APPLICATION OF CAPILLARY ELECTROPHORESIS FOR THE ASSAY OF ERYTHROMYCIN AND ITS RELATED SUBSTANCES

by

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ABSTRACT

Capillary Electrophoresis (CE) is a high resolution analytical technique that may be employed in the separation and quantification of a wide range of analytes. The enormous efficiency obtained in CE are well suited for complex mixtures in which resolution of a large number of peaks in a short time is desirable. Therefore, CE has a promising future in pharmaceutical analysis. The separation mechanism of CE is based on the differential electrophoretic mobility of the solutes inside a buffer filled capillary upon the application of a voltage. Capillary electrophoresis is especially suitable for ionic species. The full potential of this technique can only be realised through the manipulation of numerous experimental parameters.

In the present study, a CE method has been developed for the analysis of the macrolide antibiotics: erythromycin, oleandomycin, troleandomycin and josamycin. The selection of initial analysis conditions and optimisation of selectivity are reviewed. A systematic approach to method development was used to maximise analyte differential electrophoretic mobilities, by adjusting the pH. Thereafter, the influences of electrolyte molarity and electrolyte additives were investigated. In addition, some instrumental parameters, such as capillary length and diameter, applied voltage and injection conditions were varied. The effect of the sample solvent and on-capillary concentration techniques such as FASI, were investigated. Also, the influence of injecting a water plug on the quantity of sample injected was demonstrated. Full resolution was achieved with the addition of methanol to the electrolyte.

The applicability of CE for the assay of erythromycin and its related substances was investigated. Two methods were developed and successfully validated using CE: one for the quantitative determination of erythromycin alone and another for erythromycin related substances in the presence of large quantities of erythromycin A. Several related substances and impurities that result from the fermentation process used to produce erythromycin as well as degradation products are known to be present in commercial samples. These impurities include erythromycin B, C, D, E, F, erythromycin enol ether, anhydroerythromycin and N-demethylerythromycin. Currently both the USP and BP official assays for the analysis of erythromycin involve the use of microbiological assays. These methods are limited as they are unable to differentiate between erythromycin and its related substances and degradation products. Furthermore, the microbiological assays are time-consuming and tedious to perform.

The CE methods developed for the analysis of erythromycin and for its related substances were fully validated in terms of precision, linearity, accuracy, sensitivity and stability. In addition, erythromycin was subjected to six stress modes and the stressed samples were analysed. An internal standard was employed to provide acceptable precision for the migration time (< 1.80 % RSD) and peak area (< 4.44 % RSD). Optimum sensitivity was obtained using low UV wavelengths, with LOQ values of less than 10 % for the related substances. The developed method was accurate for erythromycin C, anhydroerythromycin and N-demethylerythromycin, even in the presence of large concentrations of the parent. The method for erythromycin related substances was applied to the determination of impurities in three commercial erythromycin bases. The CE methods developed were rapid, precise, specific and stability-indicating and may be used to provide additional information to augment that attained by HPLC for purity assessment and in stability studies of erythromycin.

Capillary electrophoresis is a simple, cost-effective technique that is capable of generating high quality data. This technique will become firmly established within pharmaceutical analysis for main peak and related impurity determination assays as familiarity becomes more widespread across the pharmaceutical industry and improvements in instrumentation are performed.

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AE	anhydroerythromycin
AUFS	absorbance units full scale
BICINE	N, N-bis[2-hydroxyethyl]-glycine
CD	cyclodextrin
CD-MEKC	cyclodextrin modified micellar electrokinetic capillary chromatography
CE	capillary electrophoresis
CEC	capillary electrochromatography
CGE	capillary gel electrophoresis
CIEF	capillary isoelectric focussing
CITP	capillary isotachophoresis
CMC	critical micelle concentration
CTAB	cetyltrimethylammonium bromide
CZE	capillary zone electrophoresis
DNA	deoxyribonucliec acid
DTAC	dodecyltrimethylammonium chloride
EB	erythromycin B
EC	erythromycin C
EEE	erythromycin enol ether
EKC	electrokinetic chromatography
EOF	electro-osmotic flow
EPC	European Pharmacopoeial Convention
EtOH	ethanol
FASI	field amplified sample injection
FAPSI	field amplified sample injection with polarity switching
FDA	Food and Drug Administration
GC	gas chromatography
H_2O_2	hydrogen peroxide
HC	hydroxycellulose
HCl	hydrochloric acid
HEC	hydroxyethylcellulose
HPLC	high performance liquid chromatography
HPMC	hydroxypropylmethylcellulose
ICH	International Convention on Harmonization
KOH	potassium hydroxide

LC	liquid chromatography
LOD	limit of detection
LOQ	limit of quantitation
MEKC	micellar electrokinetic chromatography
MES	2-[N-morpholino]-ethanesulphonic acid
NaOH	sodium hydroxide
NDEA	N-demethylerythromycin A
psEAEN	pseudoerythromycin A enol ether
psEAHK	pseudoerythromycin A hemiketal
PVOH	polyvinyl alcohol
RSD	relative standard deviation
SDS	sodium dodecyl sulphate
TAA	tetra-alkylammonium
TLC	thin layer chromatography
TRICINE	N-[2-(hydroxymethyl)ethyl]-glycine
TRIS	tris(hydroxymethyl)aminomethane
USP	United States Pharmacopoeia
UV	ultraviolet
UV-Vis	ultraviolet-visible
vs	versus

α	alpha
β	beta
γ	gamma
λmax	lambda maximum
µA	microampere
µl	microliter
µm	micrometer
cm	centimeter
g	gram
kV	kilovolt
M	molar
mbar	millibar
mg	milligram
min	minute
ml	milliliter
MM	molecular mass

mm	millimeter
mM	millimolar
ng	nanogram
nl	nanolitre
nm	nanometer
v/v	volume per volume
w/v	weight per volume
°C	degrees Celsius

CHAPTER ONE

INTRODUCTION

1.1 HISTORICAL BACKGROUND AND DEVELOPMENT

Electrophoresis is a powerful technique for the separation of a wide variety of chemical compounds in a mixture. It is defined as the differential migration of charged species through a fluid or gel under the influence of an electric field. This separation technique was introduced by Tiselius in 1937 [1]. It was found that when a protein mixture was placed between two electrolyte solutions in a tube, the sample components migrated on the application of an electric field. The direction and rate of migration was determined by their charge and mobility. However, thermal diffusion and convection limited the separation efficiency, thus contributing to the advancement of electrophoresis in an anti-convective media such as polyacrylamide or agarose.

The complications associated with thermal convection can be alleviated by performing electrophoresis in narrow tubes. The large surface area to volume ratio, that is characteristic of these tubes, permits efficient heat dissipation. Hence these tubes can be considered to be anti-convective. This eliminates the need for gel media and facilitates the performance of free solution electrophoresis. The first demonstration of zone electrophoresis in free solution using a quartz glass tube of inner diameter 3 mm was performed by Hjértens in 1967 [2]. Thermal convection was minimised by rotating the tube along its longitudinal axis. Mikkers et al. [3] demonstrated free zone electrophoresis on equipment designed for isotachophoretic separations, using teflon capillaries of 200 µm. Glass capillaries of internal diameter 200 µm were used by Virtanen [4] to explore the separation potential of capillary electrophoresis (CE).

In 1981, Jorgenson and Lukacs [5 - 7] conclusively demonstrated the analytical potential of capillary zone electrophoresis (CZE) by using a 75 µm fused-silica capillary. The high separation efficiency and resolving power of the method was demonstrated. In addition, the importance of experimental and instrumental design in minimising the production of excess heat was also shown. Jorgenson and Lukacs [8 - 10] also clarified the theory of electrophoresis by determining the relationship between the various operating parameters and separation quality. Further

developments in CE include the introduction of micellar electrokinetic chromatography (MEKC) by Terabe et al. [11]. This technique involves the incorporation of surfactants into the electrolyte to enhance the separation of neutral compounds. The availability of gel filled capillaries, coated capillaries and the possibility of incorporating various modifiers into the electrolyte have extended the scope of CE techniques and enhanced the efficiency and selectivity of the separation [12,13]. The popularity of electrophoresis is evident from the proliferation of techniques that have been developed to resolve a variety of complex separations.

1.2 MODES OF CAPILLARY ELECTROPHORESIS

Capillary electrophoresis (CE) is a rapidly growing, modern analytical technique that presents a new and extraordinarily powerful approach for the separation of charged compounds. It fills the void between conventional electrophoresis and high performance liquid chromatography (HPLC). Capillary electrophoresis comprises numerous modes of operation that have dramatically different operative and separative characteristics. These related techniques have emerged as CE has developed from a combination of chromatographic and electrophoretic techniques. These techniques offer complementary and orthogonal information to HPLC. Capillary electroseparation modes include capillary zone electrophoresis (CZE), capillary gel electrophoresis (CGE), capillary isotachophoresis (CITP), capillary isoelectric focussing (CIEF), micellar electrokinetic chromatography (MEKC) and capillary electrochromatography (CEC). In CZE, CGE, MEKC, and CEC a uniform electrolyte composition is maintained throughout the separation. The electric field strength and solute mobility are stable, thus resulting in analytes that migrate with constant, but differential velocities.

1.2.1 Capillary Zone Electrophoresis (CZE)

Capillary zone electrophoresis is the simplest, most versatile and thus most frequently used mode of CE. This technique is applicable to the separation of a wide variety of compounds and is performed with speed and ease. Separation is based on the differential electrophoretic migration of ionic species in the electrophoretic buffer. The mobility and consequent velocity of the ionic species is dependent on their charge-mass ratio at a given pH. The presence of the electro-osmotic flow (EOF) in uncoated fused-silica capillaries facilitates the separation of both cations and anions within a single run. This is possible as the EOF is significantly greater in magnitude than the electrophoretic mobility of the individual ions. The electrophoretic mobility of cations is augmented by the EOF as they are attracted towards the cathode, while anions migrate at a slower pace as their electrophoretic mobility is against the EOF. The order of migration is as follows: cations with the largest charge-mass ratio are followed by those with reduced ratios,

which in turn are followed by unresolved neutral solutes which migrate with the EOF. Anions with low charge-mass ratios migrate next, prior to those with high charge-mass ratios [14].

1.2.2 Micellar Electrokinetic Chromatography

Micellar electrokinetic chromatography (MEKC), introduced by Terabe et al. in 1984 [11, 16, 17, 18], is a hybrid of electrophoresis and chromatography. The experimental technique of electrokinetic chromatography (EKC) forms part of CZE, while the separation principles are classified as chromatographic [15, 16]. This mode of electrokinetic chromatography has gained universal acceptance as it is the only electrophoretic technique that is capable of selectively resolving both neutral and ionic solutes in a single run. In MEKC, ionic surfactants are incorporated into the electrolyte at concentrations above the critical micellar concentration (CMC). At these concentrations surfactants aggregate to form micelles. The micellar solution, often referred to as the pseudo-stationary phase, provides the phase for chromatographic separation.

The separation mechanism in MEKC is principally based on the differential partitioning of the solute between the micellar phase and the aqueous phase. The extent of partitioning is governed by the degree of hydrophobicity of the solute. In addition, electrostatic interactions between the solute and the surface of the micelle and charge-mass ratios of the solute combine to effect the separation. Thus micellar systems provide a multiplicity of interactions, such as hydrophobic, electrostatic and hydrogen-bonding interactions. These interactions cannot be duplicated by HPLC [18].

The charged micelles migrate either with or against the EOF. The anionic surfactant, sodium dodecyl sulphate (SDS), is most commonly employed in MEKC. Despite the attraction of the anionic micelles towards the anode, they migrate towards the cathode, as the magnitude of the EOF is greater than that of the micelle's electrophoretic mobility. This results in a fast moving aqueous phase and a slow moving micellar phase. The migration velocity of an analyte is thus dependent on its partition coefficient between the micellar and aqueous phase. Neutral hydrophilic molecules migrate with the EOF. If the analytes are charged they migrate according to their electrophoretic mobility. Hydrophobic compounds are incorporated into the micelle and migrate at the velocity of the micelle. The more hydrophobic the molecule, the greater the distribution into the micelle and the longer the migration time [18, 19].

Electrically neutral analytes migrate within the migration time window. The migration time window is defined by the difference between the electro-osmotic velocity, that is determined by the migration time of the bulk solution, and the velocity of the micelle. Modified chromatographic relationships are adopted to define the separation mechanisms in MEKC. Terabe et al. [11]

derived the capacity factor (k) in MEKC. It is defined as the ratio of the total number of moles of solute in the micellar phase to those in the aqueous phase. The capacity factor provides quantitative information about the distribution coefficient. Large values of k suggest that a substantial portion of the solute is incorporated within the micelle.

In addition to selectivity and efficiency, resolution in MEKC is dependent on the capacity factor [17]. The linear relationship between the capacity factor and surfactant concentration provides a means of improving the resolution. However, this intervention is limited by the high conductivity of such micellar solutions, which may result in undesirable thermal effects. Alternatively, the migration window may be expanded by incorporating organic modifiers into the electrolyte. This results in an improvement in efficiency and resolution of the system [20, 21].

In addition to facilitating the separation of electrically neutral compounds, MEKC is capable of providing enhanced selectivity for the separation of ionic solutes [22, 23]. Electrostatic repulsion limits the degree of solubilisation of anionic solutes by anionic micelles. The separation mechanism for cationic solutes in the presence of anionic micelles involves a combination of surface solubilisation, through ion-paring at the Stern layer of the anionic micelle followed by micellar solubilisation, and ion-pairing with surfactant monomers [24]. A correlation between the migration time of cationic solutes and the concentration of the surfactant is indicative of ion-pairing [24]. The distribution coefficient of ionic solutes is dependent on a combination of hydrophobicity and charge.

The utilisation of different surfactants, mixtures of surfactants, and the inclusion of additives such as cyclodextrins, polymer ions and microemulsions in the surfactant solution, all provide an extensive means of manipulating selectivity in MEKC separations. Different micellar systems affect selectivity by modifying the micelle size, aggregation number and geometry [24]. The wide range of selectivities available through the use of different micelles was illustrated by Burton et al. [25]. In this study a comparison of the separation of nine test solutes with both SDS and dodecyltrimethylammonium chloride (DTAC) micellar phases was demonstrated. Although a wide variety of surfactants are available, only a limited number are applicable to MEKC. The selection of surfactants is governed by their solubility and CMC. Surfactants possessing large CMC's are highly conductive, rendering them unsuitable.

Surfactants are classified as anionic, cationic, non-ionic, bile salts and chiral surfactants. In the presence of cationic surfactants the direction of the EOF is reversed. These surfactants are electrostatically attracted to the negatively charged silanol groups of the capillary wall and form a bilayer with the free cationic head groups facing the buffer solution. This results in a positive charge along the capillary wall [25, 26, 27]. Biological surfactants, of which bile salts are the most

frequently used, provide an alternative surfactant system. Bile salts are naturally occurring steroid based compounds synthesised in the liver. The most commonly used salts are sodium cholate, sodium deoxycholate, sodium dehydrocholate and their taurine conjugates. They have a unique structure and form helical micelles with a planar sheet. In addition, they possess different aggregation properties to alkyl surfactants's [28]. Nishi et al. [29] and Terabe and co-workers [30] studied the effects of bile salts on various separations. In the study of Terabe et al. [30] sodium taurocholate and sodium taurodeoxycholate were successfully employed as chiral selectors for the separation of several dansyl amino acids into their enantiomers. The optical nature of these bile salts broadens the application of CE to enantioseparations.

The inclusion of non-ionic chiral selectors, such as sodium N-dodecanoyl-L-valinate [31] and digitonin [32], into the micellar system effects the separation of enantiomers through selective hydrophobic entanglement of the solute with the micelle. Ionic surfactants are often included in the above systems to produce mixed micelles that are charged and thus possess electrophoretic mobilities. Natural surfactants such as digitonin [32], glycirrhizic acid and β-escin [33] have also been used for chiral separations. These natural surfactants are generally used in combination with SDS to produce mixed micellar systems.

The scope of MEKC is extended by the use of mixed micellar systems and by the incorporation of modifiers such as urea, alcohols, ion-pairing reagents and cyclodextrins (CD) into the micellar solution. Cyclodextrin-modified MEKC (CD-MEKC) is a branch of MEKC that arises from the inclusion of cyclodextrins into the micellar solution. Cyclodextrins are electrically neutral with hydrophilic exteriors and hydrophobic cavities. They present an additional phase to that of the micellar phase and are capable of selectively solubilising solutes in accordance to their size, shape and hydrophobicity. The chirality of the CD's extends the application of CD-MEKC to the separation of enantiomers. Enantiomeric separations by CD-MEKC were investigated by Terabe et al. [16, 34]. Nishi and co-workers [35] demonstrated the effect of including various types of CD's into a micellar solution created with SDS. It was found that chiral recognition was dependent on the nature of the CD.

The addition of high concentrations of urea (up to 8 M) into the electrolyte solution in MEKC was described by Terabe et al. [36]. Urea increases the solubility of highly hydrophobic compounds in water by diminishing the water structure around the molecules. The solubility change subsequently affects the selectivity and capacity factor. In addition, the migration window is expanded without substantially affecting the migration time. A wide migration window is beneficial for high resolution.

Organic solvents, at concentrations of up to 20 % (v/v), are used in MEKC to enhance resolution and to alter selectivity by extending the migration time window. They interact at several levels to reduce the EOF, modify the distribution coefficient, decrease the polarity of the electrolyte and hence facilitate the separation of hydrophobic compounds [20, 21, 37, 38]. Higher concentrations of organic solvents may disrupt the micellar phase slightly, thereby facilitating the separation of structurally similar compounds.

Nishi et al. [39] demonstrated the effect of incorporating tetra-alkyl ammonium salts (TAA) into the electrolyte in MEKC in an attempt to improve resolution. The migration time of anions increased as a result of the formation of ion-pairs between the analyte and the ammonium ion. This led to the incorporation of a larger portion of the complex into the micellar phase. Conversely, competition between the cationic analyte and the ion-pairing agent in pairing to the anionic micelle resulted in a decrease in the migration time of cationic analytes.

Chiral micelle polymers such as poly(sodium N-undecylenyl-L-valinate) are promising chiral selectors for the separation of enantiomers. These polymicelles are advantageous in that they possess enhanced stability and rigidity. The formation of these polymicelles is concentration-independent as they have no CMC. Resolution in the presence of these polymicelles is dependent on their concentration and the pH of the electrolyte. Polymerised micelles of compact conformation are formed at low pH while loose structures form at high pH [40].

1.2.3 Capillary Isotachophoresis (CITP)

Capillary isotachophoresis is a moving boundary electrophoretic technique that is performed in a discontinuous buffer system. The buffer system consists of leading and terminating electrolytes that have higher and lower mobilities respectively than that of the analytes. The sample component condenses between the two electrolyte constituents into consecutive sample zones. Each band migrates with the same electrophoretic mobility after equilibrium is achieved and the bands are focussed. The electric field across each zone fluctuates, but is self-adjusting, thus maintaining a steady-state velocity. Electroneutrality is preserved as ions which diffuse into neighbouring zones experience a velocity change and immediately return to their own zones. Each zone in CITP has a constant concentration which is determined by that of the leading electrolyte. The ratio of the concentration to the mobility of the ions in each zone is constant. As a result, zones that are more concentrated than the leading zone are broadened to maintain a constant ratio.

Capillary isotachophoresis has limited application since the analysis of both anions and cations within a single run is not feasible. In addition, the selection of a discontinuous buffer system that

is based on the analyte's mobility, at the desired pH, is often difficult. Conventionally, CITP is performed in the presence of a reduced EOF. This is achieved by using coated capillaries or by increasing the viscosity of the electrolyte. Detection is accomplished using a conductivity detector in which the response signal is proportional to the conductivity of the solution. The isotachopherogram that is obtained consists of a series of steps that depict the bands in decreasing order of conductivity. Quantitative analysis is based on the measured zone length, which is proportional to the amount of sample present. The step height is a qualitative parameter that is used for identification of the analyte.

Zone sharpening and concentrating effects that are apparent in CITP are determined by the concentration of the leading electrolyte. These effects minimise the extent of diffusion and enhance separation efficiency.

1.2.4 Capillary Isoelectric Focussing (CIEF)

Capillary isoelectric focussing is a separation technique in which amphoteric ampholytes are separated on the basis of their isoelectric point (pI value). The composition of the electrolyte, the electric field and analyte mobility vary along the migration path. The mechanism of separation involves the electrophoretic migration of an ampholyte in a pH gradient. The pH gradient is established by carrier ampholytes upon the application of an electric field. Charged ampholytes migrate through the medium until they reach a region of pH where they become uncharged. Migration then ceases and the ampholytes focus at that position which corresponds to their isoelectric point. After this focussing process, the zones are mobilised either by pressured flow or by the addition of salt to one of the reservoirs. Hence the migration of the bands past the detector is facilitated [41 - 43].

The use of high electric fields, high mobility slopes at the isoelectric point and a shallow pH gradient allows for the superior resolution of compounds with low diffusion coefficients. In CIEF, resolution is limited only by the quality of the pH gradient, characterised by its slope and linearity. The greater the number of carrier ampholytes, the smoother the pH gradient. Elimination or reduction of the EOF, accomplished by dynamic or covalent coating of the capillary wall, is crucial in CIEF to prevent the flushing of the ampholyte prior to analyte focussing.

1.2.5 Capillary Electrochromatography (CEC)

Capillary electrochromatography is a unique form of electrophoresis where separation is based on a combination of electrophoretic processes and sorptive interactions with a stationary phase, which is packed into the capillary [44]. The packing material consists of silica gel particles of less

than 1.5 μm in diameter (Knox and Grant [45]). The principle of EOF generation in packed capillaries is similar to that for open capillaries. However, the electrolyte is driven by electro-osmosis which originates both at the capillary wall and between the silica gel particles. Due to the tortuous nature of the flow channels in CEC, the flow profile approximates that of the plug flow attained in open capillaries. The electro-osmotic velocity is reduced in packed capillaries and may fluctuate along the length of the capillary, despite a constant zeta potential at the silica wall [46]. The separation efficiency attainable in CEC is significantly greater than that obtained in pressure-driven HPLC systems that use similar columns. This is due to the flat flow profile characteristic of CEC, as compared to the parabolic flow profile of pressure-driven systems [47].

