kV and -30 kV applied voltage, respectively. The correlation of voltage and current (Ohm's plot) was plotted for the employed buffer; the acceptable correlation coefficient R^2 = 0.9994 was found for the applied voltage of -15 kV. It has to be noted that even at highest applied voltage the resolution of analytes was not satisfactory.

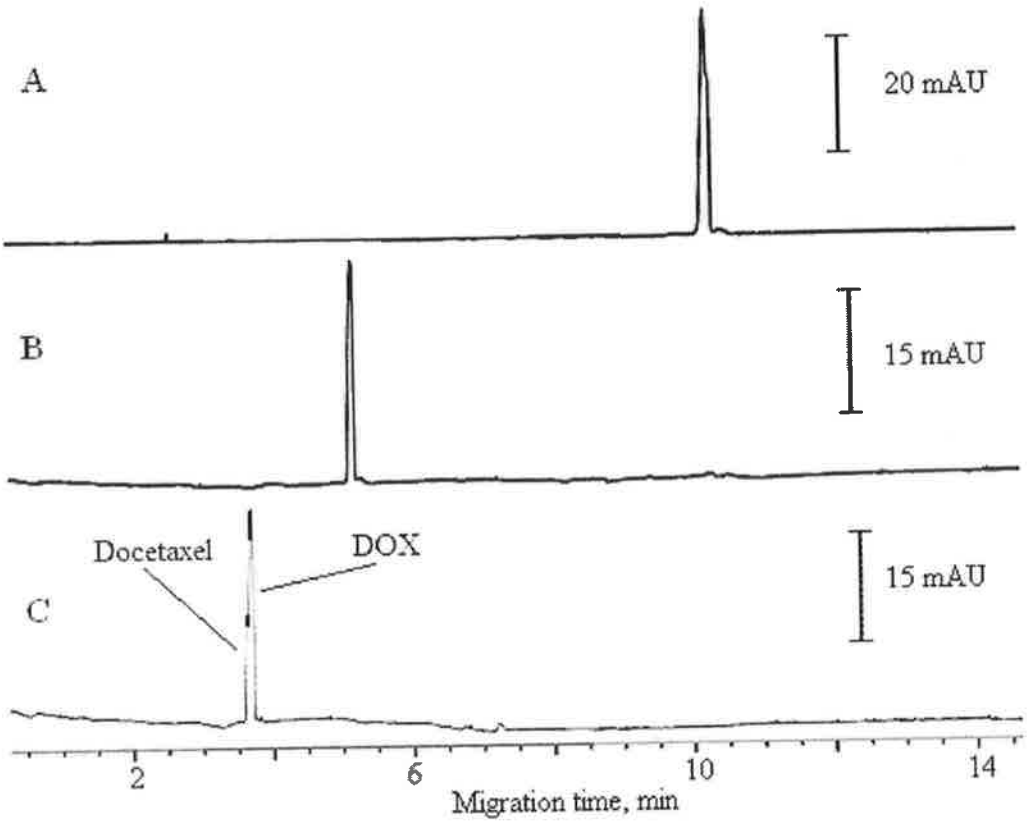


Figure 5.8. Electropherograms of MEEKC separation of docetaxel and DOX at different applied voltage. Separation cond.: 20 mM phosphate buffer pH 2.5, 0.81% w/w octane, 3.31% w/w SDS and 6.61% w/w butan-1-ol, A -15 kV, B -25 kV, C -30 kV, 20°C, hydrod. inj. 12 sec., detec. at 230 nm, capillary 56cm (48cm), 50 µm i.d. Sample: DOX 0.108 mg mL^{-1} and docetaxel 0.122 mg mL^{-1} in the run buffer.

5.3.3.2 Effect of oils on the analyte's separation

Highly hydrophobic organic compounds, such as long-chain alkanes, are generally used as the oil phase in microemulsion. The influence of the oil type on the separation of docetaxel and DOX was examined. The separation of analytes by octane and heptane were compared (data not presented). It was found that the selectivity was not affected by the carbon chain length of the oil. These results were in agreement with previously published works on MEEKC, where the nature of oil phase had little significance in the separation mechanism [101]. Octane was chosen to be the core phase for future analysis due to the greater stability of the emulsion, as suggested by Fu *et al.* [106]. This is due to the number of carbon atoms in the surfactant (SDS) being equal the number of carbon atoms in the co-surfactant (octane) plus the number of carbon atoms in the oil (butanol).

The effect of the core phase on the separation of analytes was investigated in the narrow range 0.41% to 1.21% of octane in microemulsion. It has been previously reported [107] that the stability of the oil droplets suffers at higher concentration of core phase as surfactant is not capable of maintaining core-phase dispersion in the aqueous buffer. The parameters of the separations are shown in Table 5.3. No significant changes in migration times, efficiency and resolution could be obtained from the data. The optimum efficiencies of analyte peaks were observed with 1.21% octane in the microemulsion, but the deviations from the mean value were rather high (27% and 12% RSD).

Table 5.3. Effect of the concentration of octane on the parameters of the separation. Separation cond.: 20 mM phosphate buffer pH 2.5, 3.31% w/w SDS, 6.61% w/w butan-1-ol, 25% MeOH, -20 kV, other conditions as in Figure 5.8. n = 3

% octane	Doxorubicin		Docetaxel		R
	t_m* (min)	N	t_m* (min)	N	
0.41%	13.77	41111±1619	14.48	45592±1294	2.79
0.61%	13.43	40744±2897	14.17	64591±7353	3.22
0.81%	13.53	41614±2348	14.22	56462±8079	2.93
1.01	13.83	46032±3849	14.54	59346±11716	3.08
1.21%	13.61	55112±15421	14.31	66707±8433	3.31

* Mean from three determinations with %RSD <1%.

5.3.3.3 Effect of organic solvent on the analyte's separation

Usually, the partitioning of analyte molecules between oil droplets and aqueous phase can be easily affected by the addition of a water-miscible more polar modifier. Organic solvent is more beneficial for the separation of hydrophobic compounds [102]. The effects of methanol and acetonitrile on the separation of docetaxel and DOX were investigated in this study. Figure 5.9 shows the electropherograms of the separation of analytes with the addition of either methanol or ACN to the microemulsion buffer in a range 5%-25%.

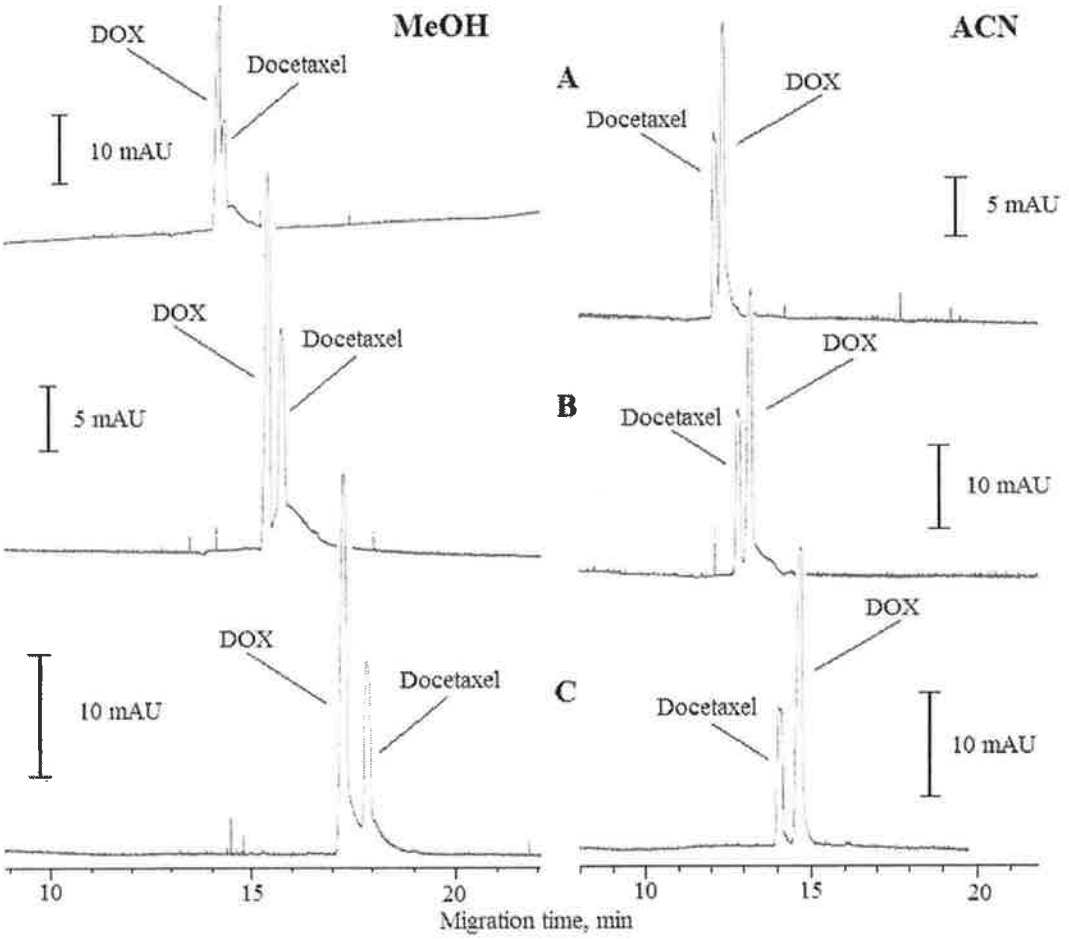


Figure 5.9. Electropherograms of MEEKC separation of docetaxel and DOX at different concentration of MeOH. Separation cond: A – 15% MeOH/ACN, B – 20% MeOH/ACN, C- 25% MeOH/ACN, -15 kV, other conditions as in Figure 5.8.

The results suggest that docetaxel and DOX were better separated when the organic modifier was added to the buffer. The presence of organic solvent decreases the degree of interaction between hydrophobic analyte and the oil droplet. The increased amount of methanol in the emulsion led to a higher resolution of analytes, although the broadening of peak tails was observed at higher concentrations of methanol. This is due to the increased solubility of analytes in the electrolyte solution and difference in the mobility of the oil droplets and analytes. Acetonitrile was found to be a better modifier for the target separation as resolution of docetaxel and doxorubicin was achieved at only 15% of ACN in the buffer. The increase in the methanol content caused the velocities of analytes to be significantly lower. This phenomenon suggests that methanol dramatically decreased the partitioning of analytes into the oil droplets. This delayed the migration of positively charged molecules of docetaxel and doxorubicin towards the anode. The presence of organic solvent can also cause changes in the degree of ionisation of analytes, thus, affecting their mobility [108]. This effect was to a slightly less degree when acetonitrile was added to the microemulsion buffer. The change in the selectivity was observed with the presence of methanol: doxorubicin migrated first, followed by docetaxel. The selectivity of the separation with ACN was identical to the one in the Figure 5.8, were no organic modifier was employed.

5.3.3.4 Effect of SDS concentration on the analyte's separation

The oil droplets in microemulsion are stabilised by coating with the ions of surfactant such as SDS. The effect of SDS content on the separation of docetaxel and DOX was investigated. The concentration of SDS was examined in a range 2.71% - 3.61% w/w. The results of the obtained separations are reported in Table 5.4. The results indicated that the migration velocities of analytes were affected only slightly by the change in SDS concentration. Even in this case it can be noted that docetaxel was affected to a higher degree than DOX. The general trend observed was a reduction in migration time with increased concentration of SDS in the buffer. It points to the fact that docetaxel had a stronger interaction with the oil droplets. At higher concentration of surfactant the number of ions of SDS associated with each droplet increases, thus increasing the charge of the droplets [109]. This can suggest that the nature of the interaction of analytes, especially docetaxel, was based not solely on partitioning but could include ion-pairing process as docetaxel held some positive charge due to the amine group in its structure. The resolution of analytes decreased from 2.76 to 2.08 for the 2.71% and 3.61% of SDS, respectively. The efficiencies of both analytes were enhanced with higher amount of surfactant and were 53438 and 55288 theoretical plates for DOX and docetaxel, respectively.

Table 5.4. Effect of the concentration of SDS on the parameters of the separation. Separation cond.: 20 mM phosphate buffer pH 2.5, 0.81% w/w octane, 6.61% w/w butan-1-ol, 25% MeOH, -20 kV, other conditions as in Figure 5.8. n = 3

% SDS	Doxorubicin		Docetaxel		R
	t_m* (min)	N	t_m* (min)	N	
2.71%	12.60	26781±2223	13.34	39801±6469	2.76
3.01%	12.13	39512±4903	12.67	44383±3909	2.37
3.31%	12.44	41576±2981	12.97	50504±4651	2.39
3.61%	12.17	53438±1970	12.58	55288±7823	2.08

* Mean from three determinations with %RSD <1%.

5.3.3.5 Effect of co-surfactant on analyte's separation

In a study by Marshal *et al.* [110] it was noted that co-surfactant is the most influential constituent on the separation selectivity of microemulsion. Co-surfactant is usually added to the microemulsion buffer to reduce levels of SDS required for the formation of droplets. The influence of the concentration of co-surfactant 1-butanol was studied in order to determine its effect on the separation of docetaxel and doxorubicin. The examined concentration range of 1-butanol was 5.61% - 7.61%. The parameters of the acquired electropherograms are presented in Table 5.5. The increase in concentration of 1-butanol was accompanied by reduced velocities of analytes, which resulted in longer migration times. When the content of co-surfactant in the buffer is at higher rates, the solution viscosity changes and the microemulsion droplets increase in size [111]. The latter is thereby reducing the ability of droplets to move faster in the direction of the anode, which obviously affects the migration of analytes in the capillary if they interact with the micelles. The resolution of analytes was quite consistent and was approximately 2.6 with 6.11% - 7.11% of 1-butanol in the buffer, but reduced to 1.89 at 7.61% 1-butanol. The efficiency of analyte peaks was found to be the best when the concentration of 1-butanol was 7.11%.

Table 5.5. Effect of the concentration of 1-butanol on the parameters of the separation. Separation cond.: 20 mM phosphate buffer pH 2.5, 0.81% w/w octane, 3.31% w/w SDS, 25% MeOH, -20 kV, other conditions as in Figure 5.8. n = 3

% butanol	Doxorubicin		Docetaxel		R
	t_m* (min)	N	t_m* (min)	N	
5.61%	12.35	44058±3331	12.91	48047±4482	2.55
6.11%	12.99	36204±202	13.63	49462±4682	2.66
6.61%	13.77	37443±4070	14.45	47385±6438	2.67
7.11%	14.14	49442±5958	14.75	62892±17393	2.65
7.61%	14.34	41378±5245	15.36	41267±15118	1.89

* Mean from three determinations with %RSD <1.5%.

5.3.3.6 The effect of temperature on the analyte's separation

The hydrophobic properties of analytes and thus their partitioning with the oil droplets are influenced by the temperature, at which the separation is performed in MEEKC. It was stated by Gong *et al.* [112] that the optimised temperature is a combination of dynamic and thermodynamic processes in the system. In terms of thermodynamics the increased temperature induces a decrease in the partitioning coefficient of analytes between the oil phase and the water phase. Regarding dynamics, the elevated temperature can speed up the transferring velocity between the oil and the water phase.

The effect of temperature on the separation of docetaxel and doxorubicin was studied at 20°C, 35°C and 50°C. The data obtained is shown in Figure 5.10. It was found that increasing the temperature from 20°C to 50°C caused the migration times of analytes to reduce from 13.28 min and 13.86 min to 9.46 and 9.58 min for doxorubicin and docetaxel, respectively. The migration time decreased when the temperature increased due to the partitioning of the insoluble compounds into the oil droplets being less as their solubility increase [113]. The EOF is higher at the elevated temperature as the viscosity of the buffer is lower; therefore, the migration time is reduced [114]. In the present work docetaxel and less hydrophobic doxorubicin were affected in a similar way by the elevated temperature. It can be seen in Figure 5.10. (A) the efficiency of both peaks have decreased dramatically when the temperature was raised. The resolution (Figure 5.10(B)) of docetaxel and doxorubicin was below 1 at 50°C. For this reason it was decided to perform further experiments at 20°C, disregarding the advantage of shorter analysis time at elevated temperature.