The use of CEC with fused-silica capillaries packed with reversed-phase materials of various dimensions was investigated by Yamamoto and co-workers [48]. To date, the use of packed capillaries is not as popular as conventional open tubular capillaries. This is possibly due to the difficulties encountered in manufacturing these capillaries. However, it is anticipated that the advantages of CEC will make it a viable addition to the existing techniques for the analysis of drugs.

1.2.6 Capillary Gel Electrophoresis (CGE)

The separation mechanism in CGE is based on molecular sieving of the analytes as they migrate through the pores of the gel-filled capillary. The use of gels for electrophoretic separation is beneficial as they provide an anti-convective media, reduce or eliminate the EOF, prevent analyte adsorption onto the capillary wall and minimise solute diffusion. Capillary gel electrophoresis is principally employed in the separation of analytes with charge-mass ratios that do not vary significantly, especially macromolecules such as proteins and DNA [13, 43, 49]. The gels employed must possess thermal stability and provide the appropriate range of pore size.

Gel polymer networks are categorised into chemical and physical gels. Chemical gel networks comprise covalently cross-linked polyacrylamides that are characterised by small pore sizes and a rigid structure. This network structure precludes the use of hydrodynamic sample injection. Physical gels form a flexible polymer network through hydrogen-bonding and Van der Waal's interactions, which are in dynamic equilibrium. Linear polymer solutions such as polyacrylamide or methylcellulose form an entangled flexible network. The concentration that is required to produce the network is inversely proportional to the analyte size. The stability, flexibility, ease of use and ability to perform hydrodynamic sample injections with low viscosity polymer solutions are advantageous factors. However, the reduced viscosity requires the use of coated capillaries to eliminate the EOF. Selectivity of CGE separations is further enhanced by incorporating chiral selectors, ion-pair reagents or other complexing agents within the polyacrylamide gel.

1.3 PRINCIPLES OF CAPILLARY ELECTROPHORESIS

1.3.1 Electrophoresis

The basic theoretical concept in CE is similar to that employed in conventional electrophoresis. The migration of a particular species in CE, under the influence of an applied potential, is the result of several driving forces. Charged species are subjected to a strong electric field, which is dependent on the molecular charge and size of the species and electrolyte viscosity. In addition, charged species are also subjected to the bulk flow of the electrolyte. The separation of a mixture into spatially discrete zones of individual substances is feasible as different species possess characteristic effective electrophoretic mobilities and thus migrate with different migration velocities.

On the application of an electric field, a charged compound that is dissolved in an electrolyte solution is subjected to both an accelerating and retarding force. During electrophoresis a state of equilibrium is achieved in which the forces are equal but opposite [50]. The charged analyte consequently migrates with a constant velocity (v_{ep}) that is defined as:

$$v_{ep} = \frac{ze_0E}{6\pi\eta r}$$

z = *charge number*
e_0 = *elemental charge*
E = *electric field strength (V cm^{-1})*
r = *Stokes radius (cm)*
η = *Newtonian viscosity (Pa.s)*
v_{ep} = *migration velocity of ion (cm.s^{-1})* 1.1

The migration velocity is proportional to the electric field, where the absolute mobility, μ_{ep}, is the proportionality factor. This is related to the migration velocity as follows:

$$\mu_{ep} = \frac{v_{ep}}{E} = \frac{ze_0}{6\pi\eta r} = \frac{v_{ep}\,L}{V}$$

μ_{ep} = *absolute electrophoretic mobility (cm^2.V^{-1}.s^{-1})*
L = *total capillary length (cm)*
V = *applied voltage (V)* 1.2

The mobility of a given ion in a medium is a constant that is characteristic of that ion and is dependent only on the viscosity of the medium. Theoretically, the above equation (Equation 1.2) is only applicable to spherical particles migrating through a non-conducting medium. However, the electrophoretic medium is a conducting solvent in which moving particles are surrounded by an ionic atmosphere of oppositely charged counter-ions. This atmosphere modifies the ionic charge, local electric field and effective radii of the species and consequently influences their

electrophoretic mobility. Incomplete dissociation of the analyte results in a reduction of the net charge of the species and thus decreases the electrophoretic mobility.

Electrostatic interactions between ions of the electrolyte and between the analyte and its counter-ions are accentuated in high concentration electrolytes, thus reducing the absolute electrophoretic mobility of the ions. A possible explanation for this is the reduction of the ionic charge and an increase in the hydrodynamic radius, due to the inclusion of the atmosphere of counter-ions. The equation for the effective electrophoretic mobility is illustrated below (Equation 1.3). The effective mobility (μ_{eff}) will always be lower than the absolute mobility in the presence of ionic interactions.

$$\mu_{eff} = \frac{Q_{eff}}{6\pi\eta R}$$

Q_{eff} = *effective charge of ion [C]*
R = *total radius of ion (cm)*
μ_{eff} = *effective electrophoretic mobility* ($cm^2.s^{-1}.V^{-1}$)
η = *Newtonian viscosity (Pa. s)* 1.3

According to Hückel [51] the effective electrophoretic mobility is related to the zeta potential as follows:

$$\mu_{eff} = \frac{\zeta\,\varepsilon}{6\pi\eta}$$

ζ = *zeta potential (V)*
ϵ = *dielectric constant of the medium* ($F.m^{-1}$)
μ_{eff} = *effective electrophoretic mobility* ($cm^2.V^{-1}.s^{-1}$)
η = *Newtonian viscosity (Pa.s)* 1.4

Von Smoluchowski [52] derived a second equation (Equation 1.5) for the effective electrophoretic mobility. This equation is based on the Debye-Huckel theory of the diffuse double layer at the surface of charged particles.

$$\mu_{eff} = \frac{\zeta\varepsilon}{4\pi\eta}$$

ζ = *zeta potential (V)*
ϵ = *dielectric constant of the medium* ($F.m^{-1}$)
μ_{eff} = *effective electrophoretic mobility* ($cm^2.V^{-1}.s^{-1}$)
η = *Newtonian viscosity (Pa. s)* 1.5

Equation 1.4 differs from Equation 1.5 by a constant factor of 1.5. According to Henry [53], the equation derived by Huckel is a more accurate representation of the electrophoretic mobility.

Ideally the electrophoretic mobility should be independent of particle size and shape. However, it is evident from Equation 1.2 that mobility is a function of solute size and charge at a given pH. Small, highly charged species have higher mobilities than large minimally charged species. The pH and ionic strength of the electrolyte affect the degree of ionisation of a species and hence influence its electrophoretic mobility by altering the charge-mass ratio. The influence of temperature the on electrophoretic mobility arises from the dependence of viscosity on temperature. Electrophoretic mobility increases exponentially with temperature as a consequence of the exponential drop in viscosity with temperature.

1.3.2 Electro-osmotic Flow

Electro-osmosis is a fundamental phenomenon in all electrophoretic separation processes. It is defined as the relative motion of the solvent to the surface charge on the interior of the capillary wall that is caused by the application of an electric field. The surface of the silica capillary when in contact with an aqueous medium of pH not lower than 2.5 hydrates to form silanol groups. These silanol groups dissociate, release positive hydrogen ions into the electrolyte solution and thus form a negatively charged silica surface. In an attempt to neutralise the surface charge, counter-ions that are present in the electrolyte solution are electrostatically attracted to the silica wall. The capillary inner surface charge is characteristic of the dissociation constant of the free hydroxyl groups of the silica surface. The charge density at the silica surface is defined by the number and density of the counter-ions and is directly related to the flow. It is in the order of 5×10^4 cm^{-2} [54].

According to Stern's model, the interface between the negatively charged silica surface of the capillary and the electrolyte consists of two layers: a compact, immobile double layer of tightly bound ions called the Helmholtz plane layer and a diffuse layer of randomly orientated counter-ions as described by the Gouy-Chapman or Debye Huckel model [55].

The thickness of the diffuse layer is concentration-dependent, while that of the Helmholtz plane layer is concentration-independent. An electric potential develops at the interface between the capillary wall and electrolyte solution due to the coulombic attraction of counter-ions to the charged silica surface [55]. Within the Helmholtz plane, the electric potential declines linearly with distance from the surface. Across the diffuse layer, the potential, known as the zeta potential (ζ), decreases exponentially with distance from the surface. The zeta potential is defined by the Helmholtz equation as follows [2]:

$$\zeta = \frac{4\pi\eta\mu_{eo}}{E}$$

ζ = *zeta potential (V)*

η = *Newtonian viscosity (Pa.s)*

μ_{eo} = *electro-osmotic mobility* ($cm^2.V^{-1}.s^{-1}$)

E = *electric field* ($V.cm^{-1}$) 1.6

A second equation (Equation 1.7) which is an approximation, relates the zeta potential to the thickness of the electrical double layer. The electrical double layer is a function of the electrolyte concentration as shown below.

$$\zeta = \frac{4\pi\delta e}{D}$$

e = *total excess charge in solution*

Z = *number of valence electrons*

D = *diffusion coefficient* ($cm^2.s^{-1}$)

C = *electrolyte concentration (M)*

δ = *thickness of electrical double layer (cm)* 1.7

where $\delta = [3\times10^7 Z\sqrt{C}]^{-1}$

The zeta potential is dependent on surface properties such as the density of charged sites on the capillary wall surface and the nature and number of ions present in the electrolyte solution that are in contact with the capillary surface [52, 55]. The zeta potential may be altered in the presence of surface active species, by changing the electrolyte pH and composition, by temperature fluctuations and by chemical derivatisation of the capillary surface [7, 47, 56, 57].

The EOF originates in the electrical double layer, which can be described by Stern's model of the double layer, on the application of an electric field. It is transmitted along the capillary by the motion of loosely bound cations. The cations are hydrated and they entrain the bulk solution within the capillary towards the cathode, thus resulting in electro-osmosis [58]. In electrically-driven systems, the liquid flow is unique and exhibits a piston-like profile. There is no pressure drop across the capillary. Consequently, the driving force is uniformly distributed over most of the cross-section of the capillary and rapidly drops to zero only near the capillary wall [8]. This plug-like profile is beneficial in that it contributes equally to the velocity component of each solute, regardless of their radial position, thus minimising zone dispersion due to laminar flow [8].

Parabolic flow profiles originate from several source and necessitate control if optimal system performance is to be preserved. A totally uniform zeta potential is a prerequisite for a plug flow profile as it ensures that the liquid at the tube wall flows at a constant velocity throughout the capillary. Variations in the flow rate are accommodated by a counteracting viscous flow. This

flow superimposes a parabolic flow profile on the plug flow and results in the deterioration of system efficiency [59]. In addition, mass transfer occurs in the presence of parabolic flow in an attempt to counteract the resulting dispersive effects of the flow profile [59]. Capillary wall contaminants disrupt the uniform wall chemistry and alter the zeta potential which results in a deterioration of system performance. This can be avoided by controlling the wall chemistry through the implementation of regular sodium hydroxide wash cycles [59]. Furthermore, it is critical to contain self-heating within the capillary by ensuring efficient heat dissipation and thus eliminating parabolic temperature profiles.

Several factors influence the magnitude and direction of the EOF. These include the composition of the capillary, the nature and concentration of the electrolyte [60, 61] and its pH [47, 56, 57, 62]. The polarity and magnitude of the zeta potential influences the EOF such that flow is enhanced with an increase in the zeta potential. In addition, compression of the thickness of the electrical double layer augments the EOF. The equations derived for the electro-osmotic mobility are similar to those for the electrophoretic mobility as both phenomena are complementary. The electro-osmotic mobility (μ_{eo}) is related to the zeta potential by the Helmholtz-Smoluchwski equation as follows [52]:

$$\mu_{eo} = \frac{\zeta \varepsilon}{4\pi\eta}$$

μ_{eo} = *electro-osmotic mobility* ($cm^2.V^{-1}.s^{-1}$)
ζ = *zeta potential* (V)
η = *Newtonian viscosity* ($Pa.s$)
ϵ = *dielectric constant* ($F.m^{-1}$) *1.8*

It is important to know the magnitude of the EOF since it contributes to system efficiency and resolution [5]. Selective control of the EOF is only possible through manipulation of the zeta potential, as alterations of the bulk variables; viscosity, dielectric constant and the electric field will equally affect both the electrophoretic mobility and EOF [63]. Ionic strength and pH are the two most prominent factors that influence the zeta potential. Adjusting the electrolyte pH dramatically influences the EOF and also affects the solute charge and electrophoretic mobility. Silanol groups dissociate to a greater extent in the presence of electrolyte solutions of alkaline pH, thus resulting in an increase in the capillary surface charge. This leads to a growth of the zeta potential and acceleration of the EOF towards the cathode [58, 64, 65]. Above pH values of 8 the zeta potential plateaus as the titration of silanol groups is complete.

As described in Equation 1.7, the zeta potential is inversely proportional to the number of ions present at the capillary surface. In the presence of high ionic strength buffers the electrical double layer is significantly compressed. This causes most of the potential drop to occur across the immobile layer. As a result, the zeta potential drops rapidly across the diffuse layer, which

subsequently contains a smaller fraction of counter-ions. The decrease in the zeta potential results in a proportional decrease in the EOF [55, 66].

Capillary wall modification, either through covalent or dynamic coating, may result in an increase, decrease or reversal of the surface charge and thus the EOF [67]. The addition of competing charged species reduces the surface charge of the capillary wall by electrostatic neutralisation and thus decreases the EOF. Organic modifiers alter the zeta potential, viscosity and analyte electrophoretic mobility. It is possible to reduce, enhance or reverse the EOF by incorporating surfactants into the electrolyte solution.

A measure of the EOF is essential as a constant value is indicative of an optimally operating system. This is accomplished by injecting a water-soluble, neutral solute which does not adsorb onto the capillary wall and which possesses a high UV absorbance. These properties are necessary to facilitate the injection and detection of small quantities [58, 68, 69]. Methanol, acetone and mesityl oxide are most commonly employed as EOF markers [57]. The magnitude of the EOF is predictable, as it is dependent on the surface chemistry of the capillary, is chemically controllable and is directly related to the observed resolution [57].

1.4 CAPILLARY ELECTROPHORESIS INSTRUMENTATION

A principle advantage of CE is the elementary nature of the instrumentation. Figure 1.1 represents a schematic of a typical CE system.

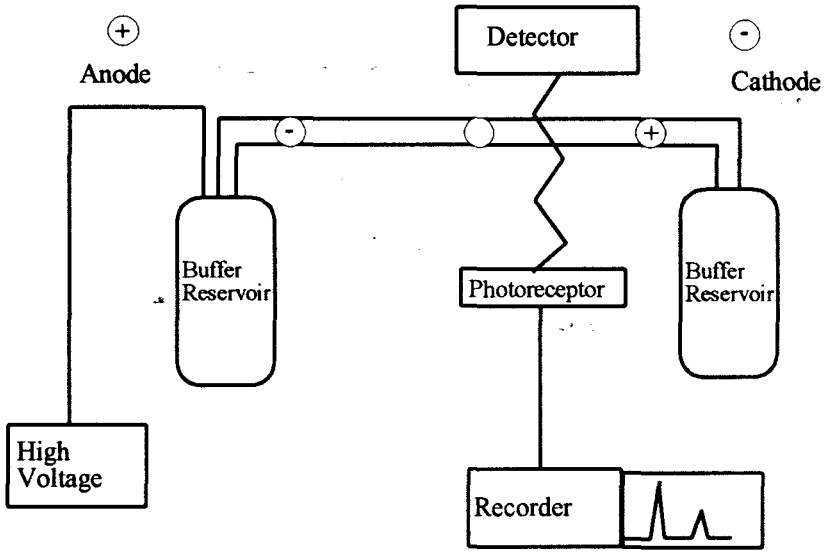


Figure 1.1 Schematic diagram of capillary electrophoresis system.

A CE system comprises a high voltage power supply that is capable of delivering between 5 kV and 30 kV, and an electrophoretic separation compartment that consists of a fused-silica polyimide coated capillary of inner diameter ranging from 10 µm to 100 µm. The capillary is connected to two buffer reservoirs. High voltage electrodes are located in the buffer reservoirs to establish electrical contact between the high voltage power supply and the capillary. Optical detection is performed directly through the cross section of the capillary wall at the cathodic end. Sample injection is accomplished by replacing the anodic buffer reservoir with a sample vial and applying either an external pressure (hydrodynamic injection) or an electric field (electrokinetic injection). Output terminals are provided at the rear of the instrument for connection to a strip chart recorder, integrator and/or data system [5].

Several commercial CE systems are designed with enhanced features such as multiple injection modes, an autosampler, a thermostated sample tray and capillary, programmable power supply, multiple detectors, fraction collectors and computer interfacing. These modifications facilitate the attainment of highly reproducible results and allow the potential of CE as an alternative and complementary quantitative separation technique to be fully appreciated [70].

The individual components of a CE instrument are described below in detail. Special emphasis is placed on their impact on the efficiency and reproducibility of this separation technique.

1.4.1 Capillary

The capillary is the fundamental element of a CE system. The principle aim of utilising a capillary is the attainment of high separation efficiency, for which high voltages are necessary. This is feasible due to the efficient heat dissipation capabilities of the narrow bore capillary [5, 7]. The tremendous interest in CE in recent years has been attributed to the availability of high quality fused-silica. Fused-silica is the material of choice owing to its intrinsic properties. These include chemical and electrical inertness, superior UV transparency, flexibility and robustness, good thermal conductance and most importantly, the feasibility of manufacturing high quality fused-silica capillaries of inner diameter below 100 µm with great precision [71 - 73].

Several potential drawbacks exist with the selection of fused-silica as the primary material. Of significance is the negatively charged silica surface, which presents a site for potential interaction with oppositely charged analytes. Electrostatic wall-analyte interactions cause peak tailing and reduce separation efficiency. Several interventions to control wall effects have been developed. These include dynamic and chemical modifications of the capillary surface. In addition, the small internal diameter that is essential to minimise heating effects, limits sensitivity. However, a gain in sensitivity can be achieved through capillary modification [71, 74].

Advances in capillary technology are apparent from the various modifications in capillary shape, and the availability of different materials and outer coatings. These improvements in capillary design have all contributed to the progress of CE. Z-shaped flow cells, bubble cells and rectangular capillaries are attempts to enhance sensitivity by extending the pathlength for optical detection without increasing the overall capillary volume.

The introduction of rectangular borosilicate capillaries with pathlengths of up to 1 mm by Tsuda et al. [75] has received limited acceptance because the capillaries are fragile due to the unavailability of a suitable protective coating. The advantages of rectangular capillaries include a significant improvement of up to twenty-fold in sensitivity. This improvement is achieved without loss in efficiency and is due to the lengthened effective pathlength. In addition, rectangular capillaries enjoy improved thermal characteristics that arise from the larger surface area to volume ratio, and reduced optical distortion that is produced by rounded capillary walls [74]. The development of protectively coated, rectangular fused-silica capillaries will offer numerous exciting possibilities, including two dimensional separations.

Chervet et al. [76] described a Z-shaped longitudinal capillary flow cell for improved UV detection sensitivity. The absorbance pathlength in the Z-shaped flow cell is increased to 3 mm. A six-fold enhancement in the signal to noise ratio can be achieved, although separation efficiency and resolution are compromised to a minor extent. The bubble cell, that is created by expanding the region of the capillary that is located in the detector to three times that of the inner diameter, offers a unique method of extending the pathlength. A three-fold increase in the signal is achieved in comparison to a straight cell [77]. The improved lower limit of detection, linear dynamic range and sensitivity attained by these capillary modifications extends the applicability of this separation technique to the quantification of trace levels of impurities.

Capillaries that are manufactured from teflon are available, although their use is not as extensive as fused-silica capillaries due to the inherent nature of the material. The difficulties in obtaining homogenous inner diameter capillaries, the poor thermal conductance of the material and the tendency for sample adsorption limit their use. However, teflon possesses the advantage of being UV-transparent, thus eliminating the need to create an optical window. In addition, teflon exhibits significant EOF [73].

A new optically-transparent coating for silica is available as an alternative to polyimide. These capillaries can be handled with ease as it is not necessary to burn the protective coating to produce a detection window. The flexibility and chemical inertness of this type of tubing will promote its use in CE [74, 78].

1.4.2 Power Supply

The majority of commercially-available CE instruments are designed with a power supply that is capable of delivering voltages of up to 30 kV or current levels of between 200 and 300 μA. Migration time reproducibility (within 0.1%) is dependent on the ability of the system to provide stable voltage regulation. The power supply should have a facility whereby field programming can be implemented to ramp the voltage at the beginning of the analysis, thereby producing voltage and current gradients. This is useful as it prevents thermal expansion and expulsion of the sample and electrolyte from the capillary ends on application of the separation voltage by avoiding rapid heating. The efficiency of the system is dramatically reduced by the loss of small, non-reproducible amounts of sample at the onset of the separation. Another desirable feature of a power supply is the ability to switch polarity, thus enabling sample introduction to be performed at either the cathodic or anodic end of the system, without the need to relocate the on-line detector. Typically, injection is performed at the anode [79].

Constant current vs constant voltage

Historically, voltage control has been used for electrophoresis as it was believed to maintain a constant field strength during the analysis. However, elevation of the electrolyte temperature is inevitable, regardless of the presence of a cooling system or thermostating of the capillary wall, especially when high salt concentrations are used. Temperature variation effects a parallel response in buffer conductivity and augments the production of joule heat. The effect is thus self-amplifying. Fluctuations in current with voltage simultaneously affect the EOF and electrophoretic mobility in such a manner that a non-linear relationship develops between mobility and voltage at high field strengths.

Tsuda et al. [80] and Lee and co-workers [81] have both argued that a constant current mode is superior to a constant voltage mode of operation as a linear relationship exists between the mobility and current, despite high values of current. If the current is kept constant, the corresponding increase in electrolyte conductivity with an inner temperature rise will induce a voltage drop across the capillary. Consequently, the fluctuations in viscosity are compensated for by a proportional change in the voltage, thus maintaining a constant migration time. This effect is more self-limiting. Current is insensitive to temperature variations and hence greater precision of the EOF and electrophoretic mobility of the solutes can be attained [79]. The precision of current control is inferior to that of voltage control, especially for small currents at high voltages (1 % compared to 0.1 %). This makes the selection of voltage control preferable.

1.4.3 Detection

Detection is presently the weakest component in any CE system. The lack of reliable, universal, sensitive detection presents a significant challenge and is often the limiting factor in the utilisation of CE in quantitative analysis. The small capillary dimensions that are necessary for separation in CE together with the minuscule volume of sample injected, demand the employment of a detector system that is capable of sensitive detection of solute zones, without introducing zone dispersion.

Detection is performed almost exclusively in an on-capillary mode as this eliminates peak broadening that is caused by joints, fittings and connections and diminishes the disturbance of the potential field across the capillary [14, 82]. Thus the inherent efficiency of this technique is preserved. Advances in detection techniques used in CE have relied on modification of state-of-the-art detection methods used in HPLC. Several detector design features need to be reviewed prior to selection. The requirements of a detector suitable for CE include the minute volume of the detection cell, high sensitivity, wide linear dynamic range, rapid detector response and contribution to peak width [83]. Furthermore, detection in CE has been hindered by the need to immerse both capillary ends in the buffer reservoir. This has resulted in the almost exclusive development of on-capillary UV and fluorescence detection.

UV- Vis absorbance is presently the most popular detection technique due to its relative universal nature, widespread availability at low cost, ease of installation for on-capillary use and adequate sensitivity for chromophoric compounds [82]. The capillary serves as a cylindrical detection cell and is mounted in the light beam. The light beam is masked by a suitable window, thus limiting the length of tube illuminated to less than 1 cm. Fused-silica, with the polyimide coating removed to form a detection window, is suitable for optical detection methods requiring UV-Vis light transparency as it has a UV cut-off around 170 nm [14].

In accordance with the Beer-Lambert Law, the intensity of absorbed light, and thus sensitivity of the separation technique, is dependent on the concentration of the analytes and the optical pathlength. The pathlength in on-capillary detection is inevitably short and is limited to the inner diameter of the capillary. This demands excellent detector design to preserve high sensitivity and a wide linear detection range. In CE, the small injection volumes (nl in CE vs µl in HPLC) and the low loadability of the capillary result in inferior sensitivity as compared to HPLC.

Compensation for the poor concentration sensitivity by increasing the volume of sample injected is inadvisable as it leads to an unacceptable loss of both efficiency and resolution [84]. The non-linear increase in the peak height with increasing amounts of sample injected onto the capillary

is due to overloading of the capillary and peak broadening. Consequently, the volume of sample injected onto the capillary is limited to 1 - 2 % of the total capillary volume. The loading capacity of the capillary can be increased by using an electrolyte of higher molarity than the sample solvent. However, the separation voltage must be reduced to prevent the generation of large quantities of joule heat. This results in longer analysis time [84].

The relative insensitivity of detection in CE, largely caused by the small volume of solute injected into the capillary and the low volume of the detection cell, can be partially compensated for by the use of low UV detection wavelengths, at which many species possess high molar extinction coefficients [85]. Detection at these low wavelengths warrants the use of electrolytes which have low UV absorptivity in solution. The presence of high background absorbance will increase baseline noise and decrease the signal [85]. Inorganic salts such as phosphate and borate are appropriate in this respect as opposed to biological buffers which absorb substantially up to 215 nm.

Conventional UV detectors have detection limits between 10^{-5} M and 10^{-6} M. Such limits are unattainable in CE because of the difficulty in transmitting sufficient light through the short optical pathlength of the capillary and due to the constraints of sample size. Quantitative detection limits in CE are regarded as being inferior to those in HPLC by at least an order of magnitude [86, 87]. On-capillary concentration techniques such as sample stacking [3, 85] and field amplified sample injection (FASI) [88], that are achieved by increasing the ionic strength of the electrolyte relative to that of the analyte solution, may increase detection sensitivity by improving the loading capacity. However, the generation of excess joule heat limits the degree of such an intervention [85].

The future of CE will be determined by its ability to detect trace amounts of a wide variety of analytes. The large number of theoretical plates that are required for separation is achieved in CE. However, this is offset by the lack of sensitivity in the detection of trace amounts of impurities [89]. Detection requirements in CE have been forced to the lower limit of current system capabilities by the small capillary dimensions [89]. As a result, insufficient detection sensitivity remains a major impediment to future applications. The ideal detector for CE should have high light throughput, suppress stray light and be easily aligned. It should be capable of providing universal detection, enhanced selectivity and high sensitivity without deterioration of separation efficiency [90].

1.5 PERFORMANCE CRITERIA

The adopted separation technique should exhibit acceptable performance that can be characterised by a series of parameters that describe the EOF rate, electrophoretic mobility and migration time. The performance criteria in CE include efficiency, resolution and selectivity. These criteria are extensions of those used in chromatography, but are modified in terms of the basic concepts applicable to electrophoresis.

1.5.1 Selectivity

Selectivity (α) is determined by the differences in effective electrophoretic velocities of the sample ions. It is a critical parameter for the optimisation of separation in CE.

$$\alpha = \frac{\Delta v}{v_{(ave)}} = \frac{\Delta t}{t_{mig(ave)}} = \frac{t_1 - t_{eo}}{t_2 - t_{eo}}$$

$v_{(ave)}$ = *average migration velocity* ($cm.s^{-1}$)
Δv = *difference in velocity between 2 zones* ($cm.s^{-1}$)
Δt = *difference in migration time (s)*
$t_{mig\,(ave)}$ = *average migration time (s)*
t_{eo} = *migration time of EOF marker (s)* 1.9

The potential of a separation technique is dependent on the number of parameters and degree to which they influence selectivity. Optimisation of separation selectivity can be achieved by varying the degree of dissociation of the sample in the electrolyte, either through physical or chemical alteration of the sample [91], or by modifying the hydrodynamics of the capillary. Selectivity can be manipulated by varying the composition of the electrolyte, by altering the concentration, pH, nature of the buffer anion or cation and incorporating organic solvents and surfactants. The organic solvents and surfactants serve as EOF modifiers [91 - 93].

Manipulation of electrolyte pH is highly effective in optimising the selectivity, while minimal improvements are observed by changing the ionic strength. EOF modifiers enhance selectivity through several mechanisms, which will be discussed in section 1.7.

1.5.2 Efficiency

System efficiency is determined from the number of theoretical plates (N) achieved by the capillary. For Gaussian peaks, efficiency can be calculated experimentally from the electropherogram by applying either of the equations below (Equation 1.10 and 1.11). Of these equations, Equation 1.11 is preferable in the presence of peak tailing.

$$N = 16[\frac{t}{w}]^2$$

t = *migration time (s)*
w = *temporal peak width at baseline (s)* 1.10

$$N = 5.54[\frac{t}{w/2}]^2$$

$w/2$ = *temporal peak width at half peak height (s)* 1.11

Jorgenson and Lukacs [5] predicted a linear relationship between the plate number and applied voltage, provided that longitudinal diffusion is the exclusive form of dispersion. The fundamental electrophoretic expression for the plate number is given below.

$$N = \frac{\mu V L_D}{2DL_T}$$

μ = *electrophoretic mobility* ($cm^2.V^{-1}.s^{-1}$)
D = *diffusion coefficient* ($cm^2.s^{-1}$)
L_D = *effective capillary length (cm)*
L_T = *total capillary length (cm)*
V = *applied voltage (V)* 1.12

The above equation is applicable under ideal conditions where longitudinal diffusion is the sole contributor to solute zone-broadening. Further requirements include the absence of analyte-wall interactions, the maintenance of plug flow throughout the entire length of the capillary and the injection of a small sample plug [59]. Unlike chromatography, capillary length plays a minimal role in separation efficiency, although it affects analysis time profoundly [8]. The inverse relationship between efficiency and molecular diffusion implies that separation efficiency is enhanced with larger molecules which possess low diffusion coefficients and thus exhibit less dispersion [89].

Peak capacity (n) gives an indication of the number of compounds that can be theoretically separated as peaks, with a resolution of 1 between the EOF marker and the last migrating compound. It is defined as:

$$n = 0.5\sqrt{N_{max}}$$

N_{max} = *maximum number of theoretical plates* 1.13

The flat flow profile and thus high theoretical plate numbers achieved in CE, as compared to HPLC are conducive to increased peak capacity [86].

A high level of peak efficiency can be attained by optimising the following factors:
A flat flow profile must be maintained during the separation by ensuring that siphoning, which produces a pressure differential across the capillary, is prevented. In addition, electromigration dispersion should be minimised by selecting the optimal concentration of both analyte and electrolyte. A minimum injection volume should be selected to inhibit capillary overload and the effects of joule heat should be controlled. Adsorption of charged analytes onto the capillary wall should be minimised by incorporating buffer additives, which compete for adsorption sites on the capillary. Finally, molecular diffusion, which is the primary contributor to zone broadening, should be limited by experimental design so that solutes spend minimal time in the capillary [94, 95].

Maximum efficiency can be attained if the optimum applied voltage with regards to the ionic strength of the electrolyte is selected and dispersion effects are contained.

1.5.3 Resolution

The ultimate goal in any analytical separation is to achieve resolution of the components through the employment of an efficient system, and manipulation of selectivity [95]. Resolution (Rs) can be experimentally determined from the electropherogram (Equation 1.14).

$$Rs = 2\frac{(t_2 - t_1)}{(w_1 + w_2)} \qquad 1.14$$

t = migration time (s)
w = temporal peak width at baseline (s)

In addition, resolution can be expressed as a function of efficiency and selectivity as derived by Giddings [96].

$$Rs = 1/4N^{1/2}\left[\frac{\Delta v}{v_{(ave)}}\right] \qquad 1.15$$

N = number of theoretical plates (efficiency)
Δv = difference in velocity between 2 zones ($cm.s^{-1}$)
$v_{(ave)}$ = average migration velocity ($cm.s^{-1}$)
$[\Delta v/v_{(ave)}]$ = measure of sensitivity

Jorgenson and Lukacs [5] derived a second equation for resolution (Equation 1.16). This equation is based on Gidding's equation, with the additional assumption that longitudinal diffusion is the only form of zone broadening.

$$Rs = \frac{1}{4}\frac{\sqrt{V}}{\sqrt{2D}}\frac{\Delta\mu_{ep}}{\sqrt{\mu_{ep(ave)} - \mu_{eo}}}$$

μ_{eo} = *electro-osmotic mobility*($cm^2.V^{-1}.s^{-1}$)

$\mu_{ep(ave)}$ = *average electrophoretic mobility*($cm^2.V^{-1}.s^{-1}$)

$\Delta\mu_{ep}$ = *difference in electrophoretic mobility between 2 zones* ($cm^2.V^{-1}.s^{-1}$)

V = *applied voltage (V)*

D = *molecular diffusion coefficient* ($cm^2.s^{-1}$) *1.16*

Resolution is a function of selectivity, column efficiency and migration time. However, the separation is primarily driven by efficiency and not selectivity as in chromatography [63]. From Equation 1.16, it is apparent that resolution decreases with the magnitude of the EOF if it is in the same direction as the electrophoretic mobility. Infinite resolution can be obtained when the electro-osmotic mobility is balanced by the electrophoretic mobility. The magnitude and sign of the ratio of the electro-osmotic mobility to that of the electrophoretic mobility (μ_{eo}/μ_{ep}) directly affects the resolution and selectivity in such a manner that if the electro-osmotic mobility is independently controlled the ratio will change [63]. This can be achieved through static or dynamic coating of the capillary walls or by applying an external electric field. However, if the electro-osmotic mobility and electrophoretic mobility are simultaneously altered by modifying either the applied voltage, pH, type and concentration of buffer or incorporating organic solvents, the ratio remains constant [63]. The operating parameters that are selected should ensure a balance between the resolution and analysis time.

1.6 DISPERSION EFFECTS

The extremely high resolving power of CE emanates from controlling the sources of dispersion, thereby enhancing the electrophoretic separation. This is possible with the advent of modern instrumentation. Meticulous selection of the experimental conditions, in conjunction with appropriate design of the instrument, facilitates control of dispersion. Several factors contribute to dispersion. Amongst the most relevant are temperature gradients induced by joule heating, electrophoretic dispersion, injection plug length, adsorption, coulombic interactions between the analyte and capillary wall, detection zone width and diffusion [3, 8, 68, 94, 95].

1.6.1 Joule Heat

The principal advantage in utilising a capillary as the separation compartment in CE is the anti-convective stability that it provides. This limits the extent of joule heating, which has traditionally restricted the progress of electrophoretic techniques. System performance factors such as efficiency, migration time and detection limit are all related and are dependent in a complex fashion on temperature [47]. Theoretical equations for efficiency (Equation 1.12) and resolution (Equation 1.16) advocate the use of high electric fields, but the benefits of this approach are ultimately limited by joule heat. The increased thermal diffusion in the presence of excessive joule heat adversely affects the resolution and contributes significantly to the plate height, thus resulting in serious deterioration of the efficiency of the system [5, 95, 97, 98].

The passage of an electrical current through an electrolyte solution generates joule heat, which in turn elevates the temperature within the capillary. While the generation of heat is homogenous across the length and cross-section of the capillary, dissipation occurs only at the periphery [8, 99]. This inevitably results in the formation of non-uniform thermal gradients across the capillary. Such gradients are problematic as they induce density gradients, alterations in the thermal conductivity of the electrolyte and local changes in electrolyte viscosity. The temperature variation across the bore of the capillary is parabolic, with a maximum temperature at the centre. The temperatures decreases exponentially in the capillary wall and the surrounding medium [99, 100, 101].

The high thermal conductance of fused-silica implies that heat is transferred more effectively across the capillary wall than through the electrolyte [99]. Experimental work conducted by Burgi et al. [102] demonstrated that the inner diameter is not responsible for efficient heat removal, as the internal temperature of three capillaries of differing diameters were found to be identical. The efficiency of heat removal is dictated by the transfer of heat between the outer capillary wall and the surrounding environment [102]. It is advantageous to use capillaries with a narrow inner radius and larger outer radius as the capillary wall acts as a heat sink [101]. The dissipation of heat through the wall occurs mainly by conductance, while radiation and convection are the mechanisms of heat transfer through the surrounding air [99]. The lower thermal conductivity of the polyimide coating limits heat transfer, to some extent, even though it is only a few microns thick [99].

The heat generated by the passage of electric current, and consequently the magnitude of the temperature gradient within the capillary, is related to a number of parameters as illustrated in Equation 1.17 [47, 100, 103]

$$W = \frac{d^2 \pi \lambda C V^2}{4L}$$

W = *rate of heat generated per unit volume* ($W.cm^{-3}$)
d = *capillary diameter (cm)*
V = *applied voltage (V)*
C = *ionic strength (M)*
L = *total capillary length (cm)*
λ = *molar conductivity* ($cm^2.\Omega^{-1}.M^{-1}$) *1.17*

Active temperature control, in terms of heat dissipation and maintaining a constant capillary temperature, is critical in attaining high efficiency, resolution, selectivity and reproducibility [45, 47, 97, 100]. A one degree Celsius change in temperature results in a 2 to 3 % change in viscosity. The viscosity change translates into an equivalent change in mobility [50]. This results in an exponential increase in the electrophoretic velocity and a corresponding reduction in migration time. Diffusion coefficients are directly proportional to temperature. Thus in the event of inefficient capillary temperature control, zone broadening is further amplified [94].

Several phenomena are indicative of excessive heat generation and probable non-uniform thermal gradients. These include deterioration of efficiency despite an increase in voltage, a disproportionate increase in current with voltage and a disproportionate increase in either the EOF or electrophoretic mobility with increasing voltage [29 - 33, 94, 101]. Significant loss of efficiency is observed, especially at high voltages, when buffers of high ionic strength and capillaries of diameter greater than 75 µm are used (Gruska et al. [98]).

Numerous mechanisms are available through which joule heat may be minimised. Efficient heat dissipation is a function of the capillary geometry and the cooling system design [100]. It is apparent from Equation 1.17 that narrow capillaries of increased length will significantly reduce the thermal load that must be dissipated by a given length of capillary [47].

Narrow bore capillaries improve efficiency through several mechanisms. Firstly, the reduction in the capillary bore maximises the inner surface area to volume ratio and enables more effective heat dissipation [8, 103]. Minimal heat is generated in small capillaries as the current decreases with the cross-sectional area of the capillary [103]. Finally, lateral diffusion of solute molecules leads to randomisation of their radial position in the capillary. This effect is more pronounced in narrow bore capillaries as solute molecules are readily able to diffuse across the entire cross-section of the capillary. Lateral diffusion results in an averaging of the migrational velocity of the zone, thus minimising zone broadening [5, 8, 99]. It is not feasible to reduce the diameter of the capillary below 25 µm due to the difficulties that arise from column loadability, capillary obstruction and detection.

The direct relationship between the quantity of heat generated, the molar conductivity and concentration of the electrolyte, illustrated in Equation 1.17, advocates the use of inorganic univalent electrolytes that possess large minimally charged ions with low ionic conductivity [90]. A reduction in the electrolyte ionic strength is accompanied by a proportional decrease in the electrical current and subsequent amount of joule heat. However, sample adsorption and peak broadening may occur with electrolytes of low molarity. Selecting low electric field strengths decreases the quantity of heat generated, although efficiency and resolution are concomitantly reduced. Thus, this latter method of controlling joule heat is not of particular use.

Cooling Systems

Capillaries that are exposed to random air currents are subjected to drastic temperature fluctuations, especially due to the large surface area to volume ratio and the heat generated by the electric current. These temperature variations manifest as excessive baseline noise and irreproducible migration times. Dramatic improvements in separation have been accomplished by employing commercial instruments equipped with temperature control facilities, such as fans or liquid cooling devices. The capillary is thus thermostated with high velocity air or liquid. As previously mentioned, the limiting factor in the removal of heat is the transfer from the outer capillary wall to the surrounding atmosphere [102]. This is facilitated by the use of a fan which circulates the ambient temperature to prevent the buildup of static air pockets [90, 97]. Although flow-through liquid baths theoretically present a significantly more effective means of heat dissipation, high velocity air cooling is sufficient for most separations [90, 102]. Temperature control of the entire capillary length is essential in minimising the formation of "hot" areas along the length [90].

The key to efficient operation of the CE system is the maintenance of a uniform temperature by minimising the amount of heat generated and maximising its dissipation.

1.6.2 Injection Plug Length

The injection plug length is primarily dependent on the geometry and length of the capillary, the sample viscosity and the product of the applied voltage or pressure and time interval. The sample plug length should be shorter than the dispersion caused by diffusion, if resolution and efficiency are to be maintained at a high level [94]. If the injection length is small, the zone length is governed by the migration time, whereas if it is large, diffusion and analyte-wall interactions are responsible for the zone length [94]. Baseline peak width can be used as an estimation of the injection plug length or the diffusion constant [94]. Axial diffusion, adsorptive interactions and injection plug length all contribute to the peak width. The injection plug length is the most significant of these factors in terms of peak broadening [94].

Prolonged injection times result in increased sample zone lengths and thus cause a deterioration in peak efficiency. Therefore, it is advisable to select short injection times, provided that the instrument is capable of loading these minute volumes reproducibly and that they are detectable. Vinther and Søeberg [104] concluded that the employment of moderate stacking conditions is preferable to prolonged injection times in improving detector sensitivity. Moderate stacking conditions are effective in narrowing the sample zone length and consequently minimising dispersion due to the sample injection width.

1.6.3 Adsorption

Reversible or irreversible coulombic interactions between the capillary wall and analyte are detrimental to separations in CE and severely compromise efficiency and reproducibility [89]. The adsorption of solutes causes irregularities in the zeta potential and thus results in variations in the EOF, which produces asymmetric peaks [94]. In severe cases, incomplete recovery occurs where elution of the entire sample from the silica wall is impossible, while in minor cases of reversible adsorption, band-broadening and tailing are apparent.

The adsorptive tendencies of fused-silica arise from the negatively charged surface. This surface presents a potential problem for the analysis of macromolecules like proteins and peptides, which possess numerous charges and hydrophobic moieties [88]. Furthermore, Liu et al. [105] demonstrated that the substantial surface area to volume ratio of narrow capillaries, which is beneficial for heat dissipation, increases the tendency for adsorptive interactions between the solute and fused-silica surface, especially at high field strengths.

Various strategies, of which some are simple to implement and very effective, are available to minimise solute-wall interactions. Increasing the electrolyte ionic strength and incorporating various additives in the electrolyte reduces the effective surface charge and thus suppresses the extent of coulombic interactions. Green and Jorgenson [106] employed large concentrations of alkali metal salts to compete for cationic exchange sites on the silica surface. However, this approach is limited by the ability of the system to dissipate the excess joule heat generated. To avoid the generation of excess joule heat, non-conducting zwitterion buffer systems have been employed at high concentrations as an alternative for minimising surface adsorption [107].

It is possible to suppress the adsorptive tendencies by selecting a pH at which the analyte and silica surface possess identical charge, thus resulting in coulombic repulsion [62]. Coating the capillary wall, either dynamically or statically, limits non-specific absorption by altering the hydrophobicity and eliminating or reversing the charge on the wall [89]. Dynamic coating simply involves the incorporation of buffer modifiers such as high concentrations of salts, zwitterions,

hydrophilic polymers or surfactants into the buffer. Adsorption is minimised by an ion exchange mechanism. The coating is continuously regenerated.

Chemical modification of the silanol groups on the capillary wall by static coating with a polymer layer is a more sophisticated, permanent method of reducing adsorption and controlling the EOF [89]. However, one major drawback of surface modification techniques is the instability of the coating over a wide pH range. Several researchers [108 - 110] have published procedures by which the silanol groups can be coated with a coating that is stable over a wide pH range. Supelco (Bellefonte, Pennsylvania) has recently introduced three bonded-phase CE capillaries: a moderately hydrophobic capillary with a C8 bonded phase (CElect H1), a highly hydrophobic capillary with a C18 bonded phase (CElect H2) and a hydrophilic capillary (CElect P1). Solute-wall interactions can be minimised by any of the above strategies or by employing a coated capillary in an attempt to limit dispersion.

1.6.4 Electrophoretic dispersion

Fluctuations in the electric field strength along the length of the capillary gives rise to electrophoretic dispersion. This manifests as distorted peak shapes. Differences in the electric field strength between the sample zone and electrolyte solution result in solute zone concentrating (low conductivity sample) or solute zone defocussing (high conductivity sample) and a temporary isotachophoretic state.

Electrophoretic dispersion originates from conductivity differences between the sample and buffer zones and differences in the concentration of the sample constituent and buffer co-ions. This results in mobility differences between the sample and buffer co-ion. Analytes dissolved in solvents of high dielectric constants readily dissociate into their constituent ions. These ions are electrically conducting and increase the conductivity of the electrolyte. This results in the distortion of the homogenous electric field and causes peak spreading [3, 8, 50].