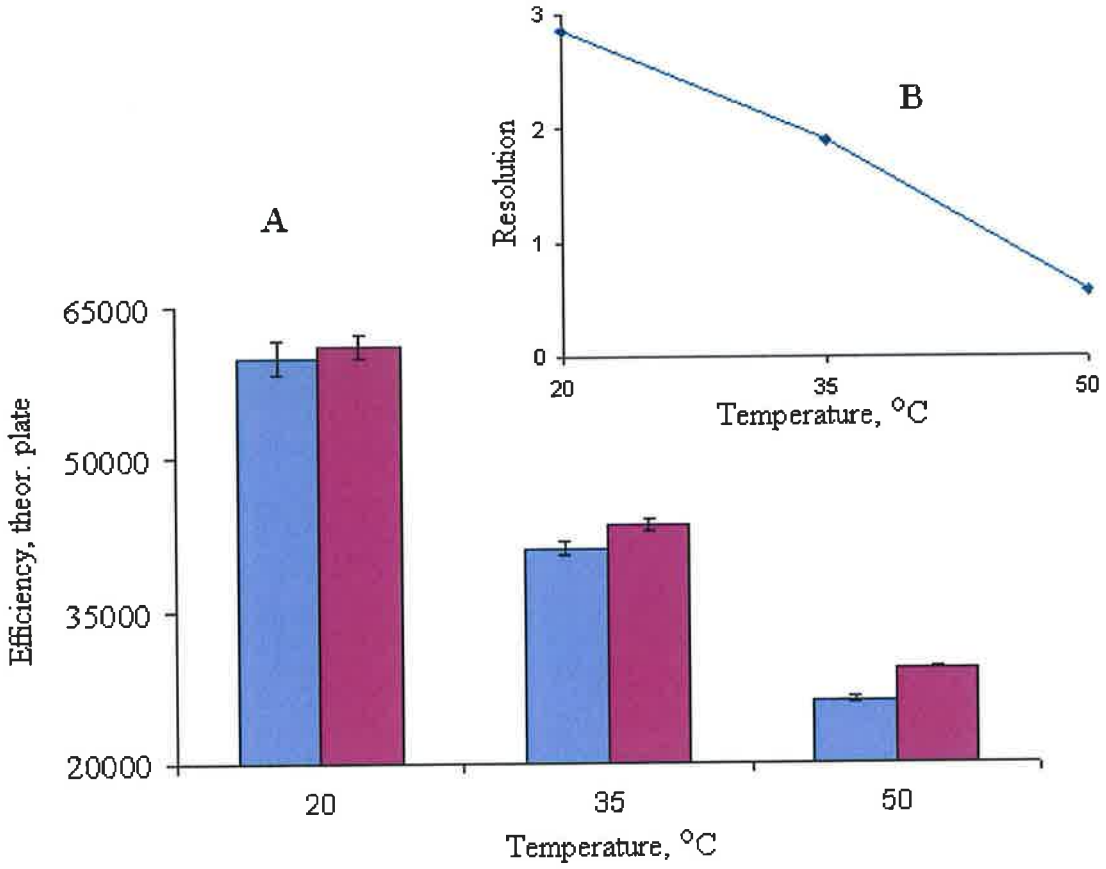


Figure 5.10. Effect of the temperature on the efficiency and resolution of analytes. Separation cond.: 20 mM phosphate buffer pH 2.5, 3.31% w/w SDS, 6.61% w/w butan-1-ol, 25% MeOH, -20 kV, other conditions as in Figure 5.8. A – efficiency of ■ - doxorubicin and ■ - docetaxel, B - resolution. n = 3

5.3.3.7 The optimum composition of microemulsion

All optimised parameters, such as the content of octane, 1-butanol and SDS, were combined in the final composition. This separation of docetaxel and doxorubicin was then compared to the separation with the original microemulsion. The parameters of both separations are shown in Table 5.6. The migration times of analytes with the optimised microemulsion were enhanced. The resolution was lower than at the separation with the original composition of microemulsion, but still acceptable. The efficiencies of analytes were within the error. Thus, both microemulsion compositions would be suitable for further application.

Table 5.6. Parameters of the separation with original composition of microemulsion and optimised composition. Separation cond.: 20 mM phosphate buffer pH 2.5, 25% MeOH, -20 kV, other conditions as in Figure 5.8. n = 3

№	Microemulsion composition	Doxorubicin		Docetaxel		R
		t_m* (min)	N	t_m* (min)	N	
1	0.81% octane, 6.61% butanol, 3.31% SDS	13.70	66701±1962	14.37	68791±6742	3.21
2	1.21% octane, 7.11% butanol, 3.61% SDS	14.41	65864±2692	14.97	63974±3708	2.62

* Mean from three determinations with %RSD <1%.

5.3.4 Separation of anthracyclines and taxanes by high-speed MEEKC

There are several ways to reduce the analysis times in CE in order to increase sample throughput in many industrial environments. These approaches include the employment of short capillaries, high voltages, high temperatures, simultaneous application of pressure and voltage, and "short end" injection [103].

The common composition of the microemulsion 0.81% w/w octane, 3.31% w/w SDS and 6.61% w/w butan-1-ol [103-105] does not allow fast separation due to the high amounts of SDS employed. The amount of added surfactant depends on the surface tension of the oil. Ethyl acetate (EA) and di-*n*-butyl tartrate are the oils that have lower surface tension and have previously been used [115, 116]. These oils require only 0.6% SDS to form the oil droplets [97].

In this study a microemulsion consisting of 0.5% w/w ethyl acetate, 1.2% w/w butan-1-ol, 0.6% SDS, was employed in a combination with a 20 mM phosphate buffer pH 2.5 [97]. The injection of the sample was performed with -50 mbar for 2 sec from the "short end", which is the end of the capillary near the detector. The effective capillary length (normally 48 cm) was reduced to 8 cm with this type of injection. In this case there was no need to reverse the voltage of separation while a low pH buffer was employed. A positive voltage of 30 kV was applied providing a stable current not higher than 30 uA.

5.3.4.1 Effect of organic solvent on the analyte's separation

The attempt to achieve the separation of docetaxel and DOX with the above conditions failed as two analytes comigrated. The previous experiments in this chapter have shown the important role of the organic solvent in the partitioning of analyte molecules between oil droplets and aqueous phase. Figure 5.11 shows the effect of the concentration of MeOH on the separation of docetaxel and doxorubicin. It was found that the analyte peaks were sharper but less resolved (R = 1.13) at 10 % MeOH. The baseline resolution (R = 3.42) was obtained with 25% MeOH in the microemulsion buffer, but the efficiencies of the analytes, and especially doxorubicin, were extremely low (N = 842 for DOX). These results suggest that MeOH decreases the partitioning of analytes into the oil droplets. The same effect of methanol on the efficiency of analytes was found with octane microemulsion (Section 5.3.3.3). Regardless of the low resolution of analytes when 10 % MeOH was added to the buffer, this concentration was chosen for further experiments as better efficiency of peaks will enable the observation of the effects of other components of microemulsion. The time of analysis in this study was significantly shorter compare to the octane MEEKC conditions and was just over 1 min.

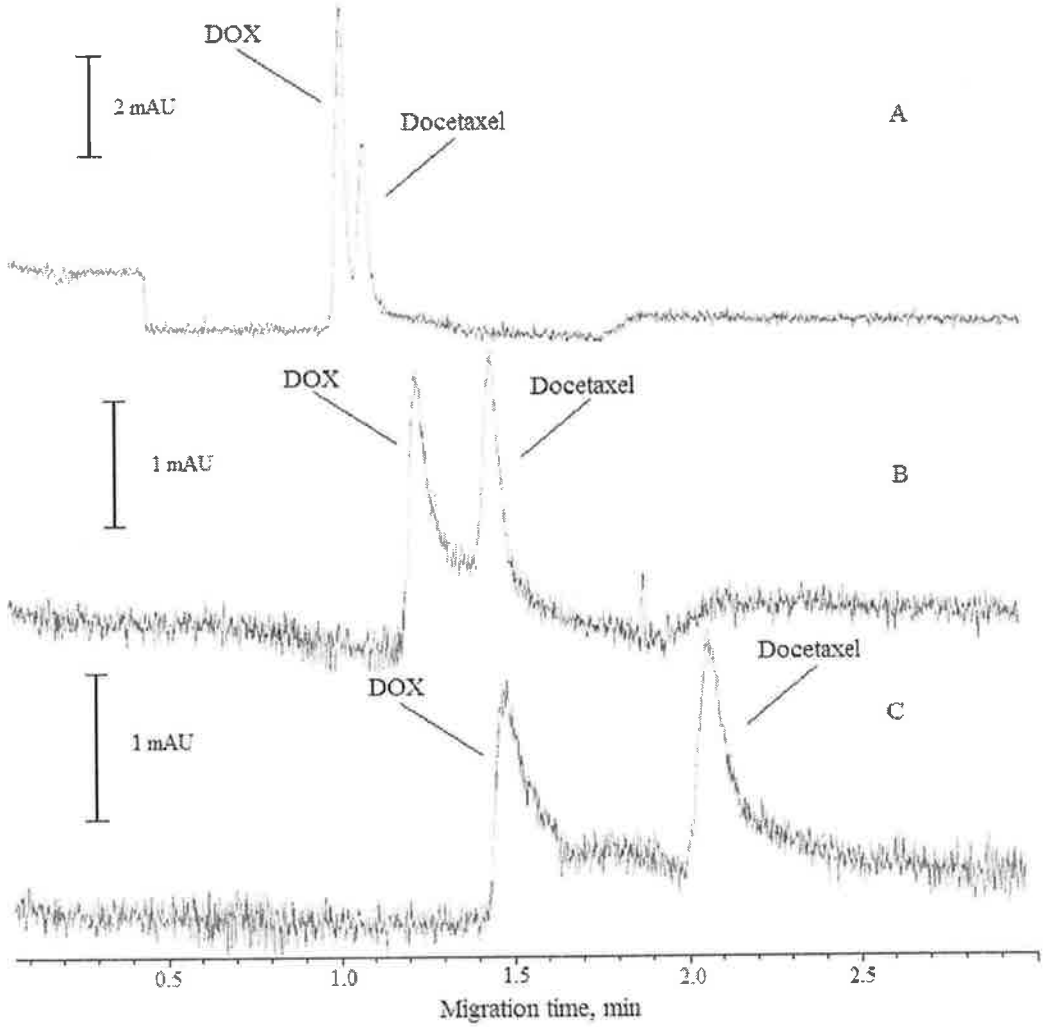


Figure 5.11. Electropherograms of high-speed MEEKC separation of docetaxel and DOX at different concentrations of MeOH. Separation cond.: 0.5% w/w ethyl acetate, 1.2% w/w butan-1-ol, 0.6% SDS, 97.7% 20 mM phosphate buffer pH 2.5, A – 10% MeOH, B – 20% MeOH, C – 25% MeOH, 30 kV, 20°C, hydrod. inj. -50 mbar for 2 sec, detec. at 230 nm, capillary 56cm (8cm), 50 µm i.d. Sample: DOX 54.1 µg mL^{-1} and docetaxel 67.6 µg mL^{-1} in the run buffer.

5.3.4.2 Optimisation of the separation

In order to optimise the separation of docetaxel and doxorubicin with high-speed MEEKC the effect of each component of the microemulsion on the separation was investigated. Table 5.7 represents the obtained parameters of the separation of docetaxel and DOX while varying the concentration of SDS, 1-butanol and ethyl acetate, respectively, in the microemulsion.

Table 5.7. Effect of SDS, 1-butanol and EA on the parameters of the separation. Separation cond.: 20 mM phosphate buffer pH 2.5, 10% MeOH, other conditions as in Figure 5.11. n = 3

Varied parameter	Doxorubicin		Docetaxel		R
	t_m* (min)	N	t_m* (min)	N	
%SDS	**Microemulsion composition: 0.5% w/w EA, 1.2% w/w butan-1-ol**				
0.4%	1.09	4160±618	1.16	3367±665	0.96
0.5%	0.99	4898±127	1.04	4765±1887	0.85
0.6%	0.98	5273±208	1.01	3992±434	0.64
0.7%	0.98	5510±749	1.00	3309±276	0.44
%butanol	**Microemulsion composition: 0.5% w/w ethyl acetate, 0.6% SDS**				
1.4%	0.98	5757±220	1.02	3992±434	0.76
1.6%	0.97	5756±327	1.01	3309±276	0.79
2.0%	1.06	5148±1694	1.11	5328±1960	0.90
%EA	**Microemulsion composition: 1.6% w/w butan-1-ol, 0.5% SDS**				
0.6%	0.94	8232±751	1.00	8215±869	1.37
0.7%	0.95	8789±1451	1.02	7938±944	1.54

* Mean from three determinations with %RSD <1.5%.

It has to be noted that the efficiencies of docetaxel and doxorubicin in Table 5.7 are significantly lower than those obtained with octane MEEKC. This is due to the difference of the effective length in two separations. The analyte migration distance was 48 cm when injection was performed from the "normal" end of the capillary. With the "short end" injection this value was 8 cm.

In general, the effect of the concentrations of SDS, butan-1-ol and ethyl acetate on the separation of docetaxel and doxorubicin was in agreement with the previously obtained data for the octane microemulsion. However due to the very short migration times and a narrow concentration range of the varied parameter, the difference in the change in migration time was quite small and the effect of the varied parameters was difficult to observe.

Thus, an increase in SDS concentration resulted in increased efficiency of doxorubicin. The efficiency of docetaxel was quite similar within the error in the range of studied concentration of SDS, only at 0.5% SDS the efficiency was higher but was accompanied by a large error. The resolution of analytes was better at low concentration of SDS in the microemulsion. The significant increase of the efficiency of docetaxel and the resolution of analytes was only noticed at 2% of butan-1-ol in microemulsion (Table 5.7). The parameters of the separation at the concentration 1.4% and 1.6% were lower than those obtained at a higher content of 1-butanol and did not differ significantly. A greater content of the core phase resulted in higher resolution of analytes and better efficiency of doxorubicin.

The optimised values of the content of ethyl acetate, 1-butanol and SDS were combined in the final composition. The separation of docetaxel and doxorubicin was compared to the separation with the original microemulsion. The parameters of both separations are shown in Table 5.8. The migration times of analytes with the optimised microemulsion (№ 2 in Table 5.8) were slightly longer. The resolution approached the acceptable value and was 2.19. The efficiencies of analytes separated with the optimised microemulsion were significantly higher than in the original microemulsion. But they were also accompanied by larger %RSD values. In general, the optimised composition of microemulsion was somewhat more advantageous over the first microemulsion and can be suggested for further development.

Table 5.8. Parameters of the separation with original and optimised composition of microemulsion. Separation cond.: 20 mM phosphate buffer pH 2.5, 10% MeOH, other conditions as in Figure 5.11. n = 3

№	Microemulsion composition	Doxorubicin		Docetaxel		R
		t_m* (min)	N	t_m* (min)	N	
1	0.5% EA, 1.2% butanol, 0.6% SDS	0.98	4898±1270	1.04	4765±1887	0.85
2	0.7% EA, 2.0% butanol, 0.4% SDS	1.13	12324±5023	1.23	10687±6026	2.19

* Mean from three determinations with %RSD <1.5%.

5.3.4.3 Choice of the internal standard

In order to ensure the repeatability of the separation the internal standard is required in the analysed sample. Naproxen, equilin and 2, 4-dichlorophenoxyacetic acid (2, 4-D) were considered as internal standards in the separation of doxorubicin and docetaxel. The electropherograms of the obtained separations are shown in Figure 5.12. All potential compounds could be used as IS for the simultaneous determination of doxorubicin and docetaxel. The mobility of naproxen, equilin and 2, 4-D differed from the mobilities of analytes at the conditions of the experiment and all the compounds were separated. It has to be noted that chosen IS possessed weak partitioning into the oil droplets as they migrated after the analytes. The separation with equilin and naproxen was short and took just over 2 min, which is preferential in terms of minimising the time of analysis.

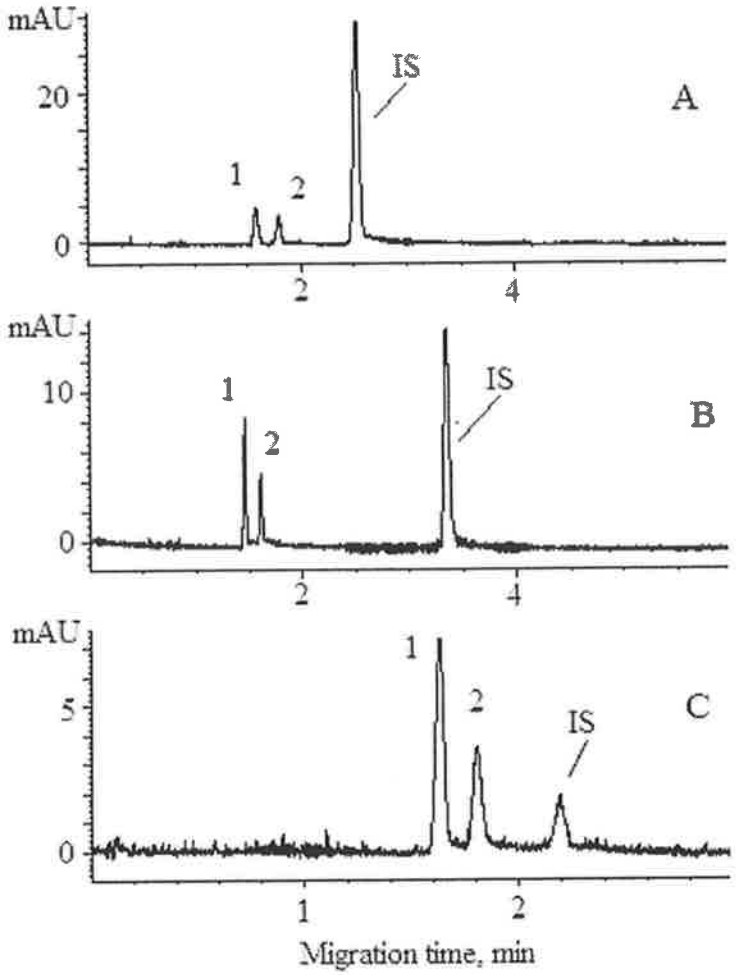


Figure 5.12. Electropherograms of separation of docetaxel and DOX with different IS. Separation cond.: 0.7% w/w ethyl acetate, 2% w/w butan-1-ol, 0.4% SDS, 97.7% 20 mM phosphate buffer pH 2.5, 10% MeOH, other conditions as in Figure 5.11. Peak identification: 1 – DOX, 2 – docetaxel, IS: A – naproxen 52.6 μg ml^{-1}, B – 2, 4-D 91.1 μg mL^{-1}, C – equilin 52.6μg mL^{-1}.

5.3.4.4 Application of the method to different anthracyclines and taxanes

The developed method was applied to the separation of various combinations of anthracyclines and taxanes. The electropherograms in Figure 5.13 show the separation of daunorubicin and docetaxel (A), daunorubicin and paclitaxel (B) and doxorubicin and paclitaxel (C).

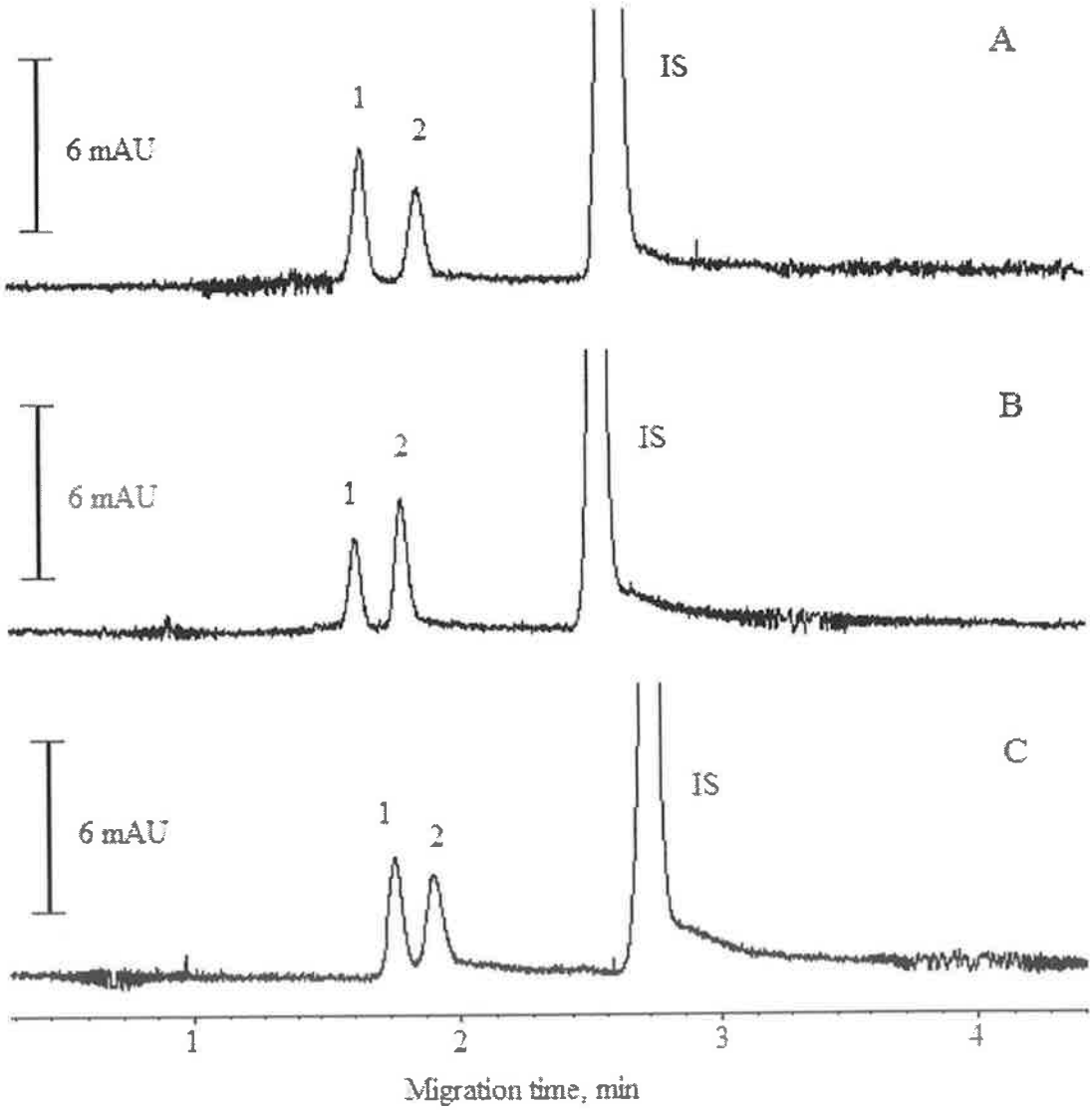


Figure 5.13. Electropherograms of separation of anthracyclines and taxanes. Separation conditions as in Figure 5.12. Peak identification: A: 1 – DAU (52.6 µg mL^{-1}), 2 – docetaxel (65.7 µg mL^{-1}); B: 1 - DAU (52.6 µg mL^{-1}), 2 – paclitaxel (65.7 µg mL^{-1}); C: - 1 – DOX (52.6 µg mL^{-1}), 2 – paclitaxel (65.7 µg mL^{-1}); IS – naproxane (55.2 6µg mL^{-1}).

It can be seen that all of the combinations of cancer drugs were separated successfully using the developed method. Daunorubicin and doxorubicin have the same electrophoretic behaviour and have the same position on the electropherogram. The method developed for docetaxel was suitable for paclitaxel. This is due to the high similarity in chemical structure of DAU and DOX and paclitaxel and docetaxel, as was discussed in 5.1.1 and 5.1.2. This experiment led to the assumption that the developed method could be a generic method for the determination of any anthracycline and any taxane.

5.3.4.5 Method precision

While the investigated method has shown a good potential for fast simultaneous separation of anthracyclines and taxanes, several issues can arise when performing statistical analysis of the data. In order to measure the precision of the developed method the repeatability of the migration time, peak area and peak height for each analyte were determine. The data was collected over 10 and 17 consequent experiments and presented in Tables 5.9 and 5.10. The combinations of DAU and docetaxel and DOX and docetaxel with equilin as an IS were investigated. Excellent repeatability of the migration times over 10 or 17 runs for all compounds could be noticed.

The %RSD of the peak area and height were higher for anthracyclines rather than for taxane and were up to 33% and 18%, respectively. Tables 5.9 and 5.10 also contain information about the peak area and height ratios in relation to the IS. The introduction of the IS into the calculation of the peak areas and heights levels out the precision of analytes and brings closer the average values between 10 and 17 consequent runs.

Due to the hydrophilic properties of anthracyclines, their interaction with hydroxyl groups of silica of the capillary walls can take place, in the same way as they can be adhered to the glass sample vial [117]. This can affect the peak shape, area and height. The capillary was often required to be replaced as the repeatability of the method was seriously suffering, but more experiments are necessary in order to establish the life time of the capillary. The employment of

several capillaries resulted in slight differences of the migration times of analytes tested on each capillary.

It was noted that precipitation occurred with anthracyclines in the microemulsion on some occasions. It is advisable therefore to prepare samples in the separation buffer directly before the analysis.

Table 5.9. Repeatability parameters of numerous separations of DOX and docetaxel.

Parameter	N	DOX		Docetaxel		IS(Equilin)	
		Avr.	%RSD	Avr.	%RSD	Avr.	%RSD
t_m	17	1.58	1.8	1.75	2.1	2.10	3.0
(min)	10	1.59	1.8	1.77	1.4	2.13	2.0
Peak	17	17.7	32.9	10.6	10.8	4.6	18.0
Area	10	14.6	34.1	10.3	9.0	4.3	20.3
Peak	17	6.3	22.8	3.6	8.4	1.4	19.3
Height	10	5.6	24.7	3.6	9.8	1.4	24.0
Area ratio	17	3.8	21.6	2.4	19.3	-	-
to IS	10	3.8	22.5	2.5	22.4	-	-
Height	17	4.6	23.0	2.6	23.1	-	-
ratio	10	4.1	25.8	2.7	27.7	-	-
to IS							

Table 5.10. Repeatability parameters of numerous separations of DAU and docetaxel.

Parameter	n	DAU		Docetaxel		IS(Equilin)	
		Avr.	%RSD	Avr.	%RSD	Avr.	%RSD
t_m	17	1.57	1.7	1.75	1.7	2.10	2.6
(min)	10	1.59	1.5	1.77	1.3	2.14	1.7
Peak	17	15.2	26.5	10.4	8.5	4.6	17.8
Area	10	16.4	26.5	10.0	7.0	4.8	17.7
Peak	17	5.8	31.9	3.7	14.9	1.4	28.0
Height	10	6.6	27.8	3.9	18.3	1.6	27.7
Area ratio	17	3.3	17.9	2.3	21.3	-	-
to IS	10	3.4	14.0	2.1	20.1	-	-
Height	17	4.1	15.3	2.7	22.7	-	-
ratio	10	4.2	13.9	2.5	21.5	-	-
to IS							

While the capillary was washed for a long period of time at the beginning of the experiments, the first run of the day was always characterised by better efficiencies of the peaks. But this was not taken into account while obtaining statistical data. This and the high values of %RSD (7-34.1% for peak area) can suggest that capillary conditioning should be investigated in more detail. At the current state of the method development the rinsing between runs consisted of 1 min was with 0.1 M NaOH, followed by MeOH for 1 min and run buffer for 1 min. Taking into the account the complex nature of target analytes, the prewashing step should be optimised.

5.3.4.6 Sensitivity of the method

The issue of sensitivity is a matter of a high priority for the analysis of cancer treatment drugs in biological samples. The levels of anthracyclines and taxanes in plasma can vary from 4.5 μg mL^{-1} to 0.02 μg mL^{-1} over a period of 24 hours after injection [46]. The working concentrations of anthracycline and taxane were 52.6 μg mL^{-1} for DAU and DOX and 65.7 μg mL^{-1} for docetaxel. Although the LODs and LOQs were not identified for the developed high speed microemulsion separation, it is clear that the method needs further improvement in relation to the sensitivity issue.

Choice of optimum wavelength is one of the ways to improve the detector response. Figure 5.14 shows the investigated separation at the employed wavelengths of 230 nm and 200 nm. It can be seen that the intensity of docetaxel at 200 nm was double the intensity at 230 nm. Most commercial detectors allow recording two wavelengths simultaneously. This is how the optimum signal for each compound can be obtained.

Another way of achieving a higher signal is a higher loading of sample onto the capillary. A hydrodynamic injection was investigated at 5 sec and 10 sec and the obtained electropherograms are presented in Figure 5.15. The resolution and efficiency suffered when 10 sec injection (B) was performed, while peak area has increased. An injection of 5 sec (A) was a better compromise for the resolution, efficiency signal of the peaks.

For further modification of the analysis with the UV detector it is possible to use an extended light pass capillary, where the diameter of the capillary at the detection window is 3 times greater than the diameter in the separation part. This was previously employed for the analysis of paclitaxel and docetaxel [83] (Table 5.1). The achieved LODs were in the required analytical range.

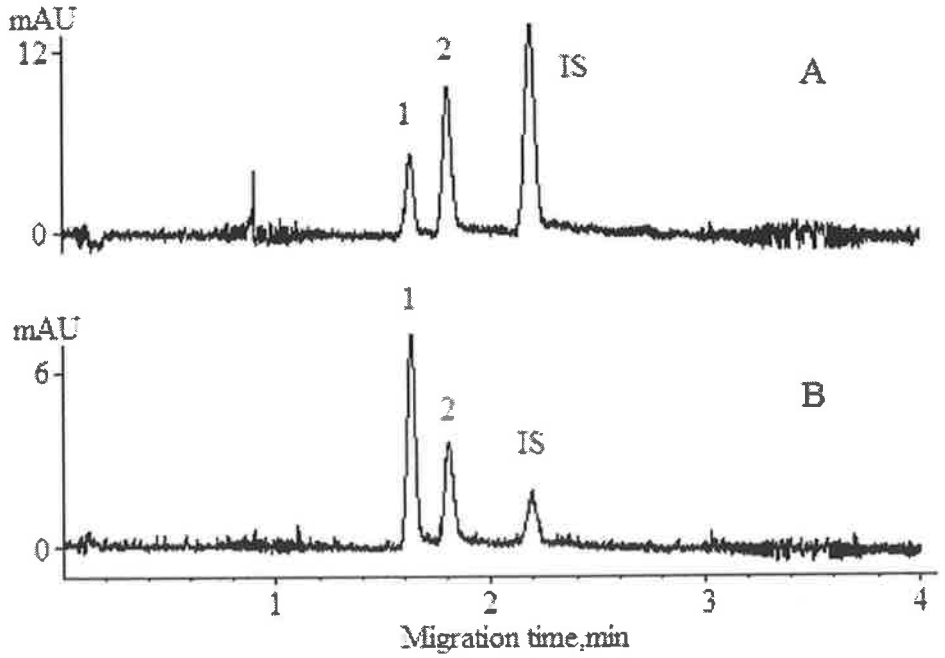


Figure 5.14. Electropherograms of separation of docetaxel and DAU at different wavelengths. A – 200 nm, B – 230 nm. Other separation conditions and peak identification as in Figure 5.12(C).

The most radical and probably most successful way of approaching the desirable detection range would be to employ a different detection technique. Thus, fluorescence detector was proven to be very sensitive for anthracyclines [61-63]. In order to perform a simultaneous determination of the anthracyclines and taxanes, a derivatization step would be required for taxanes as they do not exhibit natural fluorescence. Mass spectral detection can be universal for both compounds, but its compatibility with the microemulsion composition and the applied voltage should be investigated in details.

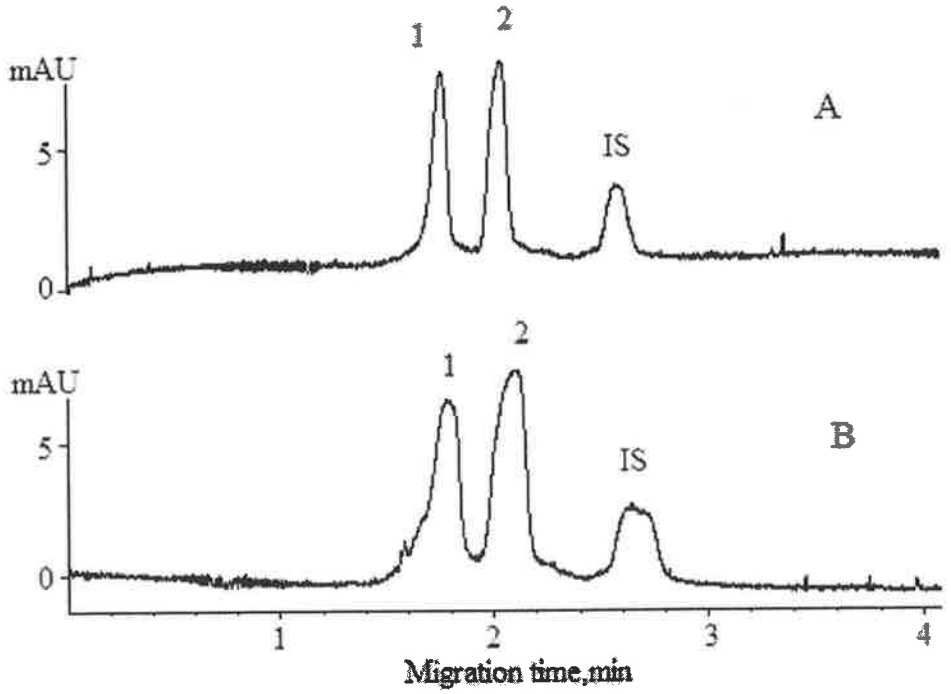


Figure 5.15. Electropherograms of separation of docetaxel and DAU at different time of hydrodynamic injection. A – 5 sec, B- 10 sec. Other separation conditions and peak identification as in Figure 5.12(C).

5.3.5 Application to plasma samples

5.3.5.1 Direct plasma injection

For the application of the method for real time monitoring, minimal preparation of the biological samples is desirable. Preliminary experiments were performed in order to test the suitability of the capillary electrophoresis simultaneous determination of taxane and anthracycline for direct injection of human plasma samples. Daunorubicin and docetaxel were used to spike a plasma sample. This was then directly injected onto the capillary and analysed with the developed EA MEEKC method. Figure 5.15 represents the electropherogram of the separation of plasma diluted with the run buffer in the ration 20 : 80 (v/v). The spiked sample (B) was compared to the blank mixture injection (A). The peaks of daunorubicin and docetaxel could be identified. Peaks were noticeably broader, compared to the peaks on the electropherogram, where analytes were injected in 100% separation buffer (Figure 5.13 (A)). It took slightly longer for daunorubicin and docetaxel to migrate. The electropherogram in Figure 5.15 (A)) shows that no interfering peaks were observed in blank plasma.