If the solute zone has a lower conductivity than that of the buffer co-ion, the electric field within the sample zone will be higher as the resistance in that zone is greater than in the buffer zone. Subsequently, the leading edge of the peak will be sharp and the trailing boundary diffuse as it has a lower velocity and thus broadens with time. The resultant peak will exhibit pronounced tailing and decrease in height. Conversely, when the conductivity of the sample constituent is higher than that of the buffer co-ion, the electric field in the sample zone will be lower than the buffer, producing peaks with a sharp trailing edge and a diffuse leading edge [111].

Samples that are more concentrated than the electrolyte may perturb the conductivity and field strength across the sample zone [95], thus causing asymmetrical peaks and zone broadening. If the ratio of analyte concentration to that of the supporting electrolyte is low (two orders of magnitude lower), conductivity changes across the zones are negligible. Consequently, a constant electric field strength is maintained along the capillary [112]. This places great demands on detection sensitivity and limits the useful concentration range of detection in CE [95, 112].

In summary, peak distortion and sample overloading effects may be suppressed by closely matching the mobilities of the sample and buffer constituents, and ensuring that the conductivity of the sample zone is lower than that of the buffer zone. This will prevent sample dilution at the separation and sampling compartment interface [90, 112]. These methods of suppression are advantageous in terms of detection sensitivity. Peak shape distortion due to electromigration are detrimental only if resolution is lost.

1.6.5 Diffusion

Axial diffusion of the sample zone during its migration through the capillary leads to peak broadening. The existence of concentration gradients within the buffer promotes diffusion as the molecules attempt to equalise the concentration. The diffusion coefficient, as defined by Fick's Law, is directly proportional to the temperature and is inversely related to the apparent hydrodynamic radius. This suggests that compounds with high molecular mass, possessing small diffusion coefficients, are particularly suited to analysis by CE as band-broadening is less pronounced [94]. Accurate temperature control is also critical. The effects of diffusion may be minimised by performing the analysis at a higher voltage.

In conclusion, separation efficiency is affected by the combination of all the dispersive effects that are present to a greater or lesser degree, depending upon the experimental conditions. Sorption-desorption kinetics become prevalent with increasing field strengths, especially in narrow bore capillaries. The contribution of thermal effects to dispersion is negligible in small bore capillaries, but become significant as the bore size increases beyond 75 μm. The selected injection plug length should ensure that dispersion and detection sensitivity are counter-balanced. The specific conductance of the background electrolyte should be slightly greater than that of the sample to eliminate broadening due to sample dilution, but should be sufficiently low to prevent excessive joule heating. Almost every dispersive effect is temperature-dependent. As a result, accurate temperature control is necessary and is achieved through optimisation of the electrolyte concentration and applied voltage and by utilising narrow bore capillaries.

1.7 OPERATING PARAMETERS

Careful selection of the operating parameters is critical to prevent band broadening effects and to attain the highest levels of peak efficiency, resolution and selectivity.

1.7.1 Capillary Dimensions

Prior consideration of the influence of heat on the quality of the separation and on the selectivity required for analysis is crucial in selecting the capillary dimensions. Capillary dimensions have an effect on several factors including the migration time, resolution, detector sensitivity, heat generated and dissipated and adsorption. Typical capillary dimensions used in CE range from 10 to 100 μm for the inner diameter, 190 to 366 μm for the outer diameter, and between 10 and 100 cm in length. The inner diameter determines the sensitivity, capillary loading capacity and the current generated, which regulates the extent of heat produced. The outer diameter regulates the heat dissipation ability and consequently influences the current level.

1.7.1.1 Capillary Length

The length of the capillary determines the magnitude of the electric field strength that is generated. Extending the capillary length establishes a higher electrical resistance which reduces the current and consequently decreases the quantity of joule heat produced. Secondly, the additional length imparts a larger surface area for the dissipation of joule heat. Resolution is directly proportional to the square root of the capillary length. At constant field strength, column efficiency is proportional to capillary length (Equation 1.12) [5]. Thus, longer capillaries provide improved resolution and efficiency because they can be operated at higher voltages. However, as analysis time is also directly related to the length (Equation 1.2) [5], additional peak broadening that results from prolonged exposure of the analytes to the silica surface and extended interaction with the buffer components limits the use of long capillaries. In the selection of capillary length, a compromise between optimal resolution, efficiency and analysis time is required. The effective length, which is the length from the anode to the detector, should be as large a percentage of the total length as possible to facilitate the application of high field strengths and to allow reduction of the capillary conditioning time.

1.7.1.2 Capillary Diameter

Manipulation of the internal diameter alters the separation performance of the system in several ways. The capillary diameter does not appear in any equations relating to system performance, however it affects the amount of heat generated and the magnitude of the thermal gradient within

the capillary (Equation 1.17). Wide bore capillaries (>100 μm) are rarely used in CE [112]. In a study conducted by Grushka et al. [98] on the dependence of column efficiency on capillary diameter, it was found that plate height is a strong function of capillary diameter. The short pathlength and distortion of light resulting from the round capillary walls impairs detection sensitivity in narrow bore capillaries. In addition, reduction of the internal volume of the capillary with the square of the radius further affects sensitivity as it is not possible to load narrow capillaries with the same volume [103]. The potential for undesirable adsorptive interactions between the solute and the fused-silica capillary wall in narrow capillaries is augmented at high field strengths as a result of a large surface area to volume ratio [103]. For these reasons and for ease of handling, the most widely used capillaries are in the 50 - 100 μm I.D. range. Wider capillaries offer shorter analysis time and improved analyte sensitivity, whereas narrower capillaries offer improved efficiency and resolution.

1.7.2 Electrolyte System

The composition and method of preparation of the electrolyte is of primary importance as it results in a particular ionic strength. This in turn, determines the migration behaviour of the solutes, the current generated and the level of the EOF. These factors all influence the selectivity of the system. The ionic strength modifies the charge density of the analyte and the thickness and composition of the electrical double layer.

The electrolytes that are employed in CE are ionisable salt solutions that permit the transfer of current when subjected to voltage. The strong dependence of the electrophoretic and electro-osmotic mobility on the electrolyte composition, especially the pH, dictates the use of buffered systems. It is imperative that various factors are considered prior to the selection of the electrolyte. Such factors include the solubility, stability and the degree of ionisation of the analyte in the electrolyte, and the influence of anions, cations, organic modifiers and other additives in the electrolyte on the electromigration of the solutes. In addition, the influence of pH and electrolyte conductivity on the generation of heat during the passage of current must be considered [113]. The selected buffer should exhibit a low UV absorbance at the detection wavelength, demonstrate a high buffer capacity over a broad pH range and show limited variation with temperature.

1.7.2.1 Electrolyte Composition

The majority of buffer systems have sufficient buffer capacity in only a limited pH range. It is therefore imperative that the pH at which the solutes are ionised is established prior to selecting the buffer. In accordance with the logarithmic definition of pH, the buffer capacity decreases by

a factor of ten for every pH unit relative to the pK_a of the compound. Therefore, effective buffering can be obtained within one pH unit of the buffering compound [113].

Of the wide variety of electrolyte systems that are utilised in CE, the majority are aqueous. Some are replicates of those used in conventional free flow or gel electrophoresis, while others have evolved empirically and have been employed for a specific separation [114]. Polybasic buffers such as phosphate and citrate have several pK_a values and can thus be used in different pH ranges (Table 1.1).

Table 1.1 pK_a Values for Some Commonly used Capillary Electrophoretic Buffers

BUFFER	pK_a VALUES
Phosphate	2.12; 7.21; 12.32
Citrate	3.06; 4.74
Formate	3.80
Succinate	4.19; 5.17
Acetate	4.75
MES	6.15
Tricine	8.15
Tris	8.30
Bicine	8.35
Borate	9.24

Biological and Good buffers such as tris-[hydroxymethyl]-aminomethane (Tris) and 1-2-[N-morpholino]-ethanesulfonic acid (MES) are particularly useful as they can be employed at high concentrations without generating significant heat due to their low conductivity. However, they exhibit strong UV absorbance which restricts their use. A beneficial characteristic of zwitterionic buffers such as N,N-bis-[2-hydroxyethyl]-glycine (bicine) and N-[tris-(hydroxymethyl)-methyl]-glycine (tricine) is their low conductivity which generates a low current draw, thus reducing the level of joule heat. This facilitates the application of high field strengths. Various studies [47, 60, 114, 115, 116] illustrated the effects of buffer anions, cations, current and hence, ion mobility and EOF on the migration time, resolution and selectivity of the separation. These studies emphasise the importance of careful buffer selection in minimising heat generation and producing optimal results.

Electrolytes that possess high buffer capacity simultaneously with low conductance are preferentially selected as they generate low current. This permits the application of high field strengths, which improves resolution [60, 115]. The mobility of the buffer constituents, which

determines the specific conductance of the electrolyte, should be considered in conjunction with the ionic strength of the buffer to avoid deterioration of the separation performance at high voltages.

VanOrman et al. [114] conducted a study that demonstrated the effect of buffer composition on EOF. It was found that the coefficient of the EOF (μ_{eo}) differed for each buffer and varied linearly with the natural logarithm of the molar concentration. These findings are consistent with results published by Altria and Simpson [57] and imply that the electrolyte selected is critical to the magnitude of the EOF and hence the quality of the separation. In addition, VanOrman and co-workers concluded that in the absence of any buffer-analyte interaction, the EOF is identical in various buffer types provided that the buffers are normalised to the same ionic strength. In a comparative study on the influence of sodium phosphate and potassium phosphate on the separation of nine dansylamino acids performed by Issaq et al. [117], it was found that resolution and selectivity improved and that shorter analysis times were achieved with sodium phosphate. Furthermore, this study clearly demonstrates the discrepancy in selectivity, resolution and migration time when different buffer salts at various concentrations are used. It is therefore imperative that the electrolyte preparation is clearly described and that the electrolyte is consistently prepared [113, 117].

Further studies were conducted by Atamna and co-workers [115] on the role of the buffer anions on the current level, electro-osmotic mobility, migration time, resolution and selectivity. It was found that the current generated at constant applied voltage differed widely depending on the anion used. Therefore, it is critical that electrolytes of low conductance are selected, as they generate the lowest current and consequently joule heat at a given applied voltage. The EOF was found to remain fairly constant in different buffer systems and changed by less than 10 %. However, large differences in migration times were noted for different buffers. This suggests that the selection of the buffer anion can be a possible means of enhancing selectivity and resolution in CE separations.

The size of the buffer cation influences the level of current generated and the resulting migration order of the analytes [60, 113, 118]. The influence of the nature of the cation on the EOF and thus resolution and the current generated was studied by Atamna et al. [118]. A linear relationship exists between the migration time and the cation size. According to the Debye-Huckel theory [50], the migration time increases with a reduction in charge density, thus resulting in a linear dependence of the electrophoretic velocity on charge density [118].

The nature of the buffer determines the equilibrium behaviour between the silica surface and the buffer. In a study conducted by Tran et al. [116], improved resolution was attained when

phosphate was selected over acetate or sulphate as the buffer of choice. This is explained in terms of the stronger interactions between the phosphate ions and the silica surface of the capillary, as suggested by McCormick [62]. Phosphate buffers are known to show slow equilibrium behaviour as the phosphate migrates into and out of the bulk silica, thus continuously changing the zeta potential of the wall as long as migration occurs. It is therefore advisable to allow for an equilibration time of at least four hours if highly reproducible results are to be obtained [116].

1.7.2.2 Electrolyte pH

The pH value of the electrolyte solution is an important parameter that can be manipulated to optimise separation in CE. The separation of compounds of a comparable nature is effected by charge differentiation, which is induced by adjusting the pH to close proximity of the pK_a value of the analyte of interest [119]. The electrophoretic mobility of the analyte is characterised by its charge-mass ratio, as depicted in Equation 1.2. The size of the ion is ascertained from its molecular mass and extent of hydration. The degree of ionisation of the solute in the electrophoretic solution regulates the charge of the ion, and is dependent on the pH of the medium and the dissociation constant of the solute. Terabe et al. [119] postulated that the optimal pH for the separation of compounds of comparable pK_a values was equal to pK_a - log 2.

The net charge on the ions of weak acids or bases is highly influenced by pH and necessitates careful and accurate preparation of the buffer. Buffer dilution should be avoided as it shifts the pH to neutrality since water is both a weak acid and base. It thus alters the inter-ionic forces and increases the activity coefficient [113]. The effect of pH on electro-osmotic mobility is observed to be substantially more prominent than the effect of buffer concentration, as illustrated by the five-fold change in ionic strength that is necessary to achieve a pH change of one unit [119].

1.7.2.3 Electrolyte Ionic Strength

Ionic strength is a parameter that can be utilised to optimise efficiency, selectivity and resolution. Numerous reports that focus on the dependence of mobility on buffer concentration have been published [56, 57, 60, 120, 121]. Wieme [50] predicted that electro-osmotic and electrophoretic mobility are both directly related to the reciprocal of the square root of the ionic strength. However, Altria and Simpson [56, 57] observed that an inverse relationship exists between the mobility and buffer concentration and that a plot of the logarithm of buffer concentration versus mobility is linear. Similar conclusions were drawn by Bruin et al. [120] and Nashabeh and El Rassi [121]. These observations contradict the theory predicted by Wieme [50] and theoretical justifications for the plotting procedure employed were not provided. Issaq et al. [60] derived an

expression for the electrophoretic mobility based on ionic strength (Equation 1.18). This expression is in qualitative agreement with Wieme's prediction [50].

$$\mu_{eo}/\mu_{ep} = \frac{e}{3\times 10^{7} Z\eta\sqrt{C}}$$

e = *total excess charge in solution*
Z = *number of valence electrons*
C = *molar concentration of solute (M)*
η = *Newtonian viscosity (Pa. s)*
μ_{ep} = *electrophoretic mobility* ($cm^2.V^{-1}.s^{-1}$)
μ_{eo} = *electro-osmotic mobility* ($cm^2.V^{-1}.s^{-1}$) *1.18*

Studies performed by Issaq et al [60] confirmed the above relationship. Plots of electrophoretic mobility versus the reciprocal of the square root of buffer concentration were linear. This implies that the migration time of analytes increases and the separation factor improves with increasing electrolyte concentration. The linear dependence of migration time on the square root of buffer concentration suggests that the migration time will double for every four-fold increase in buffer concentration [61]. Research conducted by Saloman et al. [66] demonstrated that the electro-osmotic mobility is proportionally dependent on both the double layer thickness and charge density at the silica surface.

At high ionic strength the magnitude of the zeta potential is lowered as the negative charges on the silica wall are effectively neutralised by the presence of counter-ions. This results in a decrease of the surface charge per unit area at the capillary wall buffer interface and, consequently, a decrease in the EOF. The equation below (Equation 1.19), described by Cohen et al. [49], illustrates the interrelationship between the current generated and several parameters.

$$I = \frac{r^2 \pi \varepsilon E C \zeta}{\eta}$$

I = *electrical current*
C = *molar concentration of solute (M)*
r = *capillary radius (cm)*
E = *electric field strength* ($V.cm^{-1}$)
ζ = *zeta potential (V)*
ϵ = *dielectric constant* ($F.m^{-1}$)
η = *Newtonian viscosity (Pa. s)* ***1.19***

There are many reasons that support the use of high electrolyte concentrations, but the effects of such high concentrations must be examined in the context of the separation, prior to selection of the experimental conditions. The primary step in the optimisation of any separation system is selecting the correct pH, after which the mildest values of buffer concentration, applied voltage and temperature should be ascertained. Ohm's Law and a power plot should be investigated as they provide a convenient way of determining the ionic strength limit of the system.

1.7.2.4 Electrolyte Additives

The versatility of CE can be realised by the incorporation of additives into the electrolyte solution. The additives effect different separation mechanisms and enhance the selectivity of the system. Electrolyte modifiers improve reproducibility, peak shape, resolution and detector sensitivity, possibly through the suppression of analyte-wall interactions [100]. In addition, they facilitate the separation of both chiral and non-chiral compounds.

1.7.2.4.1 Organic Solvents

The incorporation of organic solvents into the electrolyte solution broadens the applicability of CE to a wide range of slightly polar and non-polar compounds. Aqueous-organic electrolytes are considered to be particularly suitable for the analysis of compounds which demonstrate limited solubility, as the organic solvent enhances analyte solubility [122, 123]. Furthermore, organic solvents facilitate the separation of compounds that exhibit very similar electrophoretic mobilities in aqueous buffers [122].

Multifarious effects are observed with the inclusion of organic modifiers into the electrolyte solution due to modifications in the physicochemical, electrochemical and chemical properties of both the electrolyte and analyte [124]. The ranges of dielectric constant, polarity, density, viscosity and acid-base chemistry that are available in the presence of organic modifiers are extended, thus diversifying the applications of CE [124]. In addition, the analyte solubility and pK_a value, and electrolyte pH, viscosity and ionic strength are all altered. Consequently, the EOF and ion mobility are modified and result in an adjustment of the selectivity of the system [58, 66, 114, 116].

The mechanisms by which the organic solvents induce such transformations are not clearly understood. The EOF in organic-aqueous electrolytes is attributed to the dissociation of the silanol groups and specific adsorbance of the organic molecules onto the silica surface [69]. When water miscible organic solvents are added to the electrolyte in increasing proportions, the viscosity of the resultant solution increases and the dielectric constant decreases. These changes combine to reduce the zeta potential and thus the EOF [69].

The increase in the electrolyte viscosity with increasing concentrations of organic solvents cannot alone be accountable for the reduction in the EOF. If this were so, the product of EOF and viscosity would be constant, but in effect, it decreases with increasing concentrations of methanol [69]. In addition, VanOrman et al. [114] demonstrated that the viscosity of 1% (v/v) solutions of various organic solvents were found not to be significantly different from the viscosity of the bulk

water. Organic modifiers are capable of altering the migration order of analytes which further proves that the viscosity and ionic strength are not solely responsible for the apparent changes [125].

The application of non-aqueous media in CE is virtually an unexplored area. Walbroehl and Jorgenson [123] were the first to exploit pure non-aqueous media in examining acetonitrile for the separation of quinolones. Most published studies have looked at mixtures of aqueous-organic systems. Various theories [69, 126,127] have been published to explain the possible reasons for the observations encountered on the addition of organic solvents to the electrolyte. Salomen et al. [126] stated that the initial charge at the capillary wall increases in the presence of organic solvents as the total number of ionised silica groups at the capillary surface is elevated. This results as the charged ions are shielded from each other, thus minimising repulsion between the silanol groups. The decreased repulsion, inturn, permits more ions to exist in a given area [126]. Consequently, the zeta potential and EOF should increase. However, shrinkage of the compact layer adjacent to the capillary buffer interface, as a result of the enhanced solvation of the buffer ions, and the increase in buffer viscosity, reduces the EOF. Saloman et al.[126] therefore concluded that the shrinkage of the compact layer and the increase in electrolyte viscosity are more prominent and resulted in an overall suppression of the EOF.

The above explanation of the effect of organic modifiers on the separation, as proposed by Saloman and co-workers [126], contradicts that submitted by VanOrman et al [114]. VanOrman and co-workers [114] suggested that alcohols interact with the capillary wall and that the extent of the interaction is dependent on the carbon chain length of the alcohol. This interaction results in a higher apparent concentration and, thus, viscosity in the double layer. The increased viscosity causes a decrease in EOF. Furthermore, organic modifiers mask the surface charge by substituting the water molecules adsorbed onto the silanol surface, thus hindering the dissociative and non-dissociative adsorption of hydroxide ions. Their lower ability to stabilise the anions via hydrogen-bonding when compared to water, decreases the number of adsorbed hydroxyl ions. VanOrman et al. [114] concluded that the various extents to which the EOF is reduced in the presence of organic modifiers that possess the same bulk viscosity is dependent on the strength of interaction of the alcohol with the capillary wall.

Experimental work conducted by Janini et al. [128] on the effect of various organic solvents on the separation contradicts the conclusions drawn by VanOrman and co-workers [114]. Janini et al. [128] illustrated that regardless of the nature or percentage of the modifier, the EOF was constant in various electrolytes provided that the viscosities were all identical. This implies that viscosity is a major parameter affecting the EOF. However, the electrophoretic mobility is altered by the type and proportion of the organic solvent in such a manner that the higher the percentage of modifier, the greater the relative migration time difference.

The pK_a of the silanol groups on the capillary wall is shifted to higher values with increasing proportions of organic solvents due to the inability of the solvent to solvate the anionic silica surface [69]. The reduction of the negative surface charge, in conjunction with the steep decline of potential within the Stern layer, and change in the dielectric properties of the double layer in the aqueous-organic system, all culminate in the lowering of the zeta potential [69], and a consequent decrease in EOF. An almost linear decrease in zeta potential with increasing percentages of organic solvent is apparent [69].

Mixed aqueous-organic solvents facilitate the ionisation of solutes that are not easily ionised in water and extend the range over which the acidity and basicity of the buffer can be manipulated. Solvents that possess large dielectric constants or those with good proton donating and accepting properties are most appropriate as electrolytes [129]. The dielectric constants of the electrolyte declines almost linearly with incremental amounts of organic solvents [69], thus reducing the degree of ionisation of analytes. As a result the ionic charge, and subsequently, the electrophoretic mobility are reduced [69]. This results in a reduction in system efficiency as the plate numbers are dependent on analyte charge. In organic-aqueous solvents appreciable changes in selectivity are apparent.

1.7.2.4.2 Polymers

Diols, polyols and polymers such as hydroxycellulose (HC), hydroxypropylmethyl cellulose (HPMC) and polyvinyl alcohols (PVOH) are incorporated into the electrolyte at low concentrations (less than 1% w/v). They increase the electrolyte viscosity near the capillary wall and in the bulk solution, while shielding the silanol groups on the capillary and preventing undesirable adsorption. In this manner they decrease the EOF and improve separation efficiency [130]. If polymers are incorporated at a percentage greater than 0.1% (w/v), they form either a linear or entangled network. The network facilitates separation through a molecular sieving action by retarding the analytes to different extents [130].

1.7.2.4.3 Inclusion Complexes

Inclusion complexation enhances selectivity by facilitating the formation of highly selective complexes between the analyte (guest compound) and cavity of the chiral selector (host compound). Cyclodextrins (CD's) and crown ethers are particularly useful as complexing agents that extend the applicability of CE to enantioseparations. Resolution is dependent on the differences in the stability of the inclusion complex. This is governed by Van der Waal interactions, solvation effects, hydrophobic interactions and hydrogen-bonding and the size and shape of the molecules.