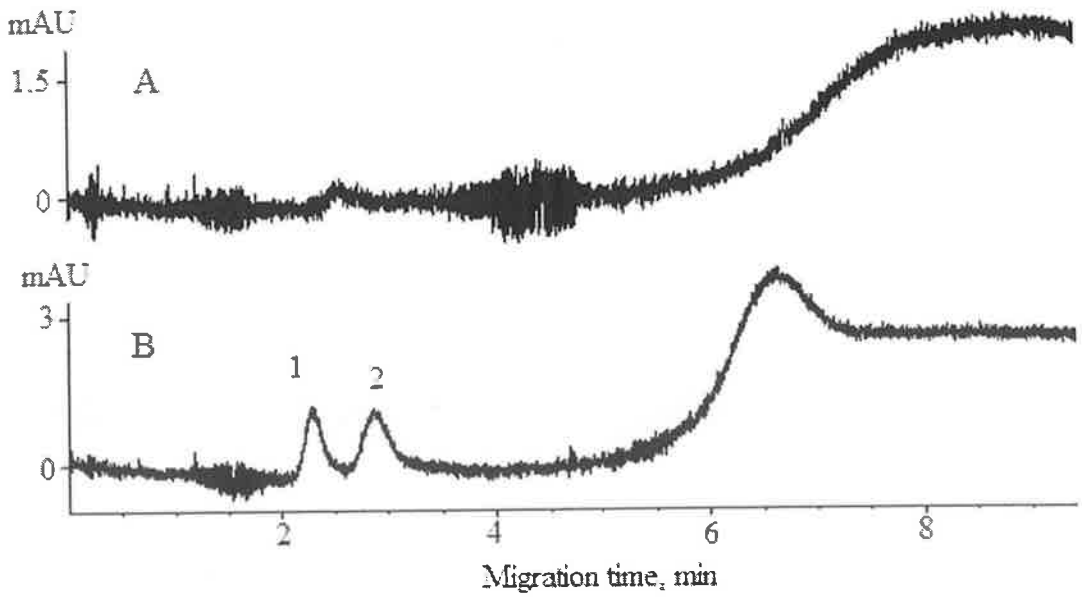


Figure 5.15. Electropherograms of direct plasma injection. Injection composition: plasma : separation buffer = 20: 80 (v/v). A – blank plasma, B – plasma spiked with docetaxel, DAU and IS. Separation conditions as in Figure 5.12. Peak identification: 1 – DAU 53.8 μg mL^{-1} 2 – docetaxel 67.2 μg mL^{-1}, IS – naproxen 11.3 μg mL^{-1}.

In the electropherogram in Figure 5.16 the plasma was diluted with the run buffer in the ration 50 : 50 (v/v). Peaks of analytes were identified and no interfering peaks were observed in blank plasma. Lowering the content of the separation buffer in the injection and increasing the plasma part of it resulted in a low efficiency and resolution of analyte's peaks. This can suggest that molecules of analytes are transferred into the oil droplets at a lower rate when the buffer part in the injection decreases. This implies that analytes undergo strong binding with proteins of plasma [118] and change their electrophoretic behaviour and, thus, do not elute from the capillary. When a combination of daunorubicin and docetaxel was injected in 100% plasma or an injection was composed with over 50% of plasma, no peaks due to analyte could be seen on the electropherograms (data is not presented).

Preliminary experiments have shown that the direct injection of plasma was possible, but required large dilutions of the sample. This is not desirable for low detection limits. Further experiments are required in order to investigate the reliability of this method.

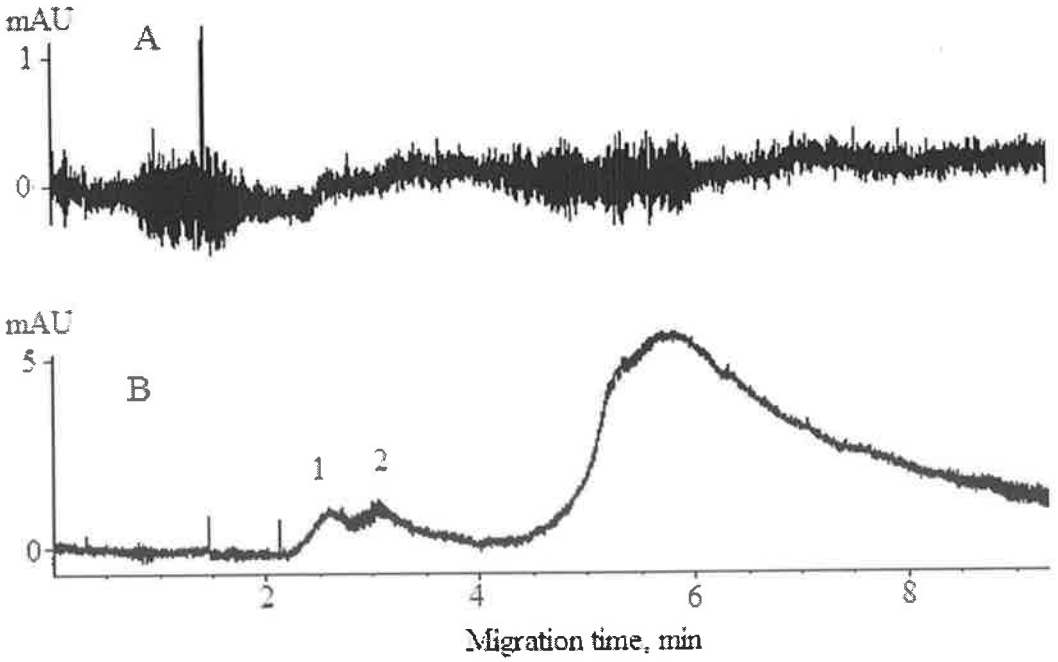


Figure 5.16. Electropherograms of direct plasma injection. Injection composition: plasma : separation buffer = 50: 50 (v/v). A – blank plasma, B – plasma spiked with docetaxel, DAU and IS. Separation conditions as in Figure 5.12. Peak identification as in Figure 5.15.

5.3.5.2 Separation of pretreated plasma

In order to disrupt the protein-drug binding so that the total amount of drug can be extracted for analysis it is advisable for a sample to undergo a pretreatment step prior to analysis [119]. Using precipitation techniques to denature the proteins, their ability to bind drugs can be destroyed depending on the binding mechanism [120]. In this study the acetonitrile precipitation of proteins was employed. The organic solvent lowers the dielectric constant of plasma protein solution, which increases the attraction between charged molecules and facilitates electrostatic protein interaction [119]. The molecules of acetonitrile also displace the molecules of water in the hydrophobic part on the protein surface. When the hydrophobic interactions between proteins are minimal, the electrostatic interactions become predominant and lead to the aggregation of proteins.

Three potential methods for the simultaneous separation of daunorubicin and docetaxel were tested in this study. The separation buffers and conditions that revealed the separation of target analytes in CZE (5.3.1), MEKC (5.3.2) and MEEKC (5.3.3) modes of capillary electrophoresis were applied for the blank plasma and plasma spiked with analytes. The precipitation of plasma proteins was performed according to the procedure described in 5.2.4. The obtained electropherograms are shown in Figure 5.17 – 5.19.

The electropherogram in Figure 5.17 (B) illustrates that the CZE method failed to determine analytes in the plasma, while the sufficient separation was obtained in a test mixture (Figure 5.4 (B)). The electropherogram of blank plasma (Figure 5.17 (A)) shows that there was a peak attributed to plasma and it interfered with the migration of analytes.

Three electropherograms in Figure 5.18 represent the separation of blank plasma (A), spiked plasma (B) and the separation of a test mixture of DAU, docetaxel and IS (equilin) (C). The latter experiment was required to be repeated as the IS was added to the mixture. It shows that the obtained MEKC method was suitable for the separation of equilin from both analytes. The mobility of equilin in the capillary was lower than the mobility of docetaxel as the interaction with the micelles was stronger. The analysis of spiked plasma sample was quite

successful, as all peaks were separated and plasma peak was resolved from analytes.

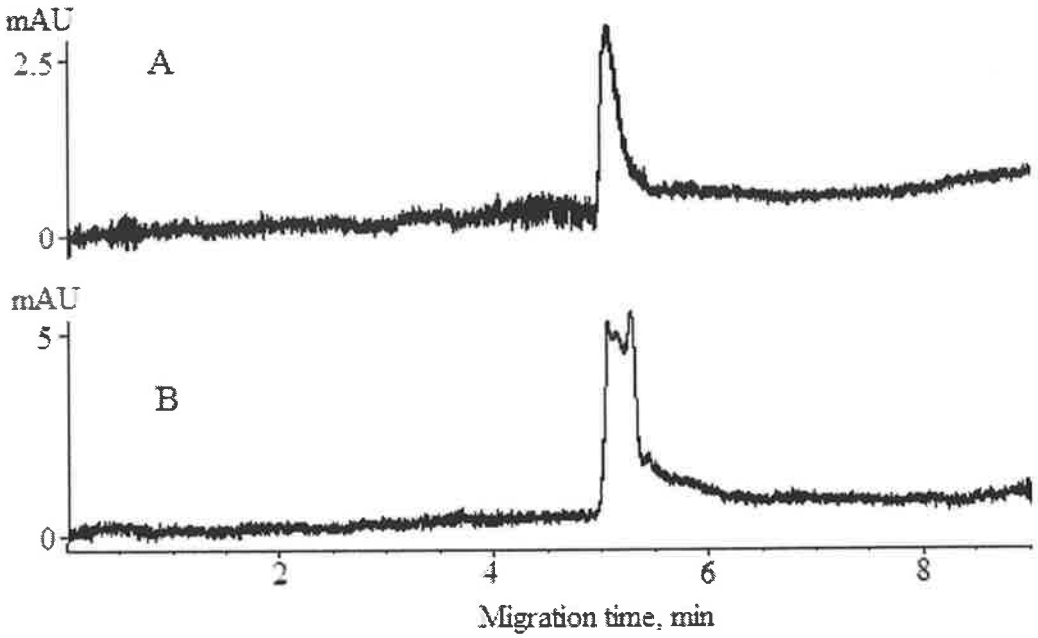


Figure 5.17. Electropherograms of the CZE separation of the pretreated plasma samples. A – blank plasma, B – plasma spiked with 45.5 μg mL^{-1} DAU, 56.8 μg mL^{-1} docetaxel and 90.9 μg mL^{-1} equilin (IS). Separation cond.: 20 mM borate buffer pH 9, 70% ACN, 30 kV, 20°C, hydrod. inj. 2 sec., detec. at 230 nm, capillary 56cm (48cm), 50 μm i.d.

A high speed microemulsion electrokinetic chromatography with the ethyl acetate as a core phase was also tested with the injection of deproteinised plasma. The electropherogram of the blank plasma extract (Figure 5.19 (A)) shows the peaks of several compounds which were present in the acetonitrile supernatant of plasma. Daunorubicin, docetaxel and IS were separated in a spiked sample, as can be seen in Figure 5.19 (B). However the experiment was not successful as the peak attributed to plasma overlapped with the anthracycline.

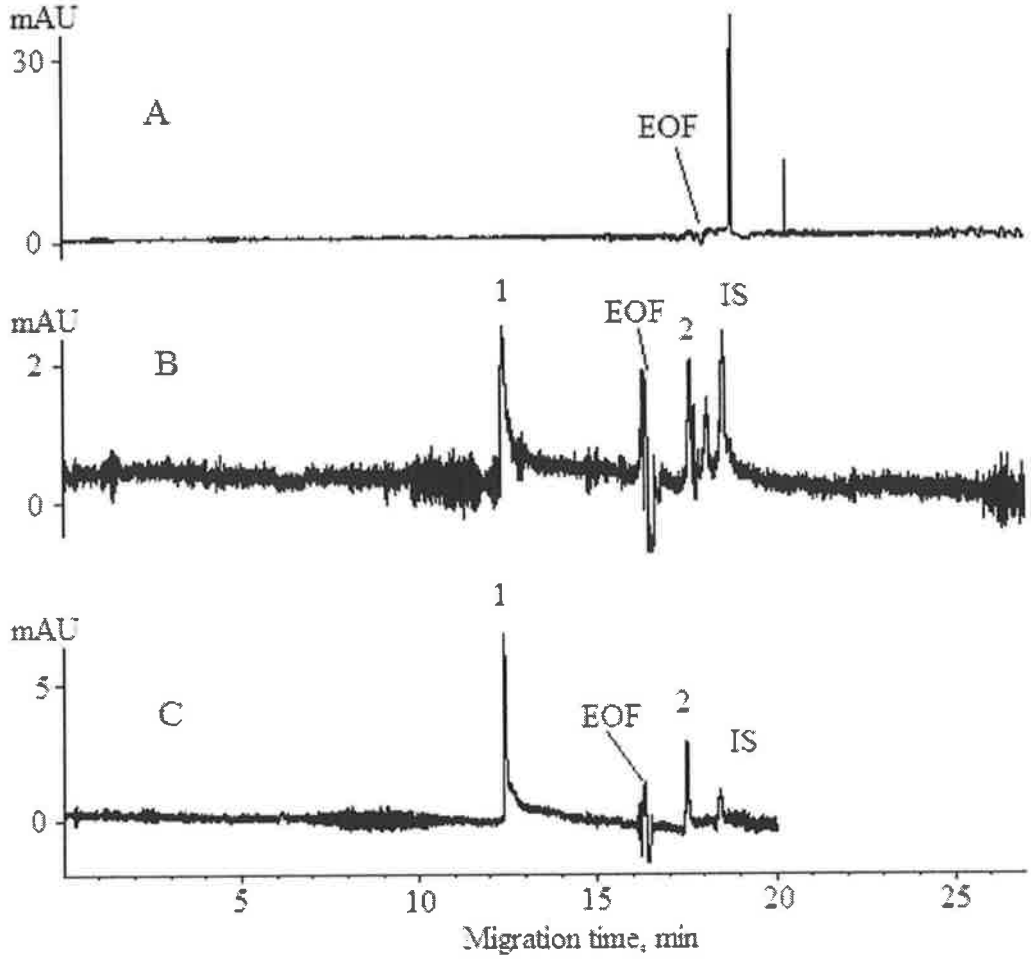


Figure 5.18. Electropherograms of the MEKC separation of the extracted plasma samples. A – blank plasma, B – plasma spiked with 45.5 μg mL^{-1} DAU, 56.8 μg mL^{-1} docetaxel and 90.9 μg mL^{-1} equilin (IS), C – test mixture of DAU 52.6 μg mL^{-1}, docetaxel 65.2 μg mL^{-1} and equilin 52.6 μg mL^{-1} the run buffer. Separation cond.: 25 mM Tris-phosphate buffer pH 9, 70% MeOH, 100% SDS, 30 kV, 20°C, hydrod. inj. 2 sec., detec. at 230 nm, capillary 56cm (48cm), 50 μm i.d.

From this experiment it can be concluded that the three analysed methods representing different modes of capillary electrophoresis were proven to be powerful in the separation of test mixtures of daunorubicin and docetaxel. However, their resolving ability was affected in a different way when real samples of plasma were analysed. CZE mode can only be effective in the separation of charged molecules. This was probably not the case in the analysis of pretreated plasma and that explains why the method had failed. MEKC and MEEKC were more successful in the application to the real samples as they are able to separate more species using partitioning mechanism.

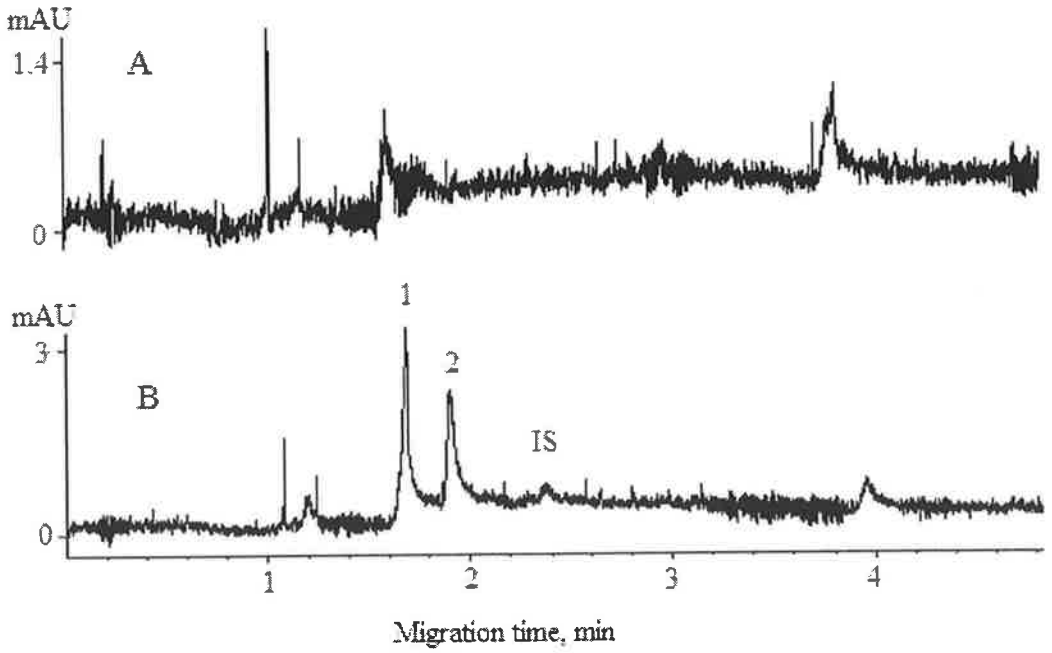


Figure 5.19. Electropherograms of the EA MEEKC separation of the extracted plasma samples. A – blank plasma, B – plasma spiked with 45.5 µg mL^{-1} DAU, 56.8 µg mL^{-1} docetaxel and 90.9 µg mL^{-1} equilin (IS). Separation cond.: 0.5% w/w ethyl acetate, 1.2% w/w butan-1-ol, 0.6% SDS, 97.7% 20 mM phosphate buffer pH 2.5, 10% MeOH, 30 kV, 20°C, hydrod. inj. -50 mbar for 2 sec, detec. at 230 nm, capillary 56cm (8cm), 50 µm i.d.