Cyclodextrins are neutral cyclic oligosaccharides composed of either six, seven or eight glucopyranose units which corresponds to the names α, β and γ cyclodextrins. They form a hollow truncated cone with a hydrophobic cavity of fixed diameter that is determined by the number of glucose units. The circumference is hydrophilic due to the presence of hydroxyl groups. Selectivity is attained through the differential inclusion of the hydrophobic portion of the analyte into the cavity and through the formation of hydrogen bonds with the chiral hydroxyl moieties.

The low solubility of β CD, in comparison to those of α and γ CD's, is problematic when high concentrations are required. The low solubility may be overcome by including organic solvents or urea (up to 8 M) [131] to enhance the solubility or by using derivatives of β CD such as methylated or hydroxyalkylated β CD. In addition to those parameters that alter selectivity in open tubular CZE, the formation and stability of the inclusion complex is influenced by several experimental parameters such as the type, charge and concentration of the CD and the analyte shape.

A second class of compounds that is capable of enhancing resolution through the inclusion - complexation mechanism are crown ethers. These macrocyclic polyethers contain electron-donating heteroatoms that are capable of forming weak dipole-dipole interactions with guest compounds within the cavity. Crown ether complexes are formed with a wide variety of organic and inorganic compounds in which the hydrophilic portion of the analyte is included within the cavity. The stability of the complex depends on matching the analyte diameter to the dimensions of the cavity.

The synthesis of enantioselective crown ethers facilitates the separation of enantiomers. The chemical structure of the analyte in conjunction with the pH of the electrolyte, organic additives and buffer composition is critical for chiral recognition.

1.7.3 Injection Conditions

The inherent high efficiency capabilities of CE can be preserved by ensuring that the injection system that is employed does not introduce significant zone broadening. The ease with which CE systems can be overloaded necessitates the use of an injection method that is capable of delivering infinitely small volumes (typically nanolitres) into the capillary, efficiently and reproducibly [94, 95, 98]. This is accomplished by utilising direct on-capillary injection methods such as electromigration [5, 132] and hydrodynamic flow [132, 133] in which one end of the capillary fulfills the role of a sample injector and eliminates the need for sample injection valves and, consequently, minimises zone broadening.

System efficiency and resolution deteriorate with sample overloading. The maximum efficiency of the system is restricted to a value that is proportional to the square of the ratio of the volume of the injected sample to the volume of the capillary (Equation 1.20) [95]. Subsequently, system efficiency can be enhanced by decreasing the injection volume or by increasing the length of the capillary.

$$N_{max} = 12[\frac{Q_c}{Q_{inj}}]^2 \qquad 1.20$$

N_{max} = *maximum number of theoretical plates*
Q_c = *total volume of capillary (nl)*
Q_{inj} = *injected volume (nl)*

In CE, the minute volume of sample injected to achieve high resolution and efficiency raises the detection limit. The injection plug length should be less than 1 - 2 % of the total capillary length. This corresponds to a few millimetres.

Conductivity differences between the sample and electrophoretic medium must be minimal to avoid electrophoretic dispersion and consequent peak distortion [90, 95]. These effects degrade system efficiency. Peak distortion can be controlled by ensuring that the conductivity of the sample solvent does not exceed 0.5 % of that of the medium [95]. This can be accomplished by decreasing the concentration of the injected sample or increasing the concentration of the electrolyte. Increasing the ion concentration of the electrolyte is detrimental to the separation, especially if the excess joule heat generated is not efficiently dissipated. Thus, in preserving system efficiency, the detection limit is raised as the volume of sample injected is minute and the concentration of the sample should be low compared to the buffer concentration [8, 95]. Conversely, sample stacking and improved peak shapes may be achieved if the sample conductivity is slightly lower than that of the buffer.

1.7.3.1 Injection methods

Quantitative sample injections can be performed by several methods of which hydrodynamic and electrokinetic injections are the most prevalent. The volume of sample loaded onto the capillary by either method is an unknown quantity. It can be calculated from the quantifiable parameters of pressure and time for hydrodynamic injections or voltage and time for electrokinetic injections. The sample introductory method that is employed influences the quantity and reproducibility of sample injection and hence determines the separation efficiency [132].

1.7.3.1.1 Hydrodynamic Injections

Hydrodynamic injection is accomplished either by the application of pressure at the injection end of the capillary, creation of a vacuum at the detector end or by hydrostatic pressure by elevating the anodic reservoir relative to the cathodic reservoir [134]. The hydrodynamic mode of injection is advantageous in that no inherent discrimination in the quantity of sample loaded, relative to the sample matrix, is apparent [14, 134]. The volume of sample injected is a linear function of both the injection pressure differential and the duration [132]. The Hages-Poiseuille equation, which describes the flow of liquid through a circular tube, is used to calculate the injection volume.

$$V_c = \frac{\Delta P \pi r^4 t}{8 \eta L_T}$$

V_c = *calculated injection volume* ($m^3 = 10^{12} nL$)
ΔP = *pressure difference (Pa)*
r = *capillary radius (m)*
t = *injection time (s)*
η = *Newtonian viscosity (Pa. s)*
L_T = *total length of the capillary (m)* *1.21*

Rose and Jorgenson [132] quantitatively studied hydrodynamic injection procedures using an automated sampling system to minimise operator error. The injection volume is dependent on the capillary dimensions and solution viscosity, which is a function of temperature. This necessitates precise temperature control of the capillary despite the small volume of the sample plug in comparison to the total volume of the capillary. Raising and lowering the sample vial during injection generates hydrodynamic pressure that results in an inadvertent injection of sample into the capillary. The amount of sample injected must be corrected for, by the time it takes to raise and lower the sample vial, if an accurate estimate is to be obtained [14]. Careful control of the compression and decompression system by use of an integrated pressure and time profile prevents irreproducible injection volumes. Inconsistent injection volumes arise from the application of instantaneous pressure to the sample vial and leads to uncontrolled up and down ramping of the actual pressure [135].

Precise control of the injection pressure in hydrodynamic injection is often difficult due to atmospheric conditions and elevation of the sample vial. This results in a lower level of precision [136]. Furthermore, degradation in system performance may occur with aging of equipment [136]. During hydrodynamic sample injection the leading edge of the flow profile resembles parabolic flow. This can potentially broaden the sample zone, but is limited by lateral diffusion of sample molecules across the capillary [132]. Siphoning effects must be eliminated by ensuring that the liquid levels of the sample and the cathodic reservoir are equal.

In hydrodynamic injection performed with gravity flow, a pressure differential is created by raising the anodic reservoir to a specific height. The volume of sample injected is calculated from the equation below [14]:

$$V_c = \frac{\rho g \Delta h \pi r^4 t}{8 \eta L_T}$$

V_c = *calculated injection volume* ($m^3 = 10^{12} nl$)
r = *capillary radius (m)*
t = *injection time (s)*
η = *Newtonian viscosity (Pa.s)*
L_T = *total length of the capillary (m)*
g = *gravitational acceleration* ($9.8\ N.Kg^{-1}$)
Δh = *height difference between liquid levels of sample and buffer vial*
ρ = *density of sample solution* ($Kg.m^3$) *1.22*

The injection height and duration are variables that directly influence the quantity of sample introduced [85]. The precision attained with gravity injections is superior to that of pressure injections as the need for accurate pressure control is eliminated. Furthermore, variables such as instrumental aging, atmospheric conditions and elevation do not affect the injection precision [136].

1.7.3.1.2 Electrokinetic Injection

Electrokinetic or electromigration injections require very little or no instrumentation besides that required to effect the separation itself [137]. Injection is performed by replacing the anodic buffer reservoir with the sample vial and briefly applying a voltage, which is usually 3 to 5 times lower than that used to effect the separation. The analytes migrate into the capillary by electromigration, which is a combination of both the electrophoretic migration of the charged ions and the pumping action of the EOF. The quantity of ions injected into the capillary is calculated as follows [47]:

$$Q = \frac{(\mu_{ep} + \mu_{eo}) V \pi r^2 C t}{L}$$

Q = *amount of ions injected into the capillary (M)*
C = *concentration of ions (M)*
t = *injection time (s)*
r = *capillary radius (m)*
μ_{eo} = *electro-osmotic mobility* ($cm^2.V^{-1}.s^{-1}$)
μ_{ep} = *electrophoretic mobility* ($cm^2.V^{-1}.s^{-1}$)
L = *total capillary length (m)*
V = *applied voltage (V)* *1.23*

Injection voltage and time are the variables that determine the quantity of sample injected. Equation 1.23 is only applicable when the conductivities of the sample solution and electrolyte are equivalent. Sample discrimination occurs for ionic solutes with the more mobile components being injected in greater quantities than the less mobile species [132, 138]. A term for the effective electrophoretic mobilities of the ions is incorporated in Equation 1.23 and thus sample discrimination is taken into account. The composition and amount of sample injected is dependent on a combination of factors. These include the EOF, electrophoretic attraction towards the electrode, electrical resistance of the medium, sample concentration, viscosity, pH, ionic charge of the solute, polarity, capillary dimensions and duration of the voltage applied [134].

The use of electrokinetic injection for quantitative analysis is limited by two forms of bias: mobility bias and conductivity bias, as discussed by Huang et al. [138]. Mobility bias is applicable to ions of opposite charge to that of the detection electrode [136], with the bias being proportional to the total mobility of each ion [138]. Ions with high mobility are loaded onto the capillary to a greater extent than less mobile ions, thus resulting in a distortion of the peak area ratios for the species [139]. Mobility bias can be corrected for if the ion mobility is known [137]. Conductivity bias can be minimised by selecting a lower sample ion concentration in comparison to the electrolyte concentration and by applying a separation voltage equivalent to that of the injection voltage [139, 140].

When electrokinetic injections are used variations are apparent in the quality and quantity of sample introduced over a period of time. The magnitude of the EOF, which is highly dependent on the capillary surface charge density, may fluctuate with time as the fused-silica undergoes modification due to ion adsorption or surface hydration. The EOF is a critical factor in controlling the quantity of sample introduced into the capillary and its magnitude is dependent on the buffer composition, ionic strength and pH [132]. Electropherograms obtained from repetitive electrokinetic injections from the same sample vial show a progressive decline in peak height and area as fewer ions are available for introduction into the capillary [141].

A further limitation of the electrokinetic sample introductory method is contamination of the sample by the formation of electrochemical reaction products as current flows through the sample solution [132, 141]. The electrokinetic mode of injection may be advantageous when velocity discrimination is necessary to enhance the selectivity, when viscous media are employed in the capillary and when hydrodynamic injection is ineffective [140].

1.7.3.1.3 Sample Stacking

Several techniques, based on differences in the field strength between the sample zone and electrolyte, have evolved to enhance detection sensitivity by on-capillary concentration during or shortly after sample injection. Stacking occurs when the conductivity of the injection sample plug is significantly lower than that of the surrounding electrolyte. This results in concentration enrichment of the analyte zone preceding separation without compromising the resolution [21, 104, 142]. The principle of stacking is attributed to the inverse relationship between the electric field and specific conductivity of the medium. The electric field in the sampling compartment is greater than that in the separation compartment due to the low concentration of ions and consequent low conductivity in that region [142, 143].

Upon the application of a voltage, a proportionally greater electric field will develop across the analyte zone. This greater electric field causes the rapid migration of ions until they reach the analyte-buffer boundary, where they slow down as the field strength decreases. Once all the ions reach the boundary, the analyte zone becomes concentrated into a narrow band with a homogeneous field and normal electrophoresis proceeds. The degree of stacking is proportional to the ratio of the concentration of the sample solvent to that of the electrolyte, provided they have a similar composition [142, 143]. The greater the concentration difference between the sample and separation compartment, the narrower the sample zone and the higher the sample peak. Both positive and negative species can be stacked into a narrow zone with the positive species stacking up in front of the sample plug and the negative ions at the rear. Neutral species are unaffected and co-migrate with the EOF [143]. Moring et al. [144] reported an increase by a factor of 10 in detectability when using sample stacking.

However, with increasing stacking power enhanced diffusion, which results in peak dispersion, becomes prominent due to hydrodynamic backflow. The pressure difference that arises from the mismatch in electro-osmotic velocity between the sample plug and bulk buffer results in a laminar flow profile, which broadens the sharp analyte zone and decreases the resolution [143]. This difference in EOF is magnified at high field strength and in sample solvents of higher conductivity than the electrolyte. Vinther and Søeberg [104] investigated the dispersive processes and found that moderate stacking conditions and applied voltage should be employed during the injection to minimise diffusion. Optimum conditions for sample stacking, as suggested by Burgi and Chien [143], are attained by dissolving the sample in a buffer of a concentration that is ten times more dilute than the running buffer and by injecting a plug length about ten times the diffusion limit peak width.

A balance between the length of the sample plug, concentration of the sample buffer, electrolyte concentration and resolution of the separation is crucial for on-capillary sample loading [143]. System efficiency and detector sensitivity are improved with stacking [86].

1.7.3.1.4 Field Amplified Sample Injection (FASI)

Field amplified sample injection (FASI) techniques have been investigated by several researchers [86, 88, 139, 145] as they lead to on-capillary enrichment of ionic analytes at the sample-buffer interface and permit the determination of low levels of sample (ng/ml) [140]. To perform FASI, the sample must be dissolved in a buffer of lower ionic strength than the electrolyte and must be injected electrokinetically onto the capillary. The low conductivity of the sample plug solvent results in the establishment of a high field strength at the injection point on the application of the injection voltage. Velocity is a product of electro-osmotic mobility and field strength. The field strength is enhanced several hundred-fold in FASI. This enhancement results in the rapid migration of sample ions into the capillary and their stacking at the sample-electrolyte interface.

Furthermore, the injection of a large number of ions into the capillary increases the sample concentration and local conductivity of the sample plug. This reduces the electric field strength in the sample compartment and amplifies the stacking effect by decreasing the velocity of the leading edge of the sample plug [88, 145]. The peak narrowing effect, that is due to sample stacking, facilitates the use of higher injection voltages or longer injection times. This narrowing effect enhances detection sensitivity without substantially broadening the peak due to the longer plug [88]. Field amplified sample injection therefore promotes on-capillary concentration in addition to introducing a larger quantity of ions into the capillary.

Effective charge discrimination is apparent when using direct FASI since only ions possessing a positive electrophoretic mobility, with respect to the EOF, will migrate into the capillary. This is because the electro-osmotic velocity is smaller in magnitude than the electrophoretic mobility. The concentration of either positive or negative ions in the capillary by direct sample injection under the enhanced electric field can be achieved by selecting the proper polarities of the electrode [142]. With reversed polarity, FASI with polarity switching (FAPSI) can be performed. However, the negative ions, that are drawn into the capillary by the high field strength will be pulled out of the capillary by the EOF. Therefore, for the concentration of negative ions the EOF must be eliminated [142].

In FASI a parabolic flow profile develops due to a mismatch in EOF between the sample and electrolyte region as described in section 1.7.3.1.3. A compromise between peak broadening and field enhancement in FASI is imperative and is achieved by selecting the appropriate sample buffer concentration, separation buffer concentration and plug length [145, 146].

1.7.3.1.5 Water Plug

The stacking effect of FASI can be further amplified by hydrodynamically injecting a plug of water into the capillary prior to sample introduction [86, 89, 142]. This results in a hundred-fold enhancement in the amount of ions injected without causing resolution to deteriorate [89]. In the absence of a water plug, the signal enhancement achieved with FASI may not be maximal, as the large number of ions that are propelled into the capillary increases the concentration within the sample zone. This increase in concentration within the sample zone causes a decrease in the electric field at the injection point and thus a deterioration in the field enhancement. The injection of a water plug ensures proper enhancement of the electric field at the injection point by providing a void region into which the sample can be injected [88, 142]. This is essential for the FASI of negative ions under reverse polarity, as the void provides a region within which the negative ions can be concentrated further from the injection point [88].

1.7.3.1.6 Extraneous Injection

Grushka and McCormick [147] demonstrated an additional source of zone broadening with the insertion, withdrawal or both actions of the capillary from the sample vial. Three mechanisms are responsible for such extraneous injections - convection movements between the buffer and sample zone due to differences in temperature, viscosity and surface tension; diffusion of analyte into the capillary and displacement of small volumes of analyte into the capillary during insertion into the sample vial. The latter effect, known as the zero injection effect, is caused by capillary action. The presence of residual analyte at the end of or on the outside of the capillary after sample injection can result in peak broadening or asymmetrical peaks as described by Lux et al. [148]. This can be minimised by simply dipping the capillary into a rinse buffer vial prior to the application of a separation voltage.

Variations in injection voltage and duration and sample conductivity for electrokinetic injection and injection pressure and time for hydrodynamic injection can be corrected for by incorporating an internal standard into the sample mixture [137]. Peak areas and heights and migration times should be reported relative to that of the internal standard.

1.7.3.1.7 Post injection

Post injection refers to the hydrodynamic introduction of a buffer plug after sample injection to improve reproducibility. On the application of the separation voltage to the capillary, a heat pulse is created. This heat pulse results in volume expansion of both the sample and buffer within the capillary. This expansion pushes the sample out of the capillary if it is located directly at the end of the capillary, and hence leads to irreproducible results, thus the need for a buffer plug.

1.7.4 Capillary Conditioning

Prior to using a capillary for the first time, a conditioning procedure should be performed to ensure that the silica surface is always identical. A typical wash sequence, as described by Lauer and McManigill [68], includes flushing the capillary with either 1 M NaOH or 1 M KOH for 20 min, followed by 0.1M NaOH for 45 min then water. The alkaline solutions are expected to be most effective in cleaning the capillary as they generate a maximum surface density of charged silanol groups by deprotonating them. Acid solutions, such as phosphoric acid, are used in conditioning, especially if the electrolyte solution is acidic. These solutions prevent hysteresis of the capillary wall charge created with base conditioning [149]. Hysteresis causes irreproducible EOF and necessitates long equilibrium times [149]. Nitric acid can be used in extreme cases to flush out analytes that are strongly adsorbed to the capillary surface.

Preconditioning between consecutive runs cleanses and equilibrates the capillary surface and ensures a consistent EOF, which is essential for high reproducibility. This step may consist of a single flush with buffer solution or may include several flushes with NaOH, water and buffer, depending on the nature of the analytes and their tendency to adsorb onto the capillary. It has also been found that preconditioning with only buffer solution produces the most reproducible conditions, as NaOH influences the status of the surface such that it is in a quasi-stable state and not in an equilibrium state. This is significantly more apparent when the electrolyte is phosphate, as it is known to adsorb onto the capillary surface and requires longer equilibration times. To minimise conditioning and maintain high reproducibility, it is advisable to dedicate an individual capillary only to a narrow pH range or for the use of specific buffers.

1.7.5 Electrolyte Replenishment

Buffer replacement is necessary in quantitative analysis as it guarantees high reproducibility. All conditions and parameters that influence the separation should be identical in every run. The replenishment system is primarily for the purpose of refreshing the buffer in the reservoir vials by simply exchanging the vial contents with fresh buffer. Chemical degradation of the buffer by electrolysis occurs during electrophoresis as a result of the application of the separation voltage. The decomposition of water leads to the production of protons at the anode. This increases the acidity of the solution in the anodic reservoir. Hydroxide ions are produced at the cathode and have the opposite effect. This alters the pH of the buffer and subsequently the EOF [150]. This effect is termed "buffer depletion" as the buffer capacity is exhausted after a number of runs [150]. The extent to which electrochemical by-products are produced depends on the analysis time and the current generated. The degree to which the pH is altered is determined by the buffer capacity, the volume of the reservoirs and whether the conditioning solutions from the capillary are flushed into the cathodic reservoir. Frequent buffer replenishment is therefore recommended.

1.7.6 Electrolyte Levelling

During injection and electrophoresis, siphoning from one reservoir to another occurs if the height of liquid in the two reservoirs is not at the same horizontal level. This results in a pressure difference between the two vials. The separation power of the CE system and reproducibility are adversely affected. The extent to which these factors deteriorate is dependent on the capillary dimensions and electrolyte viscosity. Naturally, the effect is amplified in short, wide bore capillaries. If the liquid level in the anodic reservoir is higher than that in the cathodic reservoir, the siphoning effect superimposes a laminar flow on the plug flow. This superimposition occurs in the same direction of the EOF and decreases the migration time. The reverse is also true.

Siphoning of a sample solution into the capillary occurs if the liquid level in the sample vial is elevated in comparison to that in the outlet vial, thus resulting in a larger sample loading and *vice versa*. These effects result in irreproducible sample loading, migration time and poor system efficiency.

CHAPTER TWO

MACROLIDE ANTIBIOTICS

2.1 ERYTHROMYCIN

2.1.1 Chemical Properties of Erythromycin

Discovery, Chemical Structure and Physical Properties

Erythromycin is a macrolide antibiotic that is produced by the actinomycete fungi species, *Streptomyces erythreus*. It was isolated in 1952 by McGuire et al. [151] in the Lilly Research Laboratory, USA, from a soil sample collected in the Phillipine Archipelago.

The chemical structure and stereochemistry of erythromycin was elucidated by the work of numerous researchers [152]. Figure 2.1 depicts the chemical structure of erythromycin A, which is the major component of erythromycin base. Erythromycin is a polyhydroxylactone that contains two sugars. The aglycone portion of the molecule, erythranolide, is a 14-membered lactone ring. An amino sugar, D-desosamine, is attached through a β-glycosidic linkage to the C 5 position of the lactone ring. The tertiary amine of desosamine confers a basic character to erythromycin. Through this group a number of acid salts of the antibiotic have been prepared. A second sugar, L-cladinose, which is unique to erythromycin, is attached via a β-glycosidic linkage to the C 3 position of the lactone ring [152].

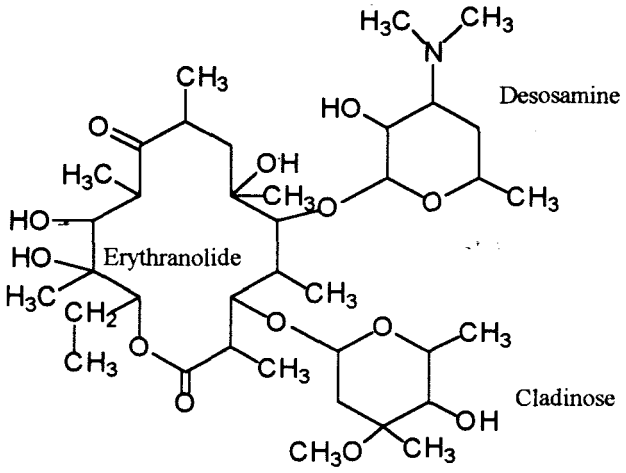


Figure 2.1 Chemical structure of erythromycin A.