The high speed MEEKC is a very attractive method from the point of short migration time – one run lasts 2.5 min. But the determination of anthracycline is difficult due to its comigration with the plasma peak. An alternative method for plasma pretreatment could be designed in order to improve the removal of undesirable plasma components. The use of solid-phase extraction can be very advantageous as a plasma pre-treatment method and preconcnetration procedure in order to achieve lower LODs. The aim of this work did not include the development of the pretreatment procedure. At this stage of the work it was found that the MEKC method for the simultaneous determination of anthracycline and taxane has the most potential in application to the plasma extracts. Nonetheless it cannot yet be used for the determination of the plasma samples from patients due to the sensitivity issue. Even though the LODs for daunorubicin and docetaxel were not determined it can be seen from the intensity of the peaks that they are greatly exceed the required values.

5.4 CONCLUSIONS

For the first time simultaneous analysis of anthracyclines and taxanes is reported. Several modes of capillary electrophoresis were screened in order to achieve the separation and investigate the electrophoretical behaviour of analytes. The separation is possible using CZE, MEKC and MEEKC techniques. The importance of the role of organic solvent in each mode of separation was noted.

While CZE and MEKC have previously been used in the separation of anthracyclines and taxanes in single runs, the MEEKC method is successfully applied to these compounds for the first time. The effects of different parameters of microemulsion on the electrophoretic behaviour of both drugs have been examined in detail.

An innovative approach of shortening the analysis time was employed. The injection was performed from the "short end" of the capillary, so that the separation was performed only on 8 cm of the effective length. With the addition of low surface tension core phase in microemulsion it was possibly to use small concentrations of surfactant and, thus, to apply high voltage for more efficient separation. This all resulted in a run time of less than 2 min.

A high speed MEEKC method have shown to be a generic method as combinations of different anthracyclines and taxanes could be analysed. This is highly beneficial for clinical analysis in cancer therapy when the combinations of prescribed drugs can be varied.

Even though at present there is need for future strategy on achieving the required sensitivity levels for analytes, MEKC and high speed MEEKC methods were proven to show good potential in their application to deproteinised plasma spiked with analytes. This is especially true for the MEKC method where all analytes and plasma components were separated. The plasma pretreatment procedure requires further development. CZE method has failed to separate anthracyclines and taxane in the deproteinised plasma. High speed MEEKC was also capable of determining the analysed drugs in the direct injection of plasma, but high dilutions with the separation buffer were required.

This work represents a novel approach in the analysis of drugs employed in combinational therapy of cancer. The sample throughput of a clinical laboratory can be significantly increased by using fast methods for simultaneous monitoring of pharmacokinetics of anthracyclines and taxanes.

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CHAPTER 6

CONCLUSIONS AND FUTURE WORK

6.1 PROGRESSION OF THE RESEARCH

The outstanding analytical strength of capillary electrophoresis lies in its vast resolving power, high speed of analysis, high efficiency and superior versatility along with minimal sample and reagents consumption. This makes it highly advantageous in the area of pharmaceutical analysis. The potential of capillary electrophoresis was investigated in this work in an attempt to provide fast, efficient and selective methods for the analysis of several pharmaceuticals.

6.1.1 CE in the analysis of alkylxanthines

Capillary electrophoresis has been shown to be a very rapid and simple method for the simultaneous determination of alkylxanthines. A full analysis could be performed in just 2 min. The method did not require rinsing of the capillary between runs as only borate buffer was employed as the background electrolyte without any additives. This is the fastest reported method for the analysis of this group of compounds. The same method can be used as for the separation of both single compounds and mixtures. The method was successfully employed in the analysis of commercial pharmaceuticals and food samples. The achieved limits of detection were satisfactory for the analysed sample and excellent repeatability of the method was reported.

6.1.2 Analysis of the association complexes of steroids and cyclodextrins

The capability of CE in the measurement of physicochemical properties was investigated. Cyclodextrins are often employed in chromatographic and elecectrophoretic methods for the determination of steroid hormones due to their high structural similarity. Fundamental knowledge of equilibrium and the association constants of analysed compounds and ligand in the system is a powerful element in the understanding of that system and in discovering ways to evaluate it. For the first time the association constants of inclusion complexes of estriol, 17β-estradiol,

ethynylestradiol, estrone, progesterone, mestranol and norethindrone and different types of cyclodextrins were investigated by capillary electrophoresis. The estimation of association constants of steroid compounds with cyclodextrins was a straightforward process due to the high efficiency and simple performance of capillary electrophoresis in a combination with three plotting methods, which were used to ensure the reliability of the data. The most powerful agent for the inclusion of the target analytes was found to be γ-CD followed by 2HP- γ-CD. Interaction with the cavity of β-CD was rather weak, while 2HP-β-CD failed to recognise a difference in structure of estriol, equilin, ethynylestradiol, 17β-estradiol and estrone. It was found that the association constant values decreased when the concentration of micelles in the run buffer was increased.

To obtain a large picture of the steroid inclusion in cyclodextrins and to investigate the mechanism of inclusion in more detail it would be beneficial to study a large number of steroids. The investigation of enatiomers of steroid compounds would be beneficial as many of them are optically active. The employment of molecular modelling and nuclear magnetic resonance technique would provide complementary data for this research.

6.1.3 Determination of montelukast sodium and related compounds

Capillary electrophoresis has shown good potential and many advantages over the HPLC method for the determination of montelukast sodium. The full analysis time was less than 9 min, which is significantly less than the 35 min analysis time of the HPLC method. A high efficiency of the montelukast sodium peak of over 900000 theoretical plates was recorded with the achieved method. The conversion of montelukast to its *cis*-isomer was investigated using the developed method. It was not possible to quantify the impurity in a fresh sample of montelukast due to their low concentration present and the insufficient sensitivity of the employed instrument (CE-UV). The employment of mass spectral detection in combination with the developed separation could potentially achieve this goal and, additionally, provide information on structural characteristic of the impurities.

6.1.4 CE for the simultaneous determination of anthracyclines and taxanes

The achievements of this research open an avenue for future improvement in the analysis of anticancer drugs used in combinational therapy. For the first time simultaneous analysis of anthracyclines and taxanes was reported, which can significantly increase a sample throughput of a clinical laboratory. Anthracyclines and taxanes were separated by CZE, MEKC and MEEKC mechanisms of capillary electrophoresis. MEEKC method was successfully applied to these compounds for the first time and was characterised by very short separation time, high efficiencies of peaks and was proven to be generic for the separation of different combinations of anthracyclines and taxanes. This is highly beneficial for clinical analysis in cancer therapy when the combinations of prescribed drugs can be varied. MEKC and high speed MEEKC methods were proven to show good potential in their application to plasma samples. Further strategy on the plasma pretreatment step should be investigated. The employment of an alternative detection technique, such as fluorescence or electrochemical detection, is the next step for this research in order to achieve the limits of detection, which are required for the monitoring of therapeutic levels of drugs in blood. Development of a microchip device for the simultaneous capillary electrophoretic separation of anthracyclines and taxanes could benefit even further the needs of high-throughput analysis in the clinical laboratory and point-of-care testing fro improved patient care.

6.2 CONCLUSIONS

In this work the high value of capillary electrophoresis in the determination of pharmaceuticals was demonstrated using the examples of several investigated methods. Taking into account the undoubted potential for high efficiency, selectivity and speed, capillary electrophoresis should be given more opportunities in analyses, where it can perform at a high level.

Appendix

PUBLICATIONS

1. "Rapid simultaneous determination of alkylxanthines by CZE and its application in analysis of pharmaceuticals and food samples" Anal. Chim. Acta 540 (2005) 103.
Yuliya Shakalisava and Fiona Regan

2. "Determination of association constants of inclusion complexes of steroid hormones and cyclodextrins from their electrophoretic mobility" Electrophoresis 27 (2006) 3048.
Yuliya Shakalisava and Fiona Regan

3. "Simultaneous determination anthracyclines and taxanes by capillary electrophoresis" Article in preparation.
Yuliya Shakalisava and Fiona Regan

4. "Separation of montelukast sodium and related impurities by capillary electrophoresis" Article in preparation.
Yuliya Shakalisava and Fiona Regan

ORAL PRESENTATION

58th Research colloquium 2006, Galway, Ireland
"A capillary electrophoretic study of the inclusion of steroid hormones in cyclodextrins"
Yuliya Shakalisava

POSTER PRESENTATIONS

1. Analytical Forum 2004, Warsaw, Poland
"Capillary zone electrophoresis for the determination of alkylxanthines"
Yuliya Shakalisava and Fiona Regan

2. European Summer School "Human environment: energy, forests, health and society" 2004, Nancy, France
"Cyclodextrin-modified capillary electrophoresis determination of endocrine disrupting chemicals with direct sample injection"
Fiona Regan, Barbara Forgaty, Anne Moran and Yuliy Shakalisava

3. MicroScale Bioseparations 2005, New Orleans, USA
"Cyclodextrin-modified capillary electrophoresis determination of steroids"
Yuliya Shakalisava and Fiona Regan

4. Analytican Forum 2005, Plymouth, UK
"Advantages of cyclodextrins in the development of novel generic methods for the determination of steroid hormones by capillary electrophoresis"
Yuliya Shakalisava and Fiona Regan

5. MicroScale Bioseparations 2006, Amsterdam, Netherlands
"Development of capillary electrophoresis method for the determination of related impurities in montelukast sodium"
Yuliya Shakalisava and Fiona Regan

6. MicroScale Bioseparations 2006, Amsterdam, Netherlands
"The potential of cyclodextrin in developing generic buffers for the separation of steroids"
Yuliya Shakalisava and Fiona Regan

7. ITP 2006, Paris, France
"Determination of antiasthmatic compounds by capillary electrophoresis"
Yuliya Shakalisava and Fiona Regan

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Rapid simultaneous determination of alkylxanthines by CZE and its application in analysis of pharmaceuticals and food samples

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Abstract

Capillary electrophoresis (CE) offers the possibility of fast, cheap and reproducible separations for pharmaceutical preparations. Alkylxanthines make up a family of compounds that are used in the treatment and prevention of bronchi asthma and chronic pulmonary disease. The group of analysed compounds include caffeine, dyphylline, theophylline, theobromine and enprofylline. This paper shows a simple capillary zone electrophoretic (CZE) method for separation of this group of xanthines. Using 20 mM borate buffer at pH 9.4 as running buffer at 55 °C it was possible to complete a total separation of a sample in 2 min. Limits of detection in the range 1.9–2.5 mg l^{-1} were achieved with %R.S.D. of 0.06–0.22% ($n=5$). The technique is applied to a range of samples containing the analytes, including tablets and chocolate. Reproducibility (%R.S.D.) of the chocolate analysis technique by CZE was less than 4.5%.

Keywords: Capillary zone elctrophoresis; Fast separation; Alkylxanthines; Chocolate

1. Introduction

A family of compounds alkylxanthines (Fig. 1) that includes caffeine, theophylline and theobromine is very well known in pharmaceutical science due to the following properties [1]: antiasthmatic, analgetic adjuvants, antitussives, bronchodilators, cardiac stimulants, cognition enhancers, diuretics, lipolytic agents, cancer chemotherapy adjuvants, treatment of cerebral ischemia, treatment of Parkinson's disease, renal failure. The frequency of human consumption of this type of pharmacologically active compounds speaks for itself. Xanthines are present in all kinds of tea, coffee, carbonated beverages, chocolate and chocolate products and chewing gum [2]. Adding all these factors together, the necessity of quality control of pharmaceuticals and alkylxanthines contents of food becomes very important.

A large amount of articles have been published on HPLC analysis of alkylxanthines [3–7]. In general, chromatographic methods for the separation of alkylxanthines require a clean-up step prior to the analysis itself and at least 20 min for the last compound to elute. It takes less, only if a single compound is analysed [6]. Usually a UV detector is employed at 214, 230, 270–275 or 280 nm [10] and the limit of detection is approximately 50 ng ml^{-1}. Relative standard deviation for caffeine ranged between 0.86 and 1.97% (intraday) and 1.04 and 3.90% (interday) [4].

While capillary electrophoresis has been reported to show successful separation of this group of compounds [8,9,11–17], lengthy capillary rinse times and pre-rinses make total analysis times long (Table 1). Due to the fact, that most of alkylxanthines are weak bases the alkali pH (Fig. 1) of several buffers (such as borate [9,18], phosphate [11,14], glycine [13,19] or a mixture of them [20]) is employed for the CE separation. Some separations use micellar electrokinetic chromatography (MEKC) [8,9,12] thereby increasing the necessity for lengthy rinses between the separations and irreversible modification of the capillary takes place. MEKC is often necessary in order to determine the neutral compounds in this group [8,9,19].

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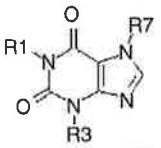

	R1	R3	R7	pKa
Theophylline	$-CH_3$	$-CH_3$	-H	8.8
Theobromine	-H	$-CH_3$	$-CH_3$	10.0
Caffeine	$-CH_3$	$-CH_3$	$-CH_3$	14.0
Enprofylline	-H	$-CH_2CH_2CH_3$	-H	8.4
Dyphylline	$-CH_3$	$-CH_3$	$-CH_2CHOHCH_2OH$	11.0

Fig. 1. Chemical structures and pK_a values of some alkylxanthines [8,9].

Each of the analytical techniques for the detrmination of alkylxanthines have advantages and disadvantages. Thus, both chromatographic and spectroscopic methods require a sample pretreatment step, while some of the CE samples can be injected directly. They can reach very low limits of detection; and this is advantageous when the concentration of target compounds is low. A disadvantage of the chromatographic technique is a long time of analysis, up to 20 min, while CE methods can take just a few minutes [11,13], and sometimes methods are complicated with the necessity to change the composition of the mobile phase [5]. Chromatographic methods require expensive equipment compared to some CE systems. The problem with spectroscopic methods is that methylxanthines give high spectral overlap [21] when determined simultaneously and that is why this technique is poorly developed. All the methods are characterized with high reproducibility.

According to all the reasons noted above, capillary electrophoresis methods have a very high potential in the separation of alkylxanthines and, in the opinion of the authors, there is more potential for further improvement.

The aim of this study was to develop a method of rapid simultaneous determination of alkylxanthines using capillary zone electrophoresis. To select the optimum conditions the following factors were investigated: buffer composition, the effects of pH, buffer concentration, voltage of separation, hydrodynamic and electrokinetic types of injection and temperature of separation. The application of the proposed method in the quality control of theobromine in chocolate samples and theophylline in commercial tablets were studied.

2. Experimental

2.1. Reagents

All analyte compounds investigated in the separations had purity 99% and were used without further purification. Caffeine, theophylline, dyphylline (7-[2,3-dihydroxypropyl]-theophyllin), enprofylline (3-propylxanthine), theobromine (3,7-dimethylxanthine), sodium phosphate (monobasic and dibasic), HCl, NaOH, methanol (HPLC grade were purchased from Sigma–Aldrich, Dublin, Ireland. Boric acid was from Riedel-de Haën, Seelze, Germany. Buffers were prepared using distilled water and adjusted using 1 and 0.1 M NaOH and 0.1 M HCL.

2.2. Equipment

All separations were performed using an Agilent CE Instrument. The PDA detector range was 190–600 nm. The CE instrument was operated using Agilent ChemStation software. The fused silica capillaries (Composite Metal Services Ltd., The Chase, Hallow, Worcs. WR26LD) were 56.3 cm long (48.3 cm to the detector) with an i.d. 50 μm.

Table 1
Comparison of analysis time of different CE methods for the determination of xanthines

Analytes	Method	Time of run (min)	Rinsing between runs (min)	Total time of analysis (min)
Caffeine, theobromine, paraxanthine, thophylline	MEKC [11]	2	Not reported	-//-
Caffeine, theophylline, dyphylline	MEKC [9]	4	8	12
Theophylline and its metabolites	MEKC [12]	30	3–6	33–36
Caffeien, aspartame, asp-phe, benzoic acid, phenylalanine	CZE [13]	2	Two volumes	–
Theophylline, ephedrine, phenobarbital	CZE [14]	9	4	13
Theophylline, enprofylline theobromine, caffeine, theophylline-7-acetic acid, dyphylline, proxyphylline, pentoxifylline	MEKC [6]	8	6	14
Caffeine, theobromine, theophylline	MEKC [15]	10	10	20
Caffeine, theobromine, theophylline	MEKC [16]	13	Not Reported	–
Caffeine, theobromine, theophylline, adenine, guanine, hypoxanthine, xanthine, uric acid	CZE [17]	16	Not reported	–

2.3. Standards

Stock solutions of 10 mM caffeine, theophylline, dyphylline, 2 mM stock of theobromine and 1 mM stock of enprofylline were prepared using distilled water. Stock solutions were stored in a refrigerator.

2.4. Procedure

All buffers were filtered through a 0.2 μm filter before use. Xanthines were detected at 200 nm, where all exhibited maximum absorption. Separations were carried out at 30 kV electrophoretic voltage and temperature 25 °C, unless otherwise stated. Conditioning between runs included a rinse of 0.1 M NaOH for 2 min, water for 5 min, running buffer for 5 min, unless otherwise stated. It is advisable to rinse the capillary with 1 M HCl, 1 M NaOH, MeOH, distilled water and running buffer once a day (before the analysis). All separations were repeated at least three times.

2.5. Sample preparation

2.5.1. Chocolate

The sample preparation technique was taken directly from [22] and was not further optimized in this work.