Erythromycin base ($C_{37}H_{67}NO_{13}.H_2O$; MM 733.90) is an odourless, bitter tasting, white crystalline compound [153]. It is a weakly basic compound (pK_a 8.8), with a UV absorption maximum at 200 nm. At room temperature it exhibits poor water solubility (2 mg/ml), which decreases with a rise in temperature [154, 155]. It is freely soluble in alcohols, acetonitrile, acetone and chloroform [152, 153].

2.1.2 Biosynthesis of Erythromycin Base

Hung, Marks and Tardrew [156] proposed two alternate pathways for the biosynthesis of erythromycin from erythranolide A. One leads to erythromycin B and C via an intermediate and the other to erythromycin A and B via another intermediate.

2.1.3 Stability of Erythromycin

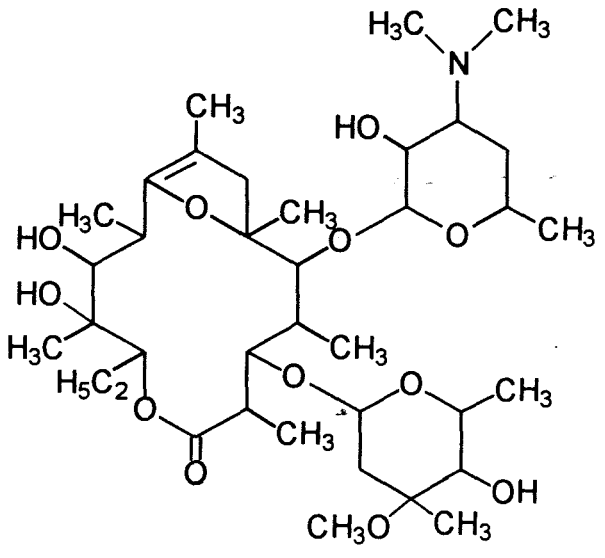
The poor stability of erythromycin in acid media is well-documented in numerous publications [157 - 165]. Until recently, the proposed mechanism of degradation was purported to follow one of two pathways, depending on the conditions of the reaction. Under mild acid conditions (glacial acetic acid) erythromycin enol ether (EEE) is formed via intra-molecular dehydration. The enol ether degrades further with subsequent acid treatment to yield a spiroketal form of erythromycin, anhydroerythromycin (AE). Under strong acid conditions (hydrochloric acid), only AE is formed (Figure 2.2) [159, 160, 162, 163, 166]. Cleavage of the glycosidic linkages have not been reported [162]. However, on further treatment of AE with acid, it is possible for a hydrolytic cleavage of AE to occur. This results in the loss of cladinose and two moles of water and the formation of erythralosamine [158, 159]. Erythralosamine possesses basic characteristics and cladinose is uncharged.

An alternative mechanism for the acid degradation of erythromycin, as proposed by Vinckier et al. [164], contradicts the above documented mechanism. Vinckier and co-workers suggested that the degradation of erythromycin is not unidirectional. It was proposed that an equilibrium exists between erythromycin and EEE, with a simultaneous pathway by which erythromycin degrades to AE, without forming EEE as an intermediate product [164, 165].

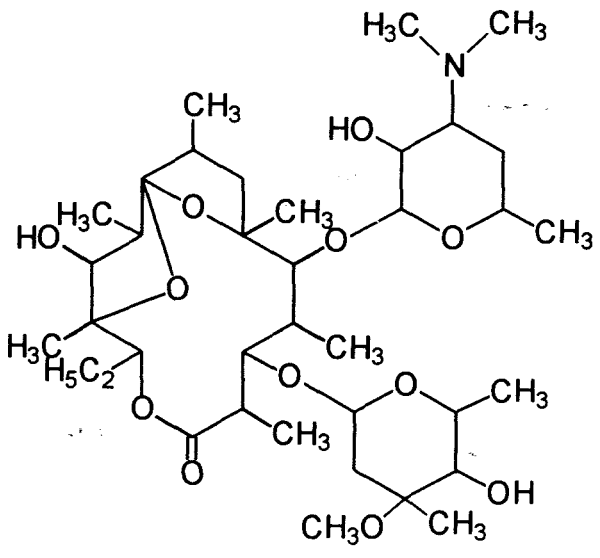
It has also been established that erythromycin is unstable in strong alkali solutions. Flynn et al. [158] and Waddell and Blizzard [167] reported that in alkaline media, erythromycin degraded to yield a zwitterionic product. This compound is polar and differs in composition from erythromycin by a molecule of water. The zwitterionic substance, known as dehydroerythromycin, contains an

acidic group with a pK_a of 4.3 and a basic function with a pK_a of 9.1 [158]. In addition, Van Den Mooter et al. [168] identified two further degradation products of erythromycin formed at pH values greater than 8; pseudoerythromycin A enol ether (psEAEN) and pseudoerythromycin A hemiketal (psEAHK) (Figure 2.3). These compounds are produced by (C_{13} - C_{11}) translactonization of erythromycin and result in the contraction of the macrocyclic lactone [163]. In a study conducted by Paesen et al. [169], it was found that psEAHK was the most prominent degradation product found at all alkaline pH values. The hydrolysis product, dihydroerythromycin, was found to be present in small quantities, while psEAEN was only detected in solutions of pH less than 9.

The rate of erythromycin degradation is primarily dependent on pH. Optimal stability is attained at pH values of between 7 and 8 [162, 170]. Furthermore, degradation is accelerated with a rise in temperature [162, 170] and in the presence of metal ions, particularly Al^{3+}, Fe^{3+} and Cu^{3+}. The stability of erythromycin and its esters in the organic solvents, acetonitrile and methanol, was investigated by Terespolsky and Kanfer [171]. Erythromycin was found to be stable in both methanol and acetonitrile, however the esters showed extensive degradation in methanol. The rate of degradation of the esters in acetonitrile was slower than in methanol and the degradation pathway was different from that in aqueous media and in methanol. Figures 2.2 and 2.3 represent the chemical structures of the acid degradation products, AE and EEE, and the alkaline degradation products dihydroerythromycin, psEAEN and psEAHK respectively.

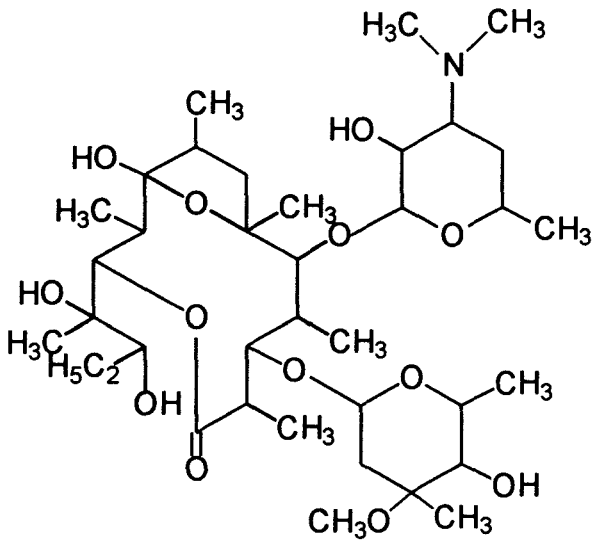


Erythromycin enol ether (EEE)

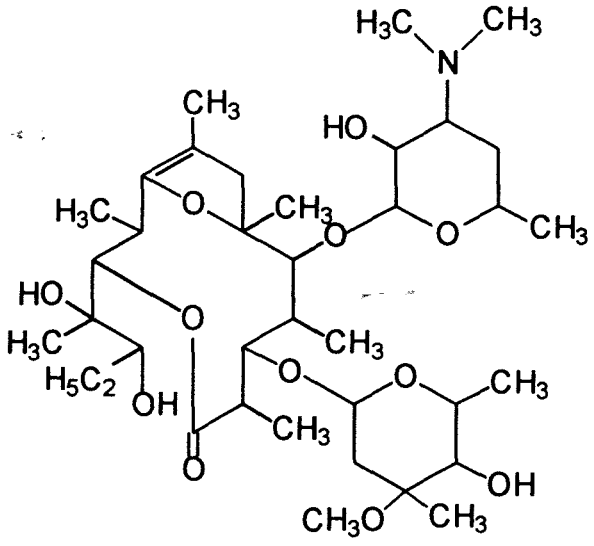


Anhydroerythromycin (AE)

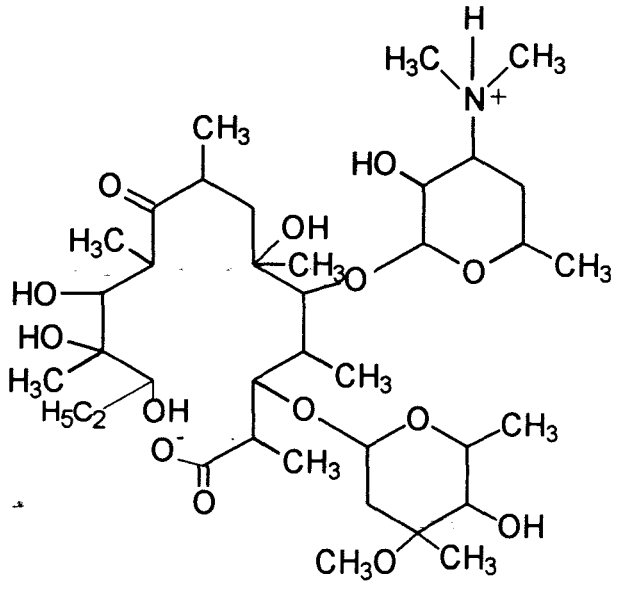
Figure 2.2 Chemical structures of the acid degradation products of erythromycin.



Pseudoerythromycin A hemiketal (psEAHK)



Pseudoerythromycin A enol ether (psEAEN)



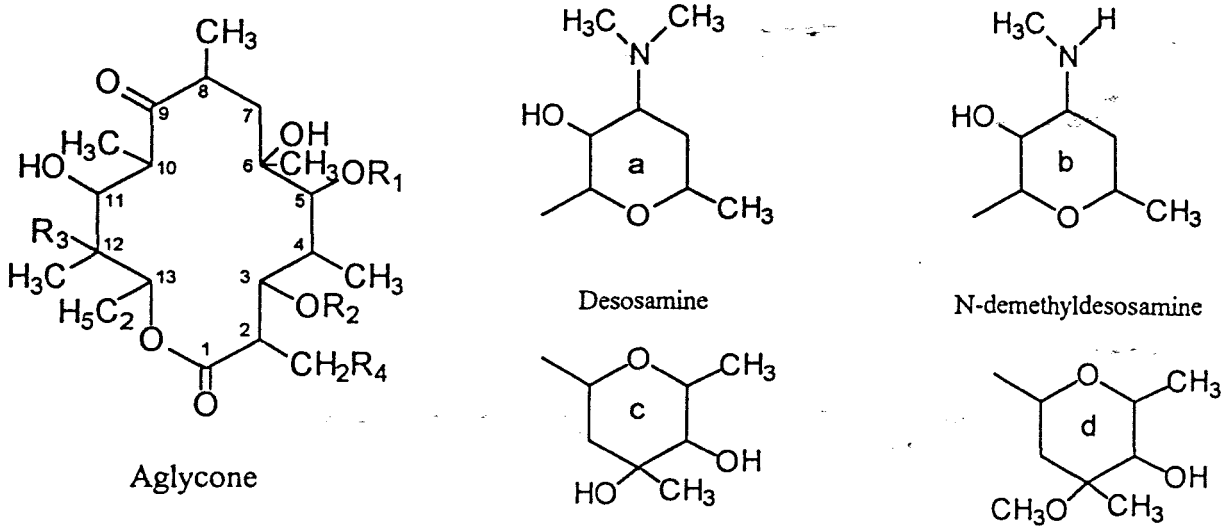
Dehydroerythromycin

Figure 2.3 Chemical structures of the alkaline degradation products of erythromycin.

2.1.4 Related Substances of Erythromycin

The fermentation process that is designed to produce commercial grade erythromycin is not entirely selective. It results in the production of small quantities of erythromycin B, C, D, E and F, in addition to erythromycin A, which is the major component [152]. Erythromycin B, C and E are the most important impurities found in commercial samples of erythromycin [172]. Erythromycin B and C possess some biological activity, while erythromycin E has low antibiotic activity [173]. In addition to the related substances, the metabolite, demethylerythromycin, and acidic and basic degradation products are also present in small quantities in commercial samples of erythromycin.

The structures of the related substances of erythromycin are illustrated in Figure 2.4. Erythromycin B differs from A by a hydrogen attached to the C 12 position. The sugar, L-cladinose, that is present in erythromycin A, is replaced by a non-methoxylated mycarose in erythromycin C [152]. The related substances are structurally very similar and differ only by hydrogen, hydroxyl and methoxy groups.



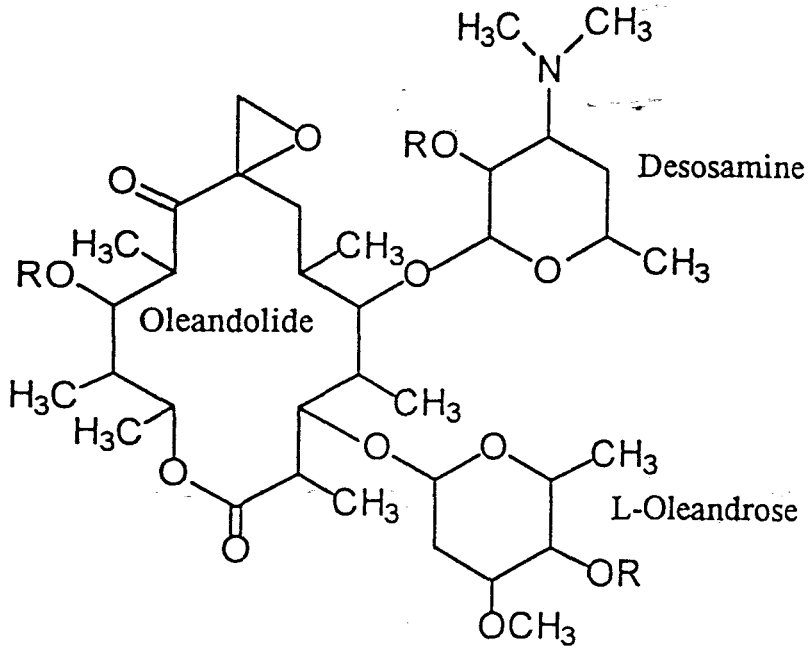
	R_1	R_2	R_3	R_4
Erythromycin A	a	d	OH	H
Erythromycin B	a	d	H	H
Erythromycin C	a	c	OH	H
Erythromycin D	a	c	H	H
Erythromycin E	a	d	OH	-O-
Erythromycin F	a	d	OH	OH
N-demethylerythromycin A	b	d	OH	H

Figure 2.4 Chemical structures of erythromycin and related substances.

2.2 OLEANDOMYCIN

Oleandomycin ($C_{35}H_{61}NO_{12}$; MM 688) has a similar structure to erythromycin. It consists of a 14-membered lactone ring, oleandolide. Two sugars, desosamine and L-oleandrose, are glycosidically attached to the lactone ring. The 14-membered ring has a complex structure and contains an exocyclic methylene epoxide on the C 8 position [166] (Figure 2.5). Oleandomycin is soluble in most organic solvents and sparingly soluble in water. It is a mildly basic compound (pK_a 8.7) and exhibits a UV absorption maximum at 200 nm.

Oleandomycin contains three hydroxyl groups that are susceptible to acylation. One hydroxyl group is found on each of the sugars and an additional hydroxyl group is present on the lactone ring. The triacetyl derivative of oleandomycin, troleandomycin ($C_{41}H_{67}NO_{15}$; MM 814), retains the antibiotic activity of the parent compound. It is practically insoluble in water and non-polar solvents and is freely soluble in alcohols.



Oleandomycin R = H

Troleandomycin R = $COCH_3$

Figure 2.5 Chemical structure of oleandomycin and troleandomycin.

2.3 JOSAMYCIN

Josamycin ($C_{42}H_{67}NO_{15}$; MM 827) is composed of a 16-membered lactone ring to which two sugar residues, mycaminose and iso-valeryl mycarose, are attached. The iso-valeryl mycarose moiety is attached via an α-glycosidic linkage to mycaminose. This in turn, is attached via a β-glycosidic linkage to the C 5 position of the lactone ring [174] (Figure 2.6). Josamycin is readily soluble in methanol, ethanol and acetonitrile and is sparingly soluble in water. The tertiary amino group at the C 3 position of the mycaminose moiety confers the mildly basic character to the molecule (pK_a values ranging from 6.7 to 7.1) [175, 176]. The diene system in the lactose ring is responsible for substantial UV absorption at 231nm.

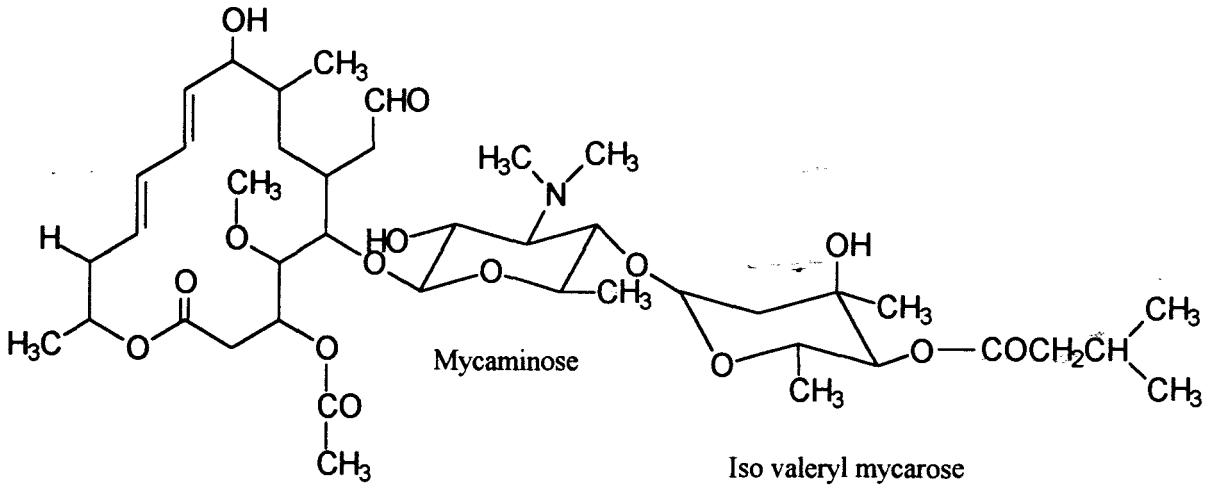


Figure 2.6 Chemical structure of josamycin.

CHAPTER THREE

ASSESSMENT OF EXPERIMENTAL VARIABLES

3.1 INTRODUCTION

A myriad of parameters can be optimised in CE to assist in achieving the desired selectivity, resolution and efficiency. Understanding the manipulative capabilities of these parameters offers a wide range of possibilities for selecting adequate conditions, thus highlighting the unlimited potential for the separation of pharmaceutical compounds [134].

The fundamental experimental parameters of applied voltage, capillary dimensions and buffer to solute concentration must all be balanced against one another in order to achieve the appropriate resolution, speed and sensitivity.

The variables in method development and optimisation in CE can be divided into three categories [177]:

System variables: capillary dimensions
temperature
applied voltage
injection system
method of detection

Sample variables: sample solution pH
solute concentration

Electrolyte variables: pH
ionic strength
electrolyte composition
buffer additives

These numerous variables exert their influence simultaneously on the separation, thus making optimisation difficult. The objective of this chapter is to examine these parameters for their effect on overall system performance for the separation and quantification of macrolide antibiotics.

3.2 Effect of pH

3.2.1 Instrumentation

3.2.1.1 Capillary Electrophoresis System

- Prince (4 tray) Electrophoresis System, Model 0500-001 (Lauerlabs, Emmen, The Netherlands).
- Butler Buffer Replenishing Device, Model 0500-001 (Lauerlabs, Emmen, The Netherlands).
- SSI 500 Variable UV-Vis Detector (Linear Instruments Corporation, Reno, NV, USA).
- Polyimide fused-silica capillary with modified Z-shaped flow cell, I.D. 75 µm, total length 81.6 cm, length from inlet to detector window 41.4 cm, pathlength 3 mm (LC Packings International, San Francisco, USA).

3.2.1.2 Additional Equipment

- Strip Chart Recorder, Model 561 (Perkin Elmer Corporation, Illinois, USA).
- Integrator, Model 3390A (Hewlett Packard, Avondale, Pennsylvania, USA).
- Electronic 5 figure Semi-microbalance, Type 2004 MP6 (Sartorius, Gottigen, Germany).
- Ultrasonic Bath, Model 8845-30 (Cole-Parker Instruments Company, Illinois, USA).
- Beckman 50 pH Meter (Beckman Instruments Inc, Fullerton, CA, USA).
- Beckman Electrode, 39849 (Beckman Instruments Inc, Fullerton, CA, USA).
- DU Series 60 Spectrophotometer (Beckman Instruments Inc, Fullerton, CA, USA).

3.2.2 Raw Materials and Reagents

3.2.2.1 Raw Materials

Erythromycin base [1]	Lot 84H0453
Josamycin base [2]	Lot JSML149
Oleandomycin phosphate [3]	Lot 406-767001B
Troleandomycin base [1]	Lot 58F0356

Sodium dodecyl sulphate [1]	Lot 42H0019
Cetyltrimethylammonium bromide [4]	85,582-0
Dodecyltrimethylammonium chloride [1]	Lot 64H02901
Heptakis(2,6-Di-O-methyl)- β - cyclodextrin [1]	Lot 52H0219
Gamma cyclodextrin [1]	Lot 52G0227

[1] Sigma Chemical Company, St Louis, MO, USA.
[2] Yamanouchi Pharmaceutical Company, Tokyo, Japan.
[3] Pfizer Pharmaceuticals Inc, Pietermaritzberg, SA.
[4] Aldrich Chemical Company, Milwaukee, WI, USA.

3.2.2.2 Reagents

All reagents utilised were of at least analytical grade quality.

Acetonitrile (distilled in glass UV grade) [1]
Methanol (HPLC grade) [2]
Ethanol (gradient grade for chromatography) [3]
Sodium dihydrogen phosphate monobasic ($NaH_2PO_4.H_2O$) [3]
Disodium hydrogen phosphate ($Na_2HPO_4.2H_2O$) [3]
Sodium hydroxide pellets (Batch 871009) [2]
Sodium tetraborate ($Na_2B_4O_7.10H_2O$) [3]

[1] Burdick & Jackson Division, Muskegon, MI, USA.
[2] British Drug House, Poole, Dorset, UK.
[3] Merck, Darmstadt, Germany.