Pieces of chocolate were placed into a mortar and pestle. Liquid nitrogen was poured over the chocolate to freeze it, allowing it to be ground into a fine powder. One gram of this chocolate powder was measured out using an analytical balance, and 5 ml of chloroform was added to a conical flask and the flask was covered. This sample was placed in a sonicator for 50 min until the solution became a suspension of very small particles. A syringe and a 0.22 μm filter were used to remove any large or precipitated particles. About 2.5 ml of the filtered extracted solution was transferred into another vial. The chloroform was evaporated by placing the vial into a warm water bath and blowing an air stream over it. About 10 ml of ethanol was added to the vial following the evaporation, and a gentle swirling action was applied to dissolve the crystals of the compounds in the ethanol. The dissolved ethanol solution was placed into an HPCE vial using a micropipette, while taking care to avoid transferring any of the cocoa butter (insoluble in ethanol) into the vial. The vial was placed into the auto sampler for analysis.

2.5.2. Pharmaceutical tablet

Each of 10 commercial tablets containing 300 mg of theophylline was weighed and ground to a fine powder. One sample of 0.01 g of each tablet powder was weighed and placed in 100 ml volumetric flasks, filled with water and then sonicated until dissolved. The solutions were filtered through a 0.22 μm filter and transferred to the vials that were placed into the autosampler for CE analysis.

3. Results and discussion

3.1. Running buffer conditions

CZE technique involves the use of a simple electrolyte buffer system for the separation of charged compounds. Borate buffer has to be employed in the separation of the mixture of compounds where dyphylline is present. Dyphylline is a derivative of theophylline with the $CH_2CHOHCH_2OH$ radical at N7 (Fig. 1). Its pK_a is 11.6, therefore it is a neutral compound and in CZE it migrates with EOF, like caffeine. Even a high pH near 12 does not deprotonate dyphylline and it cannot be separated from caffeine this way. The specific reaction of borate ions with vicinal diols (such as dyphylline) is known [23,24]. The complexation with borate ions in the BGE generates charge on the dyphylline molecules and that is how it emerged after EOF although it possessed no ionizable chemical group [8].

The effect of pH of the running buffer was investigated for the separation of caffeine and theophylline. Borate buffer was studied in its working range between pH 8 and 10. Electropherogramms showed that longer migration times resulted at high pH. Similar effect was obtained from the increase of concentration (10–100 mM borate) of the borate buffer. This is reported due to a decrease in the magnitude of the EOF that has been correlated to a decrease in the zeta potential of colloidal silica [25]. The optimum concentration of borate buffer for the separation of five compounds was 20 mM and pH 9.1. This was selected by comparing analyte resolution and migration time with each buffer composition.

A series of experiments were carried out in order to investigate the influence of varying temperature on the separation of analytes. The Agilent CE system is supplied with a thermostat, which allows the temperature of the cassette to be maintained at the 4–60 °C. The separation of the mixture was performed at 25, 35, 45 and 55 °C. It was found that an increase in the temperature strongly affects the migration parameters of the separation. The increase of the temperature of the cassette provokes the decrease in resolution of caffeine and dyphylline accompanied by peak broadening. As mentioned above, the resolution of these two compounds is based on complexation of dyphylline with borate ions; as thought, with the temperature increase this complex becomes weaker, thereby leading to poorer resolution. Peaks were found to tail due to an increase in diffusion because of overall elevated column temperatures or radial gradients in temperature. These temperature changes cause changes in the pH, mobility, distortion of the flat zone profile, and convection [26].

The positive effect of this study is that the migration time of the analytes decreases significantly with an increase in separation temperature. Further experiments showed that by increasing the pH of 20 mM borate buffer to 9.4 (previously 9.1) at 55 °C, the resolution of caffeine and dyphylline peaks reaches 1.5.

Table 2
Integration data for optimizing rinsing conditions between runs ($n=5$)

Conditions[a]	N1	N2	N3	N4	N5	R1–2[b]	R4–5
1	48907	53547	113432	106452	117494	1.6	1.7
2	67412	70238	147768	149214	162165	1.8	2
3	88326	74792	239434	210579	225465	2	2.3
4	42588	47087	101726	80278	87721	1.5	1.4
5	38345	47801	110259	102368	103683	1.5	1.7

N: efficiency; R: resolution; 1: caffeine; 2: dyphylline; 3: theobromine; 4: enprophylline; 5: theophylline.
[a] Condition: (1) rinsing before the injection with 1.5 min 0.1 M NaOH, then 1.5 min water following by 3 min buffer; (2) 1.5 min 0.1 M NaOH + 1.5 min water + 2 min buff; (3) 1.5 min 0.1 M NaOH + 1.5 min water + 1 min buff; (4) 1 min 0.1 M NaOH + 1 min water + 3 min buff; (5) no rinse, just injection.
[b] R2–3 > 5 and R3–4 > 17 in all cases.

3.2. Injection type

CE requires only minute volumes of sample to be loaded into the capillary to maintain high efficiency [27]. Typically the plug length of the sample should be 1–2% of the whole capillary length. Hydrodynamic and electrokinetic injections were investigated. A hydrodynamic injection pressure of 50 Mbar was applied in the experiment for 1–6 and 10 s. A parallel study of electrokinetic injection was curried out. The injections of 3, 5, 8, 10, 15 kV were applied for 5 s. Taking into account the resolution of caffeine and dyphylline (resolution of dyphylline and theophylline peaks is more than 10) and the efficiency of all peaks, the optimum parameters of hydrodynamic injection are 1 s at 50 Mbar and an electrokinetic injection of 3 kV for 5 s. In this case hydrodynamic injection is somewhat more preferable.

3.3. Rinsing between runs

Many studies show lengthy rinse steps in CZE (Table 1). These add to the overall analysis time and are often not reported, thereby providing an ambiguous or false impression of the actual analysis times. A series of experiments were done to study the influence of capillary rinse between runs on the parameters of separation. As stated in Section 2.4, all experiments were initially accomplished with 2 min 0.1 M NaOH, 5 min water, and 5 min buffer rinse before the injection. Rinsing time was reduced with all possible combinations of rinses and finally it was found that rinsing could be excluded altogether. Final resolution of all peaks was at least 1.5 and efficiency was not affected significantly. Some of the results are presented in Table 2 including all five target analytes. Further experiments were accomplished without rinsing between runs. It allowed a single analysis to be performed in less than 2 min. This is the shortest reported time for separation of caffeine, theophylline, dyphylline, enprophylline and theobromine by CZE to the author's knowledge.

3.4. Optimised system

Fig. 2 shows the optimised separation of caffeine, theophylline, dyphylline, theobromine and enprofylline. The calibration graphs were established with the peak-area as ordinate versus the concentration in $mg\,l^{-1}$ as axis. From the slopes of the curves the method shows greater sensitivity toward theophylline determination and least for enprofylline. The linear regression equations were obtained as follows: for caffeine assay: $y=17.18x-0.7546$, $n=5$, $R^2=0.9977$; for theophylline assay: $y=23.875x-1.0807$, $n=5$, $R^2=0.9997$; for dyphylline assay; $y=16.927x-1.6334$, $n=5$, $R^2=0.9975$; for theobromine assay: $y=20.402x+0.0001$, $n=5$, $R^2=0.9991$; for enprophylline: $y=20.091x+0.0216$, $n=5$, $R^2=0.9995$. The data indicates good linearity of the method over the studied range of 1.9–1900 $mg\,l^{-1}$ for caffeine and theophylline, 1.8–1800 $mg\,l^{-1}$ for dyphylline, 2.5–250 $mg\,l^{-1}$ for theobromine and 1.8–180 $mg\,l^{-1}$ for enprofylline. Table 3 represents data on repeatability of the method. Here three concentrations are shown (each measured in triplicate) and %R.S.D. for all measurements are below 5%. Results show that in general at higher analyte concentrations in each case %R.S.D. values improve with the exception of enprofylline. Relative standard deviations of the migration time and peak area of analytes and LODs are shown in Table 4. Here it can be noted that acceptable %R.S.D. were achieved. Limits of detection for the study varied between 1.8 and 2.5 $mg\,l^{-1}$. This range is ideally

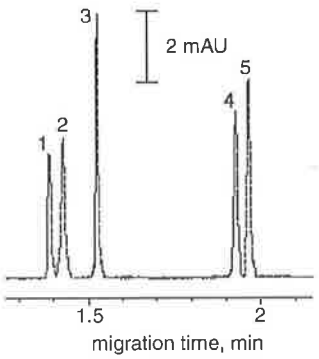

Fig. 2. Separation of mixture of five compounds. Peak identification: 1 = caffeine; 2 = dyphylline; 3 = theobromine; 4 = enprophylline; 5 = theophylline. Concentration of each compound = 0.05 mM. Separation conditions: 20 mM borate, pH 9.4, 30 kV, 55 °C, capillary 56.3 cm (48.3 cm to the detection window), 50 μm i.d., detection at λ = 200 nm. Injection conditions: 50 Mbar for 1 s.

Table 3
Data on repeatability of the method

Concentration known (mM)	Concentration found (mM)	R.S.D. (%)	95% confidence interval
Caffeine ($n=3$)			
0.5	0.525 ± 0.015	2.79	0.508–0.542
1	1.047 ± 0.032	3.06	1.011–1.083
5	5.236 ± 0.106	2.03	5.116–5.356
Theophylline ($n=3$)			
0.5	0.520 ± 0.016	3.05	0.502–0.538
1	1.045 ± 0.017	1.67	1.025–1.065
5	5.090 ± 0.056	1.10	5.028–5.154
Dyphylline ($n=3$)			
0.5	0.524 ± 0.017	3.26	0.504–0.543
1	1.048 ± 0.031	2.98	1.012–1.083
5	5.236 ± 0.098	1.86	5.126–5.347
Theobromine ($n=3$)			
0.1	0.103 ± 0.006	4.76	0.097–0.108
0.5	0.514 ± 0.021	4.15	0.490–0.539
1	1.021 ± 0.006	0.55	1.015–1.028
Enprofylline ($n=3$)			
0.1	0.107 ± 0.007	2.70	0.103–0.109
0.5	0.509 ± 0.006	1.13	0.503–0.516
0.8	0.793 ± 0.017	2.20	0.773–0.813

suited for determination of the samples under investigation, as discussed later. LOD values are selected on the basis of a signal that is three times baseline noise measurement.

3.5. Application

3.5.1. Chocolate

Methylxanthine has previously been determined in different types of food and beverages. The majority of research has focused on the analysis of methylxanthines in coffee, tea and cola beverages [28]. Less data exist on methylxanthine content of chocolate products. To the authors knowledge there is no information about the CE method being employed in the analysis of methylxanthines in chocolate. And thus this work is the first determination of theobromine in chocolate by CZE.

Eight different chocolates from three brands and one type of cocoa powder were analysed (Table 5). As expected, theobromine was determined in all samples of milk, dark chocolate and cocoa powder, but not in the samples of white chocolate. Fig. 3 shows electropherogramms of the white chocolate "Milkybar", milk chocolate "Bubbly" and dark chocolate "Bournville". It is immediately obvious from the electropherogramm that the concentration of theobromine in dark chocolate is higher than in milk chocolate.

The time of a single analysis is less than 2 min. The concentrations of theobromine in all the samples are represented in the bar chart shown in Fig. 4 and compared to the amount of cocoa solids in the chocolate bars.

The highest concentration of theobromine in cocoa powder corresponds to 100% of cocoa solids in it. Then $1.46\ mg\ g^{-1}$ concentration of theobromine in dark chocolate "Bournville" lies very close to 39% of cocoa solids. All samples of milk chocolate with 20–25% of cocoa solids have some variations in the theobromine concentration. Nestle chocolate "Aero All Bubble"(mint) contains noticeably less theobromine. This is due to the nature of this chocolate, which consists of mint part and milk chocolate outer coating. There is no data on cocoa solids contained on the product,

Table 4
Relative standard deviations of the migration time and peak area of analytes, LOD ($n=5$)

Analyte	Migration time (min)	%R.S.D.	Peak area	%R.S.D.	LOD ($mg\ l^{-1}$)
Caffeine	1.28	0.10	3.4	1.67	1.9
Dyphylline	1.32	0.06	3.5	1.42	1.8
Theobromine	1.42	0.14	4.7	1.24	2.5
Enprofylline	1.81	0.22	1.0	3.54	1.8
Theophylline	1.85	0.11	4.9	2.06	1.9

Table 5
Samples of chocolate analysed

Name of chocolate	Type of chocolate
Cadbury "Bubbly"	Milk
Cadbury "Bournville"	Dark
Nestle "Milkybar"	White
Nestle "Aero All Bubble"	Milk
Nestle "Aero All Bubble"	Milk and mint
Cadbury "Buttons"	Milk
Cadbury "Buttons"	White
Terry's "Orange"	Milk
Cadbury "Cocoa"	Cocoa powder

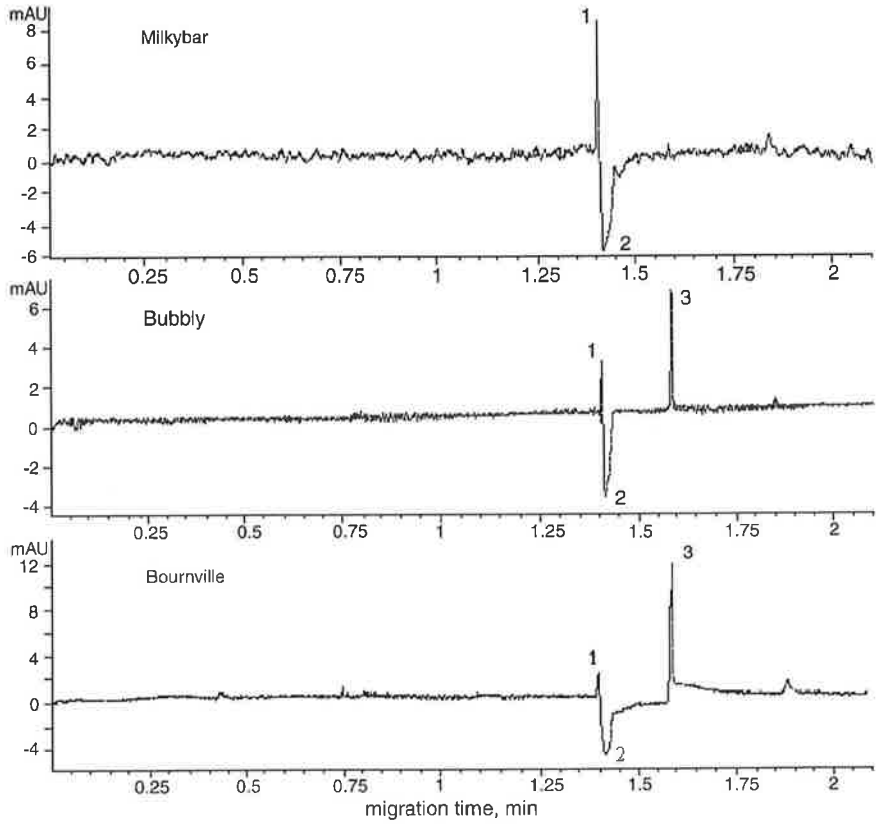

Fig. 3. Electropheroframms of theobromine determination in chocolate samples. Peak identification: 1 = caffeine/EOF; 2 = ethanol; 3 = theobromine. Separation conditions and injection conditions as in Fig. 2.

but it is obvious that it is only the chocolate part that contains it.

Table 6 represents statistical analysis of the data obtained. Relative standard deviation for peak area is less than 5%, while the %R.S.D. of the extraction is over 5%, and especially high in the case of Nestle "Aero All Bubble" (mint) (%R.S.D. 15.02). The reason for this is the heterogeneous nature of the sample. As previously stated, the extraction method was not optimised since it is not the objective of this work.

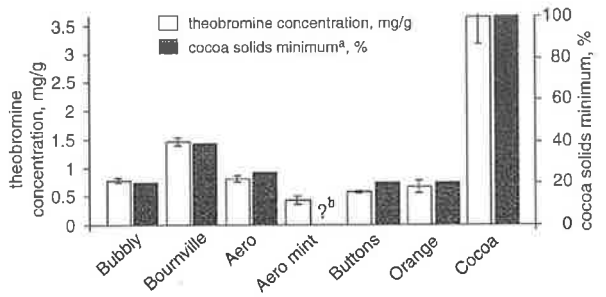

Fig. 4. Concentration of theobromine and cocoa solids in analysed samples: (a) information taken from the wrapping; (b) no data.

3.5.2. *Pharmaceutical tablets*

A tablet sample containing 300 mg theophylline was analysed using the optimized method. An electropherogramm of the commercial sample is shown in Fig. 5. A single analysis is completed in less than 2 min. Theophylline is the only peak in this separation. Analytical results for content uniformity of theophylline tablet obtained from a commercial source are shown in Table 7. The analytical values (% claimed content) obtained were found to be between 95.0 and 105.0%, which falls within the range of 94.0–106.0%, required by the USP 25 for theophylline [29].