Water of HPLC grade was used, purified by reverse osmosis through a Milli-Q purification system. (Millipore Corporation, Bedford, MA, USA).

3.2.3 Experimental Procedure

3.2.3.1 Treatment of Glassware

"A" grade glassware was used throughout the study. Prior to use, glassware was cleaned by soaking in a 0.02 % (v/v) Decon 75 concentrate (Atomic Export Company, SA) for 12 hours. The glassware was then thoroughly rinsed with tap water, followed by distilled water and finally HPLC grade water and was allowed to drip dry.

3.2.3.2 Preparation of Electrolyte

A phosphate buffer of required molarity and pH was prepared by carefully weighing the appropriate quantities of sodium dihydrogen phosphate and disodium hydrogen phosphate. The salts were transferred into a volumetric flask and were made up to volume with water. A 0.1 M sodium hydroxide solution was prepared by weighing 0.4 g of sodium hydroxide pellets into a 100 ml volumetric flask and making up to volume with water. This solution was used to adjust the pH to the required value.

The resultant buffer was deaerated under an aspirator vacuum and filtered through a 0.45 µm membrane filter (Type HVLP, Millipore Corporation, Bedford, MA, USA) prior to use. The buffer was filled into 4 ml standard inlet vials (Lauerlabs, Emmen, The Netherlands) using a 10 ml glass syringe. The vials were then capped with starburst snap caps (Lauerlabs, Emmen, The Netherlands). Buffer was filled into a 20 ml outlet vial (Lauerlabs, Emmen, The Netherlands), which replenished the cathodic reservoir. Fresh buffer was prepared on a daily basis. This method of electrolyte preparation was utilised throughout the investigations.

In the investigation of the effect of pH on separation of the macrolide antibiotics, a phosphate buffer (20 mM) was prepared at pH values ranging from 5 to 9, in increments of one unit.

3.2.3.3 Sample Preparation

Approximately 20 mg of each of erythromycin, josamycin and oleandomycin was accurately weighed into separate 20 ml volumetric flasks and made up to volume with methanol to yield a final concentration of 1 mg/ml. A mixture of the three macrolides in methanol was also prepared at a concentration of 1 mg/ml each. The sample was injected through a 0.45 µm membrane filter (Millipore Corporation, Bedford, MA, USA) into a 4 ml standard inlet vial, using a 10 ml glass syringe. The vial was sealed with a starburst snap cap to minimise evaporation losses and to prevent increases in sample concentration and irreproducible results.

3.2.3.4 Capillary Installation

The Z-shaped flow cell was securely mounted in the detector. The capillary tips were examined under a microscope to ensure that the edges were horizontal and cleanly cut. The coating at the capillary ends (0.5 cm) was carefully burnt away using a butane pencil torch (Thomas Quality Tools, Taiwan). This was performed to remove any jagged threads of polyimide and thus prevent irreproducible transfer of sample into the capillary. The end of the capillary at the anode was positioned approximately 3 mm above the base of the anode so that the electrode penetrated the septum of the vial cap first, thus facilitating ease of capillary entry and preventing damage to the fragile end.

3.2.3.5 Capillary Conditioning

Prior to use, new capillaries were flushed with 1.0 M NaOH, followed by 0.1 M NaOH and then water for 30 min each, using a pressure of 2000 mbar. Before use, the capillary was washed with 0.1 M NaOH for 10 min, water for 10 min and equilibrated with the operating buffer for 20 min. Between consecutive injections the capillary was rinsed with the operating buffer for 3 min. On completion of daily experimentation, the capillary was washed with 0.1 M NaOH and water for 10 min each. A pressure of 2000 mbar was programmed to flush the above liquids through the capillary.

3.2.3.6 Electrolyte Replenishment

The Butler system is capable of performing automated outlet buffer replenishment and exchange. It is controlled by the Prince and was programmed to replace the electrolyte in the cathode reservoir prior to sample injection at a flow rate of 3 ml/min for 1 minute. At the anodic end separate rinse and run buffer vials were placed in the sample tray. Rinsing was not performed from the separation buffer vial as the decrease in the liquid level in that vial would result in siphoning against the flow during electrophoresis. This could lead to a gradual decrease in migration time.

3.2.3.7 Electrolyte Levelling

The level of the anodic reservoir was programmed so that if the meniscus of the liquid in the vial was at the base of the vial neck, it would be horizontal with the liquid level in the outlet vial. This was performed to avoid siphoning during injection and electrophoresis. Visual alignment of solution levels in both the inlet and outlet vials was performed regularly. The horizontal position of the Prince was also adjusted with the adjustable feet at the frontside of the instrument, until a perfect horizontal level was indicated.

3.2.3.8 Temperature Settings

The section of the capillary located in the Prince is thermostated, while the remainder of the capillary is in contact with ambient temperature. In an attempt to maintain a constant temperature throughout the length of the capillary, a temperature setting of 25°C was selected, as this was in close proximity to that of the laboratory. The current was monitored throughout the experiment, as a constant electric current is indicative of a steady-state temperature.

3.2.3.9 Detector Settings

The UV absorbance spectra of erythromycin, josamycin and oleandomycin indicate that all compounds absorb strongly at low UV wavelengths, with λ_{max} of 200 nm, 231 nm and 200 nm respectively. A wavelength of 200 nm was selected at an attenuation of 0.1 Absorbance units full scale (AUFS) and a rise time of 0.3 seconds.

3.2.3.10 Injection and Separation Conditions

Prior to initiating each experimental sequence, two blank injections of sample solvent were performed to facilitate system equilibration. This ensured that the sample and electrolyte solutions in the sample tray attained a constant temperature. This is critical in achieving high precision. In addition, it facilitated equilibration of the silica surface and stabilisation of the electrolyte under electrophoretic conditions.

The mode of injection was electrokinetic; 5 kV was applied for a duration of 5 seconds. A separation voltage of 15 kV was applied to effect the separation. Controlled voltage up and down ramping for both electrokinetic injection and electrophoresis was programmed at 6 kV/s to improve the accuracy and precision of the injection and migration time. Three injections were performed from each of the macrolide sample solutions and also from the mixture of the combination.

The parameters and experimental conditions reported in the above Section 3.2.3 were preset for subsequent experiments, except when stated otherwise.

3.2.4 Results

The pH of the electrolyte was examined in the range from 5 to 9, to encompass the pK_a values of the three macrolides (erythromycin pK_a 8.8, josamycin pK_a 6.7 - 7.1, oleandomycin pK_a 8.7). The acidic region was avoided as macrolides are known to be acid-labile (Section 2.1.3). The migration time for each component was recorded and was used to calculate the electro-osmotic mobility (μ_{eo}) and electrophoretic mobility (μ_{eff}) using Equation 3.1 and 3.2 respectively:

$$\mu_{eo} = \frac{L_D L_T}{t_{eo} V}$$

L_T = *total length (cm)*
L_D = *length to detector (cm)*
t_{eo} = *migration time of neutral marker (s)*
V = *applied voltage (V)*

3.1

$$\mu_{eff} = \frac{L_D L_T}{V}\left[\frac{1}{t_{eo}} - \frac{1}{t}\right]$$

t = migration time of solute (s) 3.2

Figure 3.1 is a graphical representation of the relationship between the electrolyte pH and electrophoretic mobility and electro-osmotic mobility. The electrophoretic mobility of each component decreased with increasing pH, while the electro-osmotic mobility escalated relatively rapidly. The electro-osmotic mobility was significantly greater than the electrophoretic mobility as the experiments were conducted in uncoated fused-silica capillaries.

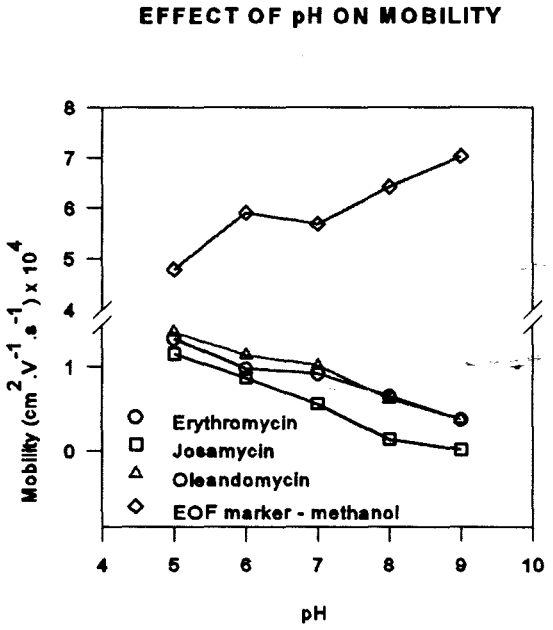


Figure 3.1 Variation of electrophoretic and electro-osmotic mobility with pH. Conditions: 20 mM phosphate buffer, pH 5 - 9, applied voltage 15 kV, injection 5 kV for 5 s. Capillary 75 μm I.D. x 81.6 cm (effective length 41.4 cm), pathlength 3 mm. Concentration of macrolides 1 mg/ml in methanol.

The effect of pH on resolution was determined from the electropherograms of the mixture of the three macrolides by applying Equation 1.14. Oleandomycin and erythromycin co-migrated over the entire pH range studied, as illustrated from the overlap of their electrophoretic mobilities. Josamycin migrated slightly after erythromycin as it possessed a lower electrophoretic mobility than erythromycin (Figure 3.1). Resolution improved with pH except that the separation of josamycin from the EOF marker, methanol, was not achieved at pH values greater than 8. The electrophoretic mobility of josamycin approached zero as the pH was increased and thus the ions co-migrated with the EOF marker. Resolution of the macrolides was found to be optimal at a pH of 7 under the above conditions.

3.2.5 Discussion

The selectivity of the separation is determined by the differences in the electrophoretic mobility of the sample ions (Equation 1.9). In CE, electrolyte pH is an important parameter for improving selectivity as it alters the degree of dissociation of the sample ions. However, the effect of pH on selectivity can only be determined if the relationship between the electrophoretic mobility and pH is known for the sample ions [177]. In addition, the pH of the electrolyte influences the ionic state of the silica capillary surface by altering the charge density [114, 149].

The increase in the EOF observed at alkaline pH (Figure 3.1), resulted from the enhanced surface charge density. This arose from the increased dissociation of silanol groups on the inner wall of the capillary and the higher concentration of dissociated ions in the electrolyte solution. As the pH was lowered, the excess hydrogen ions present in the electrolyte neutralised the anions at the capillary surface. This resulted in a lowering the zeta potential and consequently a decrease in the EOF [47, 149]. Thus, the electro-osmotic mobility varied with pH in a sigmoidal manner, changing significantly at pH values between 5 and 9.

The ionic equilibrium state of the analyte is largely dependent on pH, especially when it is close to the pK_a value of the analyte [69, 91]. From Equation 1.3, it can be deduced that the observed decrease in the electrophoretic mobility of the macrolides with increasing pH may be attributed to a decrease in the fraction of protonated species, assuming that the electrolyte viscosity and effective radius of the ion remain constant over the pH range. Differences in the degree of ionisation of the analytes confers variation in their mobility. This is crucial for attaining separation in CE.

The improvement in resolution of the macrolides with increasing pH can be explained in terms of the charge differentiation induced by adjusting the pH near the pK_a value of the compounds. The impairment of resolution at pH values greater than 8 arose from the increase in EOF and consequent decrease in separation space between the analytes. In addition, at these high pH values, the degree of ionisation of josamycin and thus its electrophoretic mobility was substantially reduced and resulted in co-elution with the EOF marker, methanol.

3.3 Effect of Applied Voltage

3.3.1 Experimental Procedure

The effect of applied voltage on mobility and resolution was studied in a range from 15 kV to 25 kV. The previously reported conditions for all other parameters were maintained as described in Section 3.2.3. A 20 mM phosphate buffer (pH 7) was prepared as reported in Section 3.2.3.2. The sample, consisting of a mixture of erythromycin and josamycin in methanol, each at a concentration of 1 mg/ml, was prepared as described in Section 3.2.3.3.

3.3.2 Results

An Ohm's plot of current verses voltage was constructed by varying the applied voltage incrementally while monitoring the current. It was found that the current doubled on increasing the applied voltage from 15 kV to 25 kV. The electro-osmotic mobility and electrophoretic mobility of each antibiotic increased linearly with applied voltage, as illustrated in Figure 3.2.

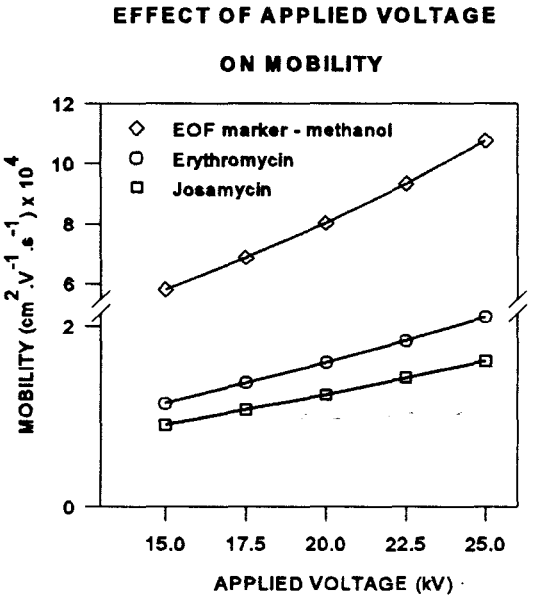


Figure 3.2 Variation of electrophoretic and electro-osmotic mobility with applied voltage. Conditions: 20 mM phosphate buffer, pH 7, applied voltage 15 - 25 kV. Other conditions as for Fig. 3.1.

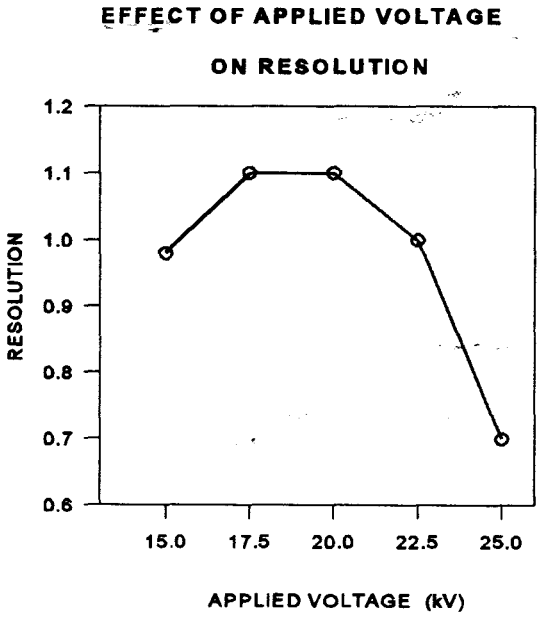


Figure 3.3 Relationship between applied voltage and resolution. Conditions as for Fig. 3.2.

The plot of resolution as a function of applied voltage is depicted in Figure 3.3. It can be observed that resolution improved with applied voltage to a maximum at 20 kV. Thereafter it deteriorated. An applied voltage of 17.5 kV was selected for the analysis of the macrolides. At

this voltage high efficiency, improved resolution and short migration times were achieved without the generation of detrimental amounts of heat.

3.3.3 Discussion

The effect of applied voltage on solute mobility and the separation parameters in CZE has been investigated by several groups [17, 57, 58, 120]. The voltage applied across the length of a capillary is the driving force behind the migration of ions in the electrophoretic medium. The optimal voltage for the separation of the macrolides in a 20mM phosphate buffer (pH 7) was determined empirically by performing an Ohm's plot. According to Ohm's Law, the plot should be linear. However, at high potential differences (22.5 kV), slight negative deviations emanating from the disproportionate generation of joule heat were apparent.

The effect of applied voltage on the electrophoretic and electro-osmotic mobility is qualitatively similar as seen from Figure 3.2. The linear correlation between the electro-osmotic and electrophoretic mobility with field strength is attributed to the stronger driving force that the ions are subjected to on increasing the applied voltage. This resulted in reduced migration time. However, at high voltages (22.5 kV) a positive deviation in the electrophoretic and electro-osmotic mobility was apparent as a result of thermal contributions to solute mobility, despite a constant field strength. A possible explanation for this increase in solute mobility at high voltages is the lowering of electrolyte viscosity that results from a rise in the temperature within the capillary. The expressions for the electrophoretic and electro-osmotic mobility (Equation 1.2 and 1.8) comprise the viscosity term in the denominator, thus both parameters are affected similarly.

Resolution improved with applied voltages up to 20 kV, beyond which it deteriorated due to the inability of the system to efficiently remove the excessive joule heat that was generated. The square root relationship between applied voltage and resolution (Equation 1.16) implies that in order to double the resolution the voltage must be quadrupled. This has serious limitations in terms of the production of joule heat. Excessive heat generation induces perturbations in the sample zone during the separation process. This results in zone broadening, thereby limiting the benefits gained from this action. Precise temperature control is essential for implementing high field strengths, as the buffer conductivity and density, solute diffusion coefficient and double layer thickness are all temperature-dependent parameters [50].

In principle, system efficiency can only be influenced by manipulating the applied voltage since mobility and diffusion coefficients are intrinsic properties of the solute. This is depicted in Equation 1.12. Theoretically, it can be deduced that the highest separation efficiencies are attainable by subjecting the capillary to a maximum possible field strength. Under these

conditions the analytes spend minimal time in the capillary and thus zone broadening due to diffusion is restricted. However, in reality, plate numbers are proportional to applied voltage for only lower values of field strength and are limited by electrical heating within the capillary. Efficiency approached a maximum as the applied voltage was increased (20 kV) and then dropped as the voltage was further increased. The electrolyte concentration and capillary dimensions determine the magnitude at which the efficiency of the system plateaus.

The direct relationship between efficiency and EOF, as illustrated in Equation 1.12, is misleading as it implies that efficiency can be improved by increasing the EOF. However, an increase in EOF reduces the analysis time and sharpens the peaks, but resolution deteriorates.

3.4 Effect of Ionic Strength

3.4.1 Experimental Procedure

In order to evaluate the effect of electrolyte ionic strength on the separation of the macrolides, phosphate buffers of molarity ranging from 10 mM to 50 mM (pH 7) were prepared as reported in Section 3.2.3.2. The injected sample comprised a mixture of erythromycin and josamycin, each at a concentration of 1 mg/ml. The sample was prepared as described in Section 3.2.3.3 and the experimental parameters selected were as specified in Section 3.2.3.

3.4.2 Results

A graphical representation of the relationship between the ionic strength and mobility is illustrated in Figure 3.4. The electro-osmotic mobility decreased with increasing electrolyte concentration, while the electrophoretic mobilities remained invariant over the concentration range studied.

Resolution improved rapidly with increasing electrolyte molarity, as illustrated in Figure 3.5. On increasing the ionic strength from 10 mM to 50 mM the current was found to increase by approximately five - fold.

The increase in peak height of both josamycin and erythromycin with high molarity electrolytes and the improvement in peak shape can be attributed to sample stacking that arose from the enhanced difference in conductivity between the sample zone and electrolyte solution.

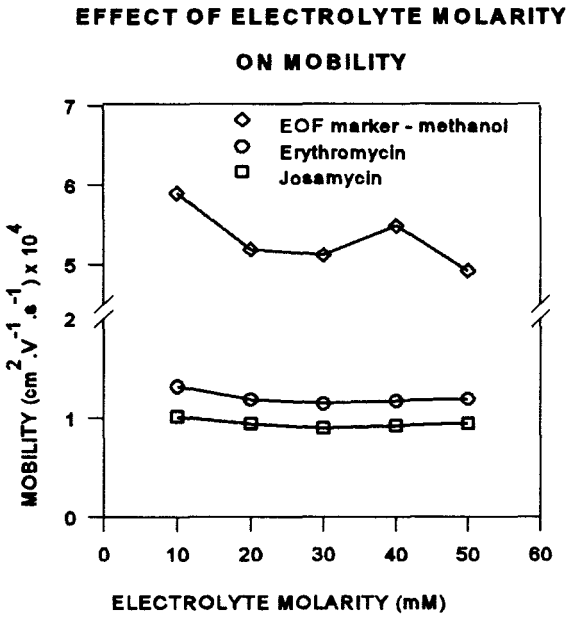


Figure 3.4 Variation of electrophoretic and electro-osmotic mobility with electrolyte molarity. Conditions: phosphate buffer 10 - 50 mM, pH 7, applied voltage 17.5 kV. Other conditions as for Fig. 3.1.

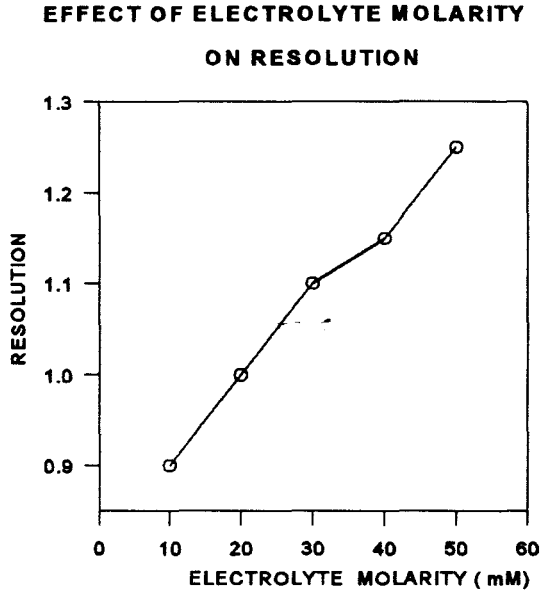


Figure 3.5. Relationship between resolution and electrolyte molarity. Conditions as for Fig. 3.4.

3.4.3 Discussion

Several researchers have reported data on the effect of electrolyte concentration on mobility [56, 57, 120, 121]. The inverse relationship between the electro-osmotic mobility and electrolyte molarity, that was derived by Issaq et al. [60] (Equation 1.18), was observed empirically in this study (Figure 3.4). The decrease in the zeta potential with increasing electrolyte concentration is well documented, however the exact relationship between the electro-osmotic mobility and electrolyte concentration has not been derived. Tsuda et al. [58] linked the reduction in the zeta potential and hence EOF with increasing electrolyte concentration to a decrease in the thickness of the electrical double layer. The thickness of the double layer is inversely proportional to the square root of the electrolyte concentration (Equation 1.7) [60]. This observation can be explained as follows: the negative charges on the silica surface are neutralised to a greater extent in the presence of high molarity electrolytes due to the abundance of counter-ions present in the electrolyte. This causes a contraction of the electrical double layer.