Table 6
Relative standard deviations of CE analysis and extraction ($n = 3$)

Sample	Run #	Area (%R.S.D.)	Extraction (%R.S.D.)
Cadbury "Bournville"	1	1.72	5.21
	2	1.79	
	3	3.32	
Nestle "Aero All Bubble"	1	2.94	5.81
	2	2.86	
	3	0	
Nestle "Aero All Bubble" mint	1	4.35	15.02
	2	0	
	3	3.46	

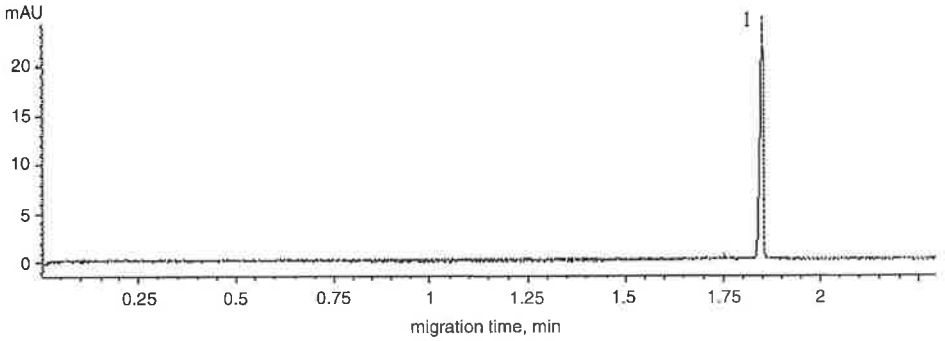

Fig. 5. Electropherogramm of commercial sample. Peak identification: 1 = theophylline. Separation conditions and injection conditions as in Fig. 2.

Table 7
Analytical results for content uniformity of theophylline tablet obtained from a commercial source

Tablet[a]	Amount found[b] (mg)	Claimed content (%)
1	292.6 ± 5.2	98
2	304.3 ± 14.9	101
3	291.9 ± 6.3	97
4	303.9 ± 20.6	101
5	297.0 ± 11.0	99
6	315.4 ± 13.0	105
7	292.6 ± 2.6	98
8	289.3 ± 5.4	96
9	293.3 ± 9.7	98
10	283.6 ± 9.6	95
Mean (%)		99
S.D.		2.9

[a] Labeled amount or theophylline in each tablet is 300 mg.
[b] Mean ± S.D. (n = 3).

4. Conclusions

The CZE method was developed for determination of caffeine, theophylline, dyphylline, theobromine and enprofylline. The main advantage of this method is a very short (less than 2 min) full time of a single analysis. It is the shortest reported time of analysis of above mentioned components by CZE to the author's knowledge. The peculiarities of the method are as follows: caffeine cannot be determined if other neutral components are present that migrate with EOF (then MEKC must be employed). If dyphylline is analysed, samples must be dissolved in the borate buffer with concentration and pH close to the run buffer (20 mM borate pH 9.4).

This method has been shown to be a good alternative technique to HPLC and will be useful for quality control in pharmaceutical and food industries.

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Research Article

Determination of association constants of inclusion complexes of steroid hormones and cyclodextrins from their electrophoretic mobility

CE estimation of the association constants of several steroid hormones with β-CD and γ-CD and their hydroxypropyl derivative is presented. Estriol, 17β-estradiol, ethynylestradiol, estrone, progesterone, mestranol and norethindrone are among the target analytes. The calculation of the cyclodextrin:analyte association constants were performed from the electrophoretic mobility values of steroids at different concentration of CDs in the run buffer. The reliability of the final data was ensured by employing three different linearisation plots (double reciprocal fit, *Y*-reciprocal fit and *X*-reciprocal fit). The highest inclusion affinity of target analytes was observed towards γ-CD and its hydroxypropyl derivative, which is demonstrated by high association constant values and corresponding good linearity of the plots. The affinity of steroids towards a particular CD type based on physical and structural characteristics is explored.

Keywords: Association constants / Cyclodextrins / Steroid hormones
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1 Introduction

There is considerable analytical interest in the determination of steroids. This interest is due to their physiological functions in the body and the potential danger in disruption of the endocrine system. Structures of the target steroid analytes are shown in Fig. 1. A number of different techniques have been previously employed in the separation of steroids; these include immunoassays [1], chromatographic [2, 3] and CE [4] techniques from a variety of sample matrices. An LC technique does not require compounds to be volatile or to have high molecular weight like those requiring GS-MS determination. The limitations of LC techniques include high solvent volumes and the need for gradient elution.

Several publications show the enhanced selectivity of HPLC methods for steroids when CDs are employed as a chiral stationary phase [5, 6] or in the mobile phase [7, 8].

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Abbreviation: HP, hydroxypropyl

Recent studies [9, 10] in CE have shown the advantage of CDs in the run buffer to aid separation of closely eluting compounds. Thus Munro and co-workers [9] investigated the enhancement in sensitivity of CE for the analysis of 17β-estradiol, estrone and estriol when charged CD-mediated sample stacking was employed. Work carried out by Poole [10] studied separation of ten steroids with α-CD, β-CD and γ-CD in the run buffer. The addition of 20 mM α-CD to the 20 mM sodium borate–sodium phosphate buffer (pH 8) containing 50 mM SDS did not improve resolution of any ten analytes. While 20 mM β-CD in the run buffer separated nine peaks out of ten almost to baseline resolution. When 20 mM γ-CD was used in place of β-CD significant changes in the migration order of analytes were observed and baseline resolution of all ten estrogens was obtained. It is difficult to judge the migration times of peaks from the figure in the paper but most of the compounds had shorter migration times with γ-CD, while 17α-dihydroequilin and equilin had longer migration time. Chan *et al.* [4] have shown the separation of ten estrogens with 20 mM γ-CD in the run buffer. Even though the separation of all analytes was achieved the reason for choosing the concentration of γ-CD to be 20 mM was not explored as well as the change in the migration order of analytes with β- and γ-CD.

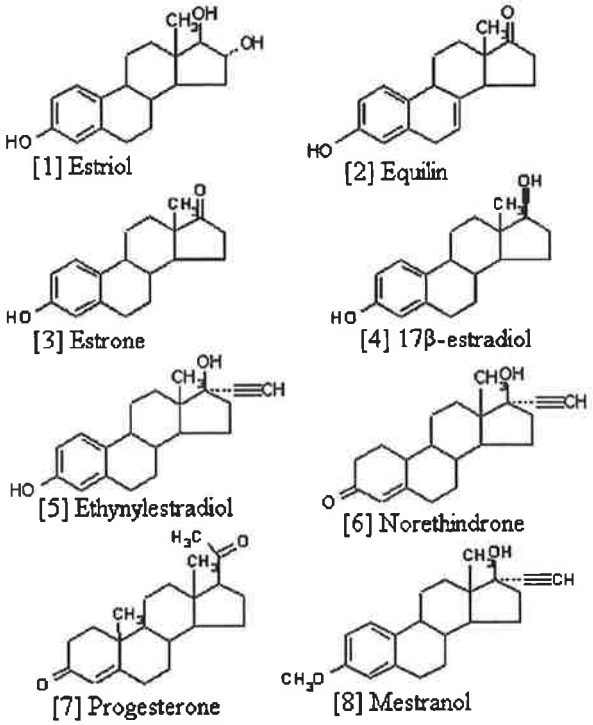

Figure 1. Structures of steroids.

CDs are truncated cone-shaped molecules with a hollow, tapered cavity, which posses a hydrophobic property. It is known that various organic molecules form inclusion complexes with CD [11]. Fundamental knowledge of equilibrium and the association constants of analysed compounds and ligand in the system is a powerful element in the understanding of that system and in discovering ways to evaluate it.

The association of CD C and analyte molecules A, and the dissociation of the formed complex AC are controlled by equilibrium for a 1:1 complexation scheme [12]:

$$A + C = AC \tag{1}$$

where A, C and AC are the free analyte, CD and the complex, respectively.

The model of that complexation was reviewed in the literature [12, 13]. The mobility of analyte μ_a at any given concentration of the ligand [C] equals

$$\mu_a = \frac{1}{1+K[C]}\mu_f + \frac{K[C]}{1+K[C]}\mu_c \tag{2}$$

where μ_f is the free analyte mobility, μ_c is the complexed analyte mobility, and K is the association constant.

This expression can be rearranged in order to linearise it in a several ways [14]:

(A):

$$\frac{1}{(\mu_a-\mu_f)} = \frac{1}{(\mu_c-\mu_f)K}\frac{1}{[C]} + \frac{1}{(\mu_c-\mu_f)} \tag{3}$$

The data can be transformed into a double-reciprocal plot of $1/(\mu_a - \mu_f)$ *versus* 1/[C].

From this plot the apparent binding constant K = intercept/slope.

(B):

$$\frac{[C]}{(\mu_a-\mu_f)} = \frac{1}{(\mu_c-\mu_f)}[C] + \frac{1}{(\mu_c-\mu_f)K} \tag{4}$$

This equation leads to *Y*-reciprocal plot of $[C]/(\mu_a - \mu_f)$ *versus* [C]. From there K = slope/intercept.

(C):

$$\frac{(\mu_a-\mu_f)}{[C]} = -K(\mu_a-\mu_f) + K(\mu_c-\mu_f) \tag{5}$$

This leads to the *X*-reciprocal plot of $(\mu_a - \mu_f)/[C]$ *versus* $(\mu_a - \mu_f)$, where $K = -$slope.

The association of steroid hormones with certain types of CD was studied by Sadlej-Sosnowska [14, 15] using HPLC and the binding constants of analyte-CD equilibrium were determined. Several papers [16–18] have demonstrated that different techniques for the determination of association constants give rather different results, which is likely to be due to the differences in the experimental conditions required by each one. To the authors' knowledge the association constants for steroids and CDs have not previously been determined from CE separation data. The aim of this work is to fill this gap, as this data has a high importance in the understanding of the electrophoretic behaviour of analytes in the presence of CDs in the run buffer.

2 Materials and methods

2.1 Reagents

All analyte compounds investigated in the separations had purity of 99% and were used without further purification. Estriol, estrone, 17β-estradiol, ethynylestradiol, equilin, norethindrone, mestranol, progesterone, β-CD, γ-CD, 2-hydroxypropyl (HP)-β-CD (degree of substitution ~4–10), 2HP-γ-CD (molecular substitution ~0.6), sodium phosphate (dibasic), HCl, NaOH, methanol (HPLC grade), SDS were purchased from Sigma-Aldrich (Dublin, Ireland). Buffers were prepared using distilled water and adjusted to the desired pH using 1 M and 0.1 M NaOH.

2.2 Equipment

All separations were performed using an Agilent Technologies ^{3D}CE system (Agilent Technologies). Data acquisition and signal processing were performed using Agilent Technolodies ^{3D}CE ChemStation (rev. A.09.03, Agilent Technologies). The fused-silica capillaries (Composite Metal Services, UK) were 53.7 cm long (45.5 cm to the detector) with an internal diameter of 50 μm.

2.3 Standards

Stock solutions of estriol, estrone, ethynylestradiols, 17β-estradiol, equilin, norethindrone, mestranol and progesterone were prepared in methanol in a concentration of 1 mM. Stock solutions were stored in the dark and eveporation of methanol from the flasks was prevented.

2.4 Separation conditions

The electrophoretic buffer consisted of 20 mM phosphate pH 11.5 and 10 mM SDS. β-CD, γ-CD, 2HP-β-CD, 2HP-γ-CD were added to the buffer separately in a range of five concentrations from 1 mM to 10 mM. Separations were carried out at 30 kV electrophoretic voltage and at a temperature of 20°C. Injections were carried out hydrodynamically at 50 mbar for 2 s. UV detection was at 214 nm and 254 nm. The concentration of each analyte in a mixture was 0.125 mM in methanol. Conditioning of the CE capillary between runs was performed with 0.1 M NaOH for 2 min and run buffer for 2 min. All separations were repeated at least three times.

2.5 Data evaluation

All migration times were converted to the mobility using following equation [19]:

$$\mu_a = l/tE = lL/tV \quad (6)$$

where $\mu_a = \mu_e + \mu_{EOF}$, V is the applied voltage, l is the effective capillary length (to the detector), L is the total capillary length, t is the migration time and E is the electric field. The mobility data plots were calculated using Microsoft Excel.

3 Results and discussion

The theory of the formation of inclusion complexes was reported to be only valid for cases of 1:1 stoichiometry [12]. A number of steroid hormones, including four of the target analytes in this work (estriol, 17β-estradiol, estrone and ethynylestradiol), have been investigated by Sadlej-Sosnowska [14, 15]. The conclusion was that 1:1 stoichiometry applies to the complexes of studied steroids with β-CD and γ-CD. Due to the high structural similarity of steroids it was assumed in this paper that inclusion of equilin, mestranol, norethindrone and progesterone follows the same scenario.

Throughout the series of experiments, the run buffer composition was held constant (except for CD type and concentration which were varied). The presence of surfactant (SDS) and pH 11.5 requires explanation. Compounds 1–5 (as in Fig. 1) were in their deprotonated form at this pH because their pKa value lies between 10.26 and 10.71 [20, 21]. The structures of mestranol, norethindrone and progesterone pointed to even higher p*K*a values (p*K*a = 13.1 for mestranol [22]) and thus they were neutral at the conditions of separation. Such close p*K*a magnitudes and molecular weights of steroids did not allow their separation in a simple mode of capillary zone electrophoresis. All target analytes were quite hydrophobic. The calculated logK_{ow} values are 2.81, 3.22, 3.43, 3.94, 4.12, 4.68, 2.99 and 3.67, for estriol, equilin, estrone, 17β-estradiol, ethynylestradiol, mestranol, norethindrone, and progesterone respectively (http://www.syrres.com/esc/kowwin.htm). The latter provokes their interaction with the micelles in the run buffer as well as CDs when added. Thus two equilibria were involved, the analyte-micelle and analyte-CD interaction. The presence of the micelles in the run buffer was necessary in order to observe the difference in the mobility of analytes while adding neutral CDs. The top electropherogram in Fig. 2A represented the separation of target estrogens at primary conditions, *e.g.* without CD. It could be seen that analytes reached the detector window after the electro osmotic flow; association with negatively charged micelles was the reason for their migration towards the anode, which was against the EOF in this separation mode.

According to Rekharsky and Inoue [11] any differences in the free analyte's interaction with the buffer prior to inclusion complexation did not contribute measurably to the complexation thermodynamics. Since the concentration of SDS was held constant at every separation, the true effect of CD was seen while adding it at different concentrations. The separation with 1 mM of each CD is shown in Figs. 2B–E. The change of the migration time of analytes clearly demonstrated the occurrence of analyte-CD interaction. The mobility changes were more significant in the case of γ-CD and 2HP-γ-CD than β-CD and 2HP-β-CD. Analyte mobility was increased due to the interaction with neutral CDs and the attraction to the anode was lower.

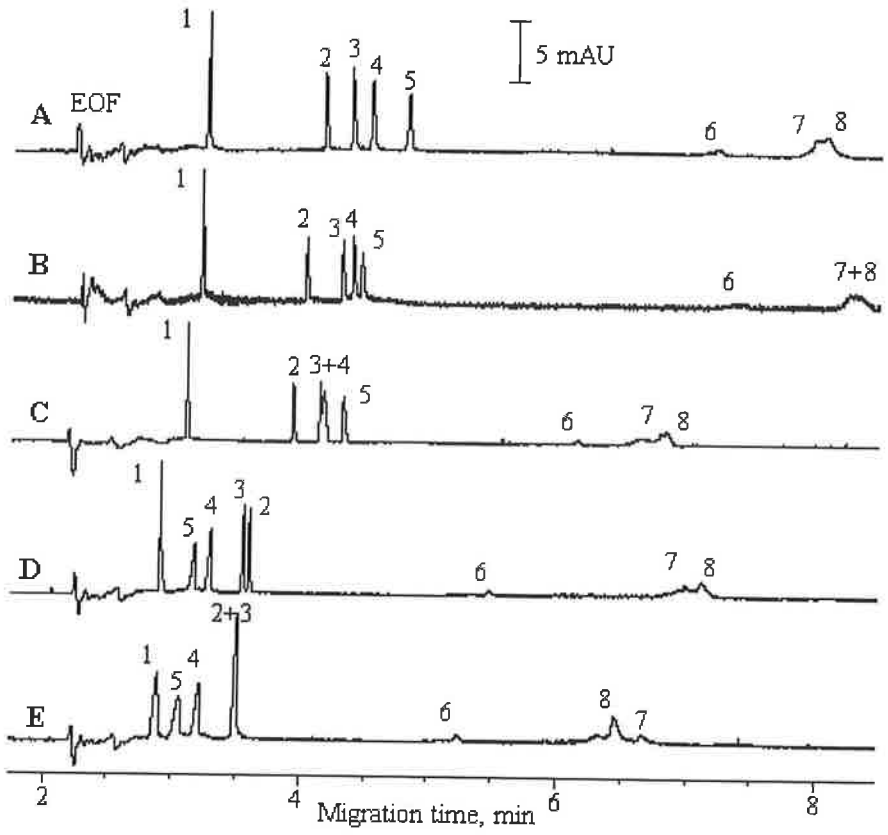

Figure 2. Electropherogram of separation of 8 steroid hormones. Separation conditions: 20 mM phosphate buffer pH 11.5 and 10 mM SDS, (A) no CD added, (B) 1 mM β-CD, (C) 1 mM 2HP-β-CD, (D) 1 mM γ-CD, (E) 1 mM 2HP-γ-CD; separation voltage 30 kV; capillary 53.7 cm (45.5 cm is effective length), 50 μm id; 20°C; hydrodynamic injection 2 s; UV detection 214 nm. Concentration of each analyte is 0.125 mM in methanol. Peak identification: 1 – estriol, 2 – equilin, 3 – estrone, 4 – 17β-estradiol, 5 – ethynylestradiol, 6 – norethindrone, 7 – progesterone, 8 – mestranol.

Association constants were calculated from the mobility values of estrogens, which were determined at 1 mM, 3 mM, 5 mM, 7 mM and 10 mM of each CD in the run buffer. The calculated values of the constants determined by three linearisation methods are given in Tables 1–4 along with R^2 of each plot and % RSD. The association constants for a single compound obtained using the plotting methods show quite different values. Regardless of that fact, the same relative order of the constants within the same plotting method was observed for each compound. The exception to this case is shown in Table 1. For 17β-estradiol and estriol the relative values for the double reciprocal fit data differed from *Y*-reciprocal and *X*-reciprocal fit. It was shown previously by Rundlett and Armstrong [23] that at low ligand concentrations (in this case CD) the double reciprocal fit masks deviations from linearity. The data obtained in this work indeed proved this to be the case as R^2 values for the double reciprocal fit for each complexation shows good linearity. The negative association constant values however, pointed to the lack of inclusion of the analytes (progesterone and mestranol) with the CD. This leads to the conclusion that the *Y*- and *X*-fits are more reliable in assessing analyte (steroid)-ligand interaction.

3.1 γ-CD

From this study it was found that the highest magnitudes of association constants belong to the inclusion of target steroids in γ-CD (Table 1). The diameter of the inside cavity of γ-CD was 10 Å, which is more sterically suitable for the 4-ring conventional structure of analytes than β-CD [24] with the diameter 8 Å. Large association constants of 331 M^{-1} (*Y*-reciprocal fit) for equilin and up to 1770 M^{-1} (*X*-reciprocal fit) for ethynylestradiol were observed.

The double reciprocal fit and the Y-reciprocal fit were found to have R^2 values in the range of 0.9797–0.9989. The relative standard deviations were observed to be less than 3.5% for the association of compounds 1–5. The latter demonstrated that the analyte inclusion process took place with the CD and its stoichiometry was 1:1. It should be noted that the R^2 value for the *X*-reciprocal fit for those compounds lay in a lower range (R^2 = 0.8969–0.9665) of linearity. The effect of varying the CD concentration on the mobility was most pronounced using the *X*-reciprocal fit compared with other linearity approaches. The latter is due to the plot parameters of $(\mu_a - \mu_f)/[C]$ *versus* $(\mu_a - \mu_f)$. The concentration range chosen for the plots also affects the R^2 values obtained.

Table 1. The value of association constants of target steroids and γ-CD, R^2 for each linearisation method, % RSD (n = 3) and literature values of constants

	K (M^{-1})	% RSD	R^2	K (M^{-1}) [15]
Estriol				3200 ± 370
Double reciprocal fit	1282	0.95	0.9585	
Y-reciprocal fit	798	2.75	0.9936	
X-reciprocal fit	1088	1.32	0.8981	
Equilin				
Double reciprocal fit	477	3.44	0.9812	
Y-reciprocal fit	331	1.15	0.9821	
X-reciprocal fit	387	2.28	0.8969	
Estrone				2550 ± 230
Double reciprocal fit	642	2.33	0.9797	
Y-reciprocal fit	461	0.83	0.9901	
X-reciprocal fit	545	1.64	0.9162	
17β-Estradiol				7100 ± 400
Double reciprocal fit	1231	1.61	0.9844	
Y-reciprocal fit	953	1.73	0.9978	
X-reciprocal fit	1137	1.40	0.9583	
Ethynylestradiol				10600 ± 960
Double reciprocal fit	1873	1.57	0.9842	
Y-reciprocal fit	1506	2.22	0.9989	
X-reciprocal fit	1770	1.51	0.9665	
Norethindrone				
Double reciprocal fit	91	4.64	0.9980	
Y-reciprocal fit	91	2.58	0.8669	
X-reciprocal fit	75	3.31	0.7375	
Progesterone				
Double reciprocal fit	−44	13.56	0.9984	
Y-reciprocal fit	−42	4.92	0.6164	
X-reciprocal fit	−52	2.44	0.7235	
Mestranol				
Double reciprocal fit	−62	12.12	0.9984	
Y-reciprocal fit	−44	7.29	0.5591	
X-reciprocal fit	−56	4.20	0.6997	

Table 2. The value of association constants of target steroids and β-CD, R^2 for each linearisation method, % RSD (n = 3) and literature values of constants

	K (M^{-1})	% RSD	R^2	K (M^{-1}) [15]
Estriol				4700 ± 400
Double reciprocal fit	43	71.23	0.9976	
Y-reciprocal fit	57	35.86	0.8621	
X-reciprocal fit	56	39.09	0.7621	
Equilin				
Double reciprocal fit	57	17.82	0.9973	
Y-reciprocal fit	68	8.81	0.9213	
X-reciprocal fit	97	9.52	0.8376	
Estrone				3100 ± 600
Double reciprocal fit	−43	20.67	0.9942	
Y-reciprocal fit	−17	24.34	0.2645	
X-reciprocal fit	−16	21.54	0.2577	
17β-Estradiol				6830 ± 680
Double reciprocal fit	75	10.61	0.9981	
Y-reciprocal fit	79	5.43	0.9384	
X-reciprocal fit	78	6.50	0.8801	
Ethynylestradiol				7600 ± 770
Double reciprocal fit	126	6.30	0.9982	
Y-reciprocal fit	120	2.33	0.9675	
X-reciprocal fit	123	2.36	0.9307	
Norethindrone				
Double reciprocal fit	−181	12.33	0.9592	
Y-reciprocal fit	−129	13.53	0.5087	
X-reciprocal fit	−90	3.56	0.9513	
Progesterone				
Double reciprocal fit	−250	2.95	0.9103	
Y-reciprocal fit	−196	23.86	0.2786	
X-reciprocal fit	−96	2.11	0.9682	
Mestranol				
Double reciprocal fit	−201	13.50	0.9416	
Y-reciprocal fit	−150	19.30	0.4113	
X-reciprocal fit	−93	2.90	0.9687	

Negative values of constants for progesterone and mestranol, along with poor linearity and precision, pointed to the fact that these steroids did not interact with γ-CD or the nature of interaction was something other than inclusion. Inclusion of norethindrone was characterised by the association constant 75 M^{-1} (*X*-reciprocal fit) with % RSD less than 5% and linearity 0.73.

Rekharsky and Inoue [11] attributed the following contributions to the stabilisation of CD complexes from a thermodynamic point of view: a) penetration of the hydrophobic part of the guest molecule into the CD cavity, b) dehydration of the organic guest, c) hydrogen-bonding interaction, d) the release of the water molecules originally included in the CD cavity to bulk water [25–27] and e) the conformational changes or strain release of the CD molecule upon complexation [28–30].

According to the factors listed above the possible reasons for strong inclusion of compounds 1–5 and weak or absence of inclusion for compounds 6–8 should be explained by referring to the differences in structural characteristics of the complexes, *i.e.* structures of steroids incorporated into a CD type, which was kept constant. According to the literature hydrophilic groups, such as hydrohxyl, amino and carboxyl [31], remain exposed to the bulk solvent even after inclusion of the hydrophobic moiety [11]. An exception to this general rule is the

Table 3. The value of association constants of target steroids and 2HP-γ-CD with two run buffers containing 10 mM SDS and 20 mM SDS, R^2 for each linearisation method, % RSD ($n = 3$).

	10 mM SDS			20 mM SDS		
	K (M^{-1})	% RSD	R^2	K (M^{-1})	% RSD	R^2
Estriol						
Double reciprocal fit	592	4.58	0.9971	395	6.10	0.9996
Y-reciprocal fit	505	9.32	0.9961	410	4.64	0.9996
X-reciprocal fit	565	5.77	0.9800	403	5.41	0.9968
Equilin						
Double reciprocal fit	288	4.71	0.9994	97	28.16	0.9994
Y-reciprocal fit	318	7.31	0.9978	102	14.06	0.9861
X-reciprocal fit	297	4.20	0.9907	99	16.70	0.9694
Estrone						
Double reciprocal fit	372	3.41	0.9982	134	20.08	0.9995
Y-reciprocal fit	432	7.12	0.9977	137	10.23	0.9938
X-reciprocal fit	389	3.26	0.9824	134	12.60	0.9842
17β-Estradiol						
Double reciprocal fit	911	2.97	0.9988	405	3.57	0.9993
Y-reciprocal fit	937	6.25	0.9997	388	2.34	0.9995
X-reciprocal fit	918	3.09	0.9954	396	2.83	0.9957
Ethynylestradiol						
Double reciprocal fit	1642	7.96	0.9984	638	6.78	0.9994
Y-reciprocal fit	1429	6.85	0.9997	610	4.34	0.9999
X-reciprocal fit	1630	3.32	0.9954	628	5.64	0.9973
Norethindrone						
Double reciprocal fit	48	10.26	0.9889	−12	88.51	0.9992
Y-reciprocal fit	154	5.27	0.8341	8	99.32	0.1742
X-reciprocal fit	86	4.41	0.4344	4	182.67	0.0880
Progesterone						
Double reciprocal fit	−81	6.49	0.9894	−39	39.32	0.9999
Y-reciprocal fit	−23	20.97	0.1156	−40	21.13	0.9571
X-reciprocal fit	−36	8.83	0.2584	−38	17.89	0.9749
Mestranol						
Double reciprocal fit	−49	13.05	0.9916	−49	46.51	0.9997
Y-reciprocal fit	11	53.63	0.0419	−42	30.99	0.9126
X-reciprocal fit	−10	42.06	0.2584	−41	28.74	0.9316

aromatic hydroxyl group, which can penetrate deeply into the CD cavity where it hydrogen-bonds to one of CD's peripheral hydroxyl groups [32]. Ring A of compounds 1–5 is identical; it was aromatic with a hydroxyl group on it. The difference was in the ring D (and nonsaturated bond in ring B of equilin) where hydrophilic hydroxyl and ketone groups and hydrophobic ethilin groups attached. According to the literature estriol, equilin, estrone, 17β-estradiol and ethynylestradiol penetrated γ-CD and form a hydrogen bond with the hydroxyl group of CD. The structural difference of ring D and $\log K_{ow}$ values explained the different K values obtained. Norethindrone and progesterone did not contain any aromatic ring with a hydroxyl group on it and the aromatic hydroxyl group of mestranol was methylated. Ring D of the latter compounds contained hydrophilic and hydrophobic groups. However, neither constituent of ring A nor ring D appeared to be the reason for the inclusion as the calculated association constants were negative. A low degree of interaction was observed in the case of norethindrone but as to which one of the above mentioned contributions or their combination was responsible for the association constant of 75 M^{-1} (*X*-reciprocal fit) was not clear.

Table 1 contains some literature association constant values for several steroids, obtained from HPLC data. To the authors knowledge association constants for other investigated steroids and other CD types are not avail-

able. It was reported previously [16–18] that equilibrium constants determined by different experimental methods for the same reaction can significantly deviate from each other. The HPLC constants were much greater than the data obtained in this work but notably the relative order of the association constants was the same. The mechanism of the separation method influences the magnitude of the association constant values. In CE, the binding constant values are determined mainly by the charge/mass ratio. In HPLC the distribution of analyte between the mobile and stationary phases play an important role. The effect of SDS on the association constants for CD in CE is discussed later.

3.2 β-CD

From this investigation a significant decrease in the magnitude of the association constants was found for the interaction of the selected steroids with β-CD (Table 2). Association constants values as low as 56 M^{-1} (*X*-reciprocal fit) for estriol and 120 M^{-1} (*Y*-reciprocal fit) for ethynylestradiol were found. The association constants were accompanied by a slightly increased deviation from linearity (R^2) for estriol, equilin and 17β-estradiol and a large decrease in linearity for estrone and norethindrone. Relative standard deviation of the obtained *K* was quite high for estriol (over 30%). The small diameter of β-CD (8 Å [25]) did not allow penetration of steroids to the same degree as the cavity of γ-CD. It is likely that the inclusion of only the most hydrophobic part of the molecules took place. The rest of the structure remained in the bulk solvent. Ethynylestradiol had the highest association constant among β-CD complexes followed by 17β-estradiol, equilin and estriol. It is interesting to note that the last three compounds had the same *K* values by three linearisation methods within experimental error. Partial interaction of the same structural fragment of the three molecules took place. Estrone and norethindrone did not undergo any interaction with β-CD, which was supported by negative constants. It has to be noted that in case of γ-CD, equilin possessed less interaction than estrone. This was not true for inclusion into β-CD. It is likely that the double bond of the B-ring of equilin induced a different interaction with the CD.

3.3 2HP-γ-CD and 2HP-β-CD

Modification of the structure of the CD has many beneficial effects, contributing to its solubility or affinity for analytes. In this study two HP functionalised γ-CD and β-CD were investigated. It was found that a general decrease of association constants could be noted for the inclusion of target steroids into 2HP-γ-CD (Table 3). It was reported that HP substituents elongate the actual CD cavity when attached through primary or secondary hydroxyl groups of the glucose ring [12]. Molecular substitution of the employed 2HP-γ-CD was 0.6 but it was not controlled and thus the actual position of HP groups was unknown. The comparison of the association constants for 2HP-γ-CD with parent γ-CD would suggest that this substitution caused some structural difficulties for steroid molecules to penetrate the cavity and/or the hydrogen bonds were not able to form between analytes and substituted hydroxyls on the CD.

The data in Table 4 shows the association constants of steroids with 2HP-β-CD. A slight deviation from linearity of the plots could be noticed here, which was similar to the linearisation plots for natural β-CD. There was noticeably improved linearity for estrone accompanied by increased association constants. Even though the % RSD of the calculated constants was high, a general increase of the constant value was noted with the functionalised CD compared to the natural β-CD. HP groups are oriented so that penetration of the part of analytes was quite similar, thereby not distinguishing types clearly. Due to the fact that there is only a slight difference between constant values for several compounds (Table 4), it is evident that the HP-substituted β-CD (degree of substitution ≈ 4–10) does not recognise the structural variations of analytes to the same degree as γ-CD for instance.

3.4 The effect of SDS

While it is clear from the association constants determined that the concentration and type of CD is important in selecting particular steroids, a study of the effect of SDS was carried out to investigate the impact of SDS on the analyte interaction with the CD. The effect of SDS concentration in the run buffer was investigated using 2HP-γ-CD. The value of association constants of target steroids and 2HP-γ-CD with two run buffers containing 10 mM SDS and 20 mM SDS are presented in Table 3. The steroid-micelle interaction is strong due to high hydrophobicity of analytes ($\log K_{ow}$ values of each analytes were mentioned in Section 3). It was found that by increasing the concentration of SDS, this leads to the equilibrium being moved toward increased micelle interaction.

The latter reason provoked the decrease of the inclusion of steroids into the CD cavity when the concentration of SDS was 20 mM. The values of the association constants at 20 mM SDS were two to three times less than at 10 mM SDS (Table 3). It has to be noted that *K* value for estriol was affected significantly less than the rest of the constants. The value for estriol was even higher than the

Table 4. The value of association constants of target steroids and 2HP-β-CD, R^2 for each linearisation method, % RSD (n = 3)

	K (M^{-1})	% RSD	R^2
Estriol			
Double reciprocal fit	114	20.42	0.9908
Y-reciprocal fit	74	11.88	0.9404
X-reciprocal fit	81	14.32	0.8926
Equilin			
Double reciprocal fit	96	31.79	0.9940
Y-reciprocal fit	63	21.31	0.9369
X-reciprocal fit	69	24.29	0.8881
Estrone			
Double reciprocal fit	57	58.59	0.9941
Y-reciprocal fit	31	45.43	0.8616
X-reciprocal fit	33	48.71	0.8206
17β-Estradiol			
Double reciprocal fit	102	30.55	0.9971
Y-reciprocal fit	84	17.82	0.9875
X-reciprocal fit	88	21.39	0.9689
Ethynylestradiol			
Double reciprocal fit	105	28.78	0.9969
Y-reciprocal fit	84	17.16	0.9922
X-reciprocal fit	88	20.54	0.9775
Norethindrone			
Double reciprocal fit	−35	102.46	0.9970
Y-reciprocal fit	−47	25.48	0.7930
X-reciprocal fit	−48	18.03	0.8680
Progesterone			
Double reciprocal fit	−17	278.04	0.9925
Y-reciprocal fit	−53	23.22	0.6962
X-reciprocal fit	−59	12.96	0.8611
Mestranol			
Double reciprocal fit	−10	466.88	0.9919
Y-reciprocal fit	−50	23.73	0.6558
X-reciprocal fit	−58	12.59	0.8472

association constant for 17β-estradiol (*Y* and *X* fits). Even though 17β-estradiol is the least hydrophobic compound of all target analytes it strongly included into the 2HP-γ-CD cavity. This result demonstrated that in this case, the inclusion is due to the strong hydrogen bonding of the vicinal hydroxyl groups on the D ring with hydroxyls of the CD. As expected, the increase in the concentration of SDS in the run buffer did not affect the association constants of the compounds 6–8, which remain negative.

4 Concluding remarks

The interaction of several steroids with different CD types was characterised and steroid-CD association constants were determined. The most powerful agent for the inclusion of the target analytes was found to be γ-CD followed by 2HP-γ-CD. High values of association constants were accompanied by good linearity of the plots. The constants values obtained for individual analytes correlate with their structural characteristics contributing to their inclusion. Interaction with the cavity of β-CD was rather weak while 2HP-β-CD failed to recognise difference in structure of estriol, equilin, ethynylestradiol, 17β-estradiol and estrone. It was found that the association constant values decreased when the concentration of micelles in the run buffer was increased.

In general the estimation of association constants of steroid compounds with CDs was a straightforward process due to high efficiency and simple performance of CE in a combination with three plotting methods used.

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