Salomon et al. [126] derived a second relationship between the electro-osmotic mobility and electrolyte concentration. In this model the decrease in the electro-osmotic mobility is linked to both a contraction of the electrical double layer and a reduction of the surface charge per unit area, due to the increased coverage of the sites on the silica wall. The zeta potential and EOF

are subsequently both reduced. An increase in the production of joule heat at high ionic strengths warrants effective temperature control. This is essential if the advantages of employing high molarity electrolytes are to be realised.

The effective ionic charge of the macrolide ions was reduced in the presence of high molarity electrolytes. This effect is due to the compression of the electrical double layer surrounding the macrolide ions in the presence of excess counter-ions. However, this reduction of ionic charge resulted in a negligible decrease in the electrophoretic mobility of the macrolides (Equation 1.5).

The migration time of the macrolides increased and resolution improved with molarity, primarily due to the reduction of EOF. In addition, the enhancement of resolution at high ionic strengths can be partly due to the augmented differentiation in the electrophoretic mobility of the components [126]. However, in this study the differential mobilities of the macrolides remained unchanged with increasing electrolyte concentration, thus the observed improvement in resolution must be solely due to the reduction of the EOF (Equation 1.16). The effect of electrolyte concentration on efficiency and resolution is not as dramatic as that of applied voltage [126]. At moderate applied voltages, high ionic strength buffers enhance capillary selectivity, efficiency and resolution of the separation. These effects occur as a result of the minimised wall interactions and thus increased mass recovery [134].

3.5 Effect of Ionic Strength and Applied Voltage

3.5.1 Experimental Procedure

To ascertain the maximum voltage that can be applied at each molarity without generating excess joule heat, the electrolyte molarity and applied voltage were simultaneously modified. Applied voltages ranging from 10 kV to 25 kV and buffer molarity ranging from 10 mM to 50 mM were selected. The experimental parameters described in Section 3.2.3 were followed. Phosphate buffers of molarity 10, 20, 30, 40 and 50 mM at pH 7 were prepared as specified in Section 3.2.3.2. The sample, consisting of a mixture of josamycin and erythromycin (1 mg/ml of each), was prepared as described in Section 3.2.3.3.

At each electrolyte concentration, an Ohm's Law plot of current versus voltage was plotted as shown in Figure 3.6. From each plot, the maximum voltage at which the curve deviated from linearity was recorded and plotted as a function of molarity (Figure 3.7). This represents a guide for the selection of applied voltage and electrolyte concentration for phosphate buffers.

3.5.2 Results

From the Ohms Plot (Figure 3.6) it is apparent that as the electrolyte molarity increases the applied voltage at which deviation from linearity occurs decreases. The suggested voltages for analysis in electrolyte of high molarity is low to prevent the generation of excess joule heat which is detrimental to the separation efficiency (Figure 3.7).

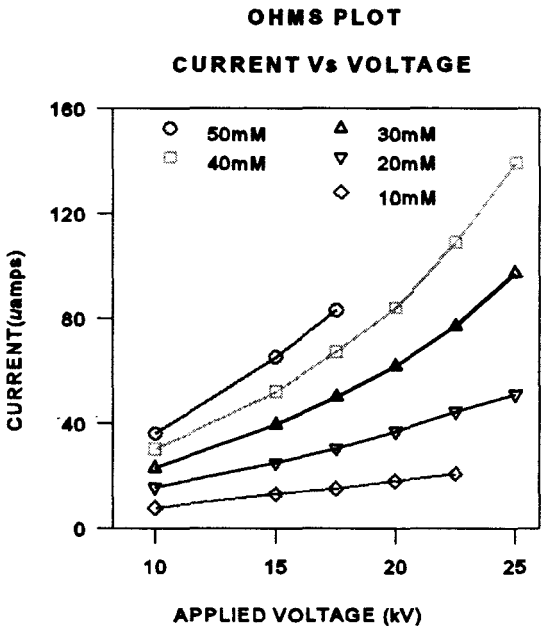


Figure 3.6 Ohm's Plot. Conditions: Phosphate buffer 10 -50 mM, pH 7. Other conditions as for Fig. 3.1.

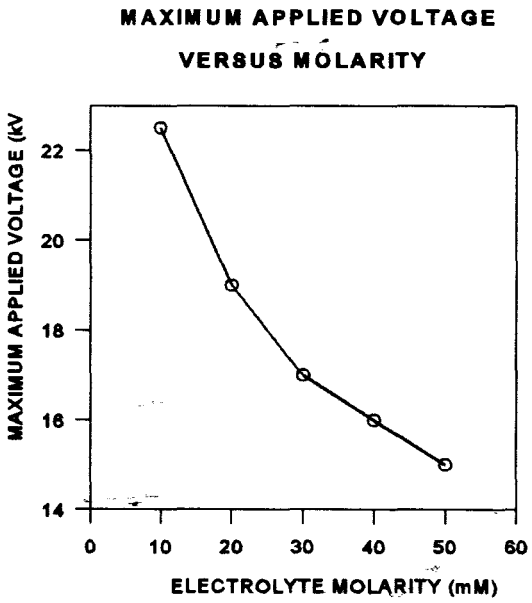


Figure 3.7 Maximum applied voltage versus electrolyte molarity. Data taken from Fig. 3.6.

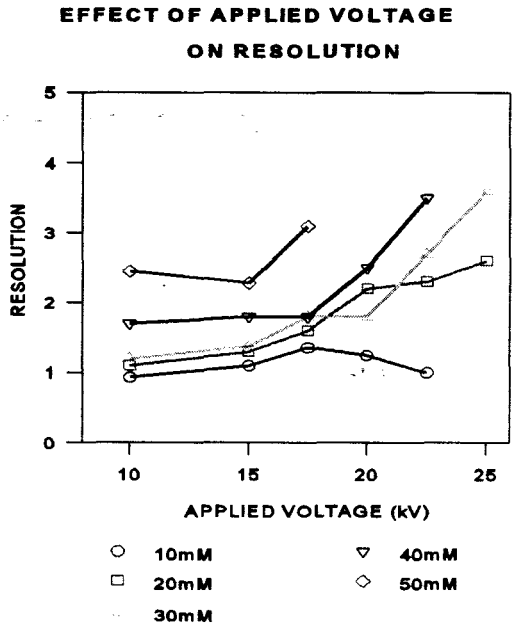


Figure 3.8 Relationship between applied voltage and resolution. Conditions as for Fig. 3.6.

Resolution, plotted as a function of applied voltage at different molarity, is represented in Figure 3.8. No specific trend in resolution was observed with increasing applied voltage for electrolytes at each molarity. With increasing electrolyte molarity at each applied voltage, the peak shape improved, became narrower and increased in height due to sample stacking. This possibly explains the improvement in resolution with applied voltage.

3.5.3 Discussion

The heat generated in CE is directly proportional to the electrolyte concentration and the square of the applied voltage, as indicated in Equation 1.17. This relationship implies that it is advisable to select electrolytes of moderate molarity and use reasonable applied voltages to maintain a high separation efficiency and resolution.

For the analysis of the macrolides a 20 mM phosphate buffer and an applied voltage of 17.5 kV were found to yield satisfactory resolution and migration time without the generation of excess joule heat.

3.6 Injection Conditions

3.6.1 Experimental Procedure

The effect of varying the injection conditions on the sample load in the capillary was studied following the methodology reported in Section 3.2.3. A mixture of erythromycin and josamycin, each at a concentration of 1 mg/ml, was prepared as described in Section 3.2.3.3. The analysis was performed in a 20 mM phosphate buffer (pH 7), prepared as specified in Section 3.2.3.2. The injection pressure was varied between 25 mbar and 100 mbars for 5 seconds, while the injection voltage was varied between 2.5 kV and 10 kV for 5 seconds and the injection time was varied between 5 and 10 seconds at 5kV. A separation voltage of 17.5 kV was selected.

3.6.2 Results

The effect of the injection pressure, voltage and time on the peak height and normalised peak area is represented in Figures 3.9 to 3.14. The mean and standard deviations of the peak height and normalised peak area are plotted as a function of the injection conditions. All peak areas reported are normalised. This involves dividing the peak area by the migration time for that peak. The peak height and area increased non-linearly with prolonged sampling conditions as greater quantities were loaded onto the capillary.

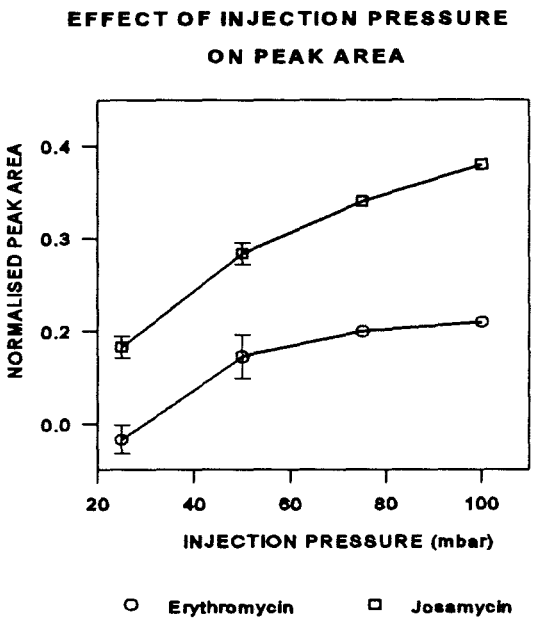


Figure 3.9 Hydrodynamic Injection
Peak area as a function of injection pressure. Conditions: 20 mM phosphate buffer, pH 7, injection conditions 25 - 100 mbar for 5 s, applied voltage 17.5 kV. Other conditions as for Fig. 3.1.

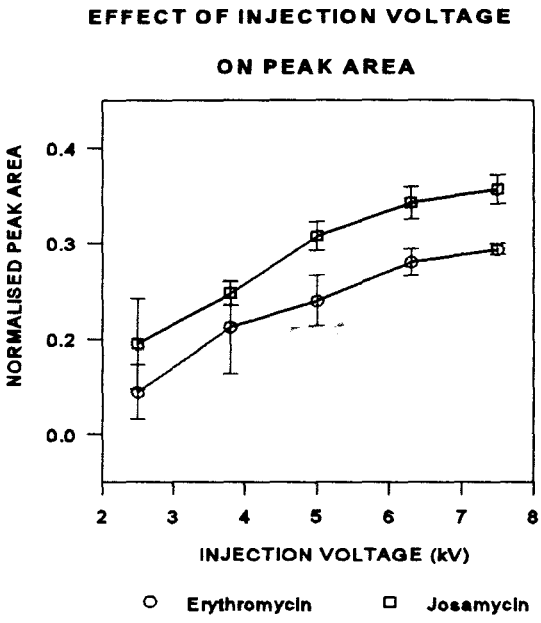


Figure 3.10 Electrokinetic Injection
Peak area as a function of injection voltage. Conditions: injection voltage 2.5 - 7.5 kV for 5 s. Other conditions as for Fig. 3.9.

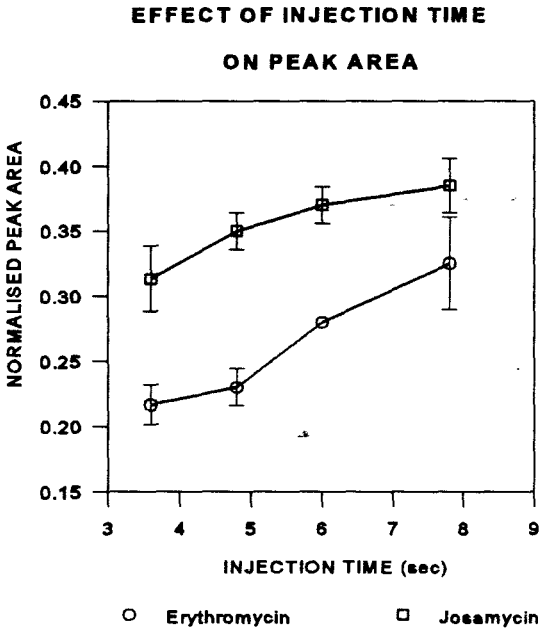


Figure 3.11 Electrokinetic Injection
Peak area as a function of injection time. Conditions: injection time 5 - 10 s at 5 kV. Other conditions as for Fig. 3.9.

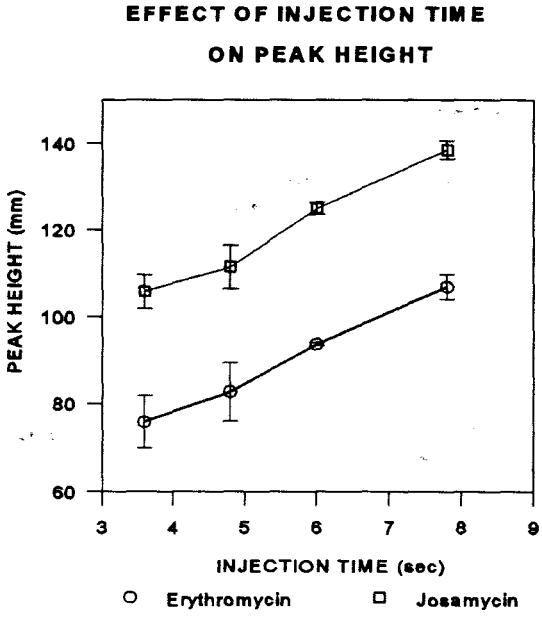


Figure 3.12 Electrokinetic Injection
Peak height as a function of injection time. Conditions as for Fig. 3.11.

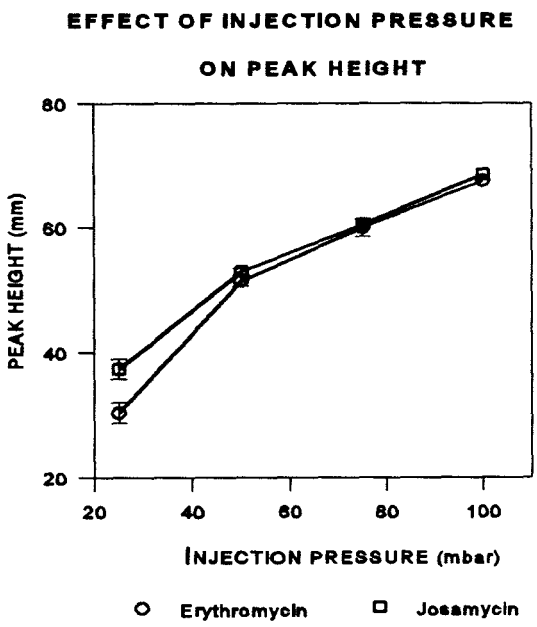


Figure 3.13 Hydrodynamic Injection
Peak height as a function of injection pressure. Conditions as for Fig. 3.9.

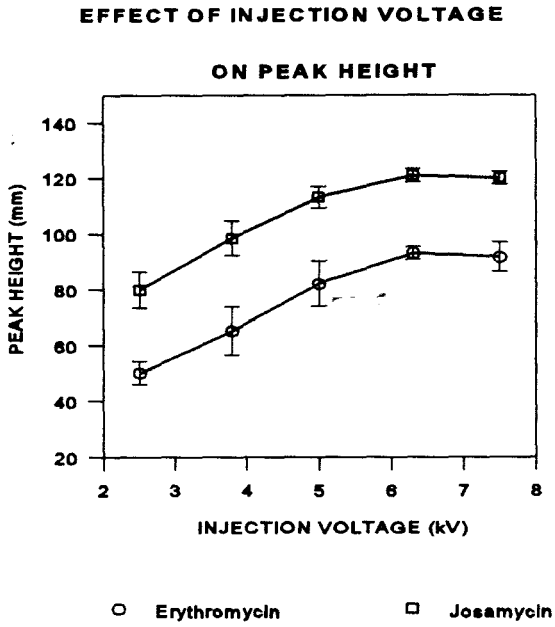


Figure 3.14 Electrokinetic Injection
Peak height as a function of injection voltage. Conditions as for Fig. 3.10.

3.6.3 Discussion

Sample introduction is a very important aspect of CE performance as it relates directly to quantitative precision. The volume of sample injected hydrodynamically is a function of the pressure differential and duration of injection. In addition, the capillary dimensions and electrolyte viscosity also influence sample volumes. Consequently, a linear increase in peak area is expected as the amount of sample introduced into the capillary increases. The amount of sample injected into the capillary hydrodynamically is a discrete volume of composition identical to that of the sample matrix. Thus, hydrodynamic flow introduction does not discriminate between sample components based on mobility [132]. Therefore, a possible explanation for the non-linearity observed in Figure 3.9 could be due to errors in the peak area reported. This arises from the inability of the integrator to accurately respond to the rapidly migrating peaks.

The amount of each solute component of the sample matrix injected into the capillary when using the electrokinetic mode of injection is dependent on the component's migration velocity and the sample buffer ionic strength [132]. In addition, the injection voltage and duration, capillary dimensions and sample concentration influence the amount of sample injected. Theoretically,

the peak area should increase directly with either the injection voltage or duration, however deviations from linearity were apparent (Figure 3.10 and 3.11). These deviations can be attributed to a continuously changing ionic strength in the sample matrix with successive injections from the same vial. This results in a sample solvent of variable conductivity and thus fluctuations in the electric field over the zone are apparent [3]. As the amount of solute electrokinetically injected into the capillary is a function of the electrophoretic mobility of the solute, electrokinetic injection leads to sample discrimination [132]. In addition, a variation in the EOF with time, due to modifications of the capillary surface, is partly responsible for the non-linear relationship.

The hydrodynamic mode of injection is preferable for quantitative analysis as it yields higher peak area and peak height precision. However, it is not possible to increase detection sensitivity using on-capillary concentrating techniques such as FASI with hydrodynamic injection. If the objective of the study is to quantitate the analytes, the hydrodynamic mode of injection is preferable. However, if the study involves the detection and identification of compounds in a sample, it is advisable to use the electrokinetic mode of injection as FASI can be employed to detect components present in low concentrations. For the purpose of investigating the various experimental parameters electrokinetic injection was selected.

On electrokinetic injection the larger peak area of josamycin, in comparison to erythromycin (Figure 3.10) implies that josamycin has a higher mobility than erythromycin. However, the peak area of josamycin was larger than that of erythromycin when the sample was injected hydrodynamically (Figure 3.9). There is no inherent discrimination in the quantity of sample loaded relative to the matrix with hydrodynamic sample injections. Thus a more plausible explanation for the discrepancy in peak area is probably the higher molar absorptivity of josamycin.

Ideally peak height should be a linear function of the injection voltage, pressure or time. The non-linear portion of the plot observed at high sampling pressure, voltage or time can be attributed to peak broadening (Figures 3.12 - 3.14). This results from the extended injection plug length [94]. System efficiency is adversely affected by sample overloading, thus warranting careful selection of the injection conditions. Generally. peak area, as opposed to height, is used in quantitative analysis as it accurately exemplifies the relationship between the quantity of sample injected and injection conditions [85]. In addition, it is recommended to use relatively high sample loadings to obtain good peak area precision [133].

3.7 Effect of Injection Solvent

3.7.1 Experimental Procedure

The effect of the injection solvent on the sensitivity of detection was investigated according to the procedure specified in Section 3.2.3. The dimensions of the capillary that was used were as follows: total length, 108.2 cm; length to detector, 75 cm; I.D., 75 µm and pathlength, 3 mm. A 50 mM phosphate buffer (pH 7.5) was prepared as reported in Section 3.2.3.2. An electrolyte of higher molarity and pH was selected for this study as the capillary length was increased and therefore the conditions of the experiment were slightly altered. The sample solvents investigated were methanol and acetonitrile in concentrations ranging from 20 to 100 % (v/v). A mixture of erythromycin and josamycin, at a concentration of 1 mg/ml each, was prepared in the various diluents as described in Section 3.2.3.3. The inclusion of at least 20 % (v/v) organic solvent in the sample diluent was necessary to maintain the solubility of the macrolides.

3.7.2 Results

The on-capillary concentration technique of electrokinetically injecting a sample prepared in either a highly diluted electrolyte, water or solvent of lower conductivity is termed field amplified sample injection (FASI). The influence of the various sample solvents on peak height and area are presented in Figures 3.15 - 3.18.

Optimum sensitivity, as determined from the peak height, was attained with the inclusion of 20% (v/v) of either acetonitrile or methanol in the sample diluent. It is evident from Figure 3.17 that the peak areas of both components decreased with incremental percentages of methanol in the sample solvent. However, with the incorporation of acetonitrile into the sample diluent, the peak area of josamycin increased, while that of erythromycin decreased with increasing concentrations. (Figure 3.18)

The peak heights of both compounds decreased with increasing proportions of methanol and acetonitrile in the sample solvent (Figures 3.15 and 3.16). However, the decrease in peak height in acetonitrile was significantly less than in methanol. In addition, the peak heights of erythromycin were lower than that of josamycin when acetonitrile at 20 % (v/v) and 40 % (v/v) was used.

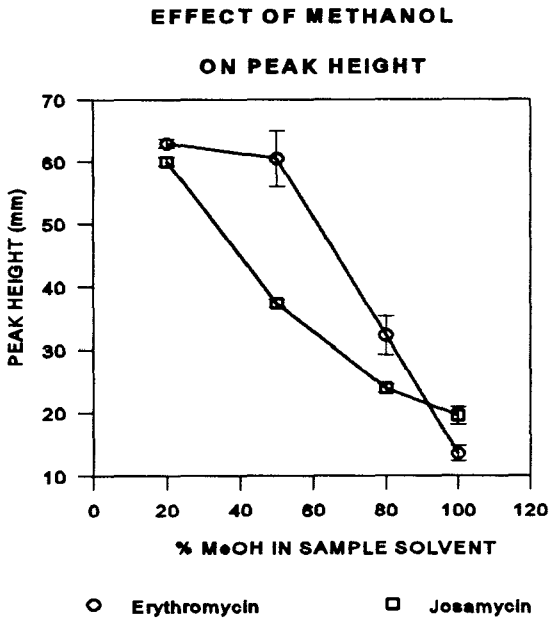


Figure 3.15 Peak height as a function of methanol concentration in the sample diluent. Conditions: sample solvent methanol, concentration 20 - 100 % (v/v), 50 mM phosphate buffer, pH 7.5, applied voltage 17.5 kV, capillary 75 µm x 108.2 cm (effective length 75 cm), pathlength 3 mm.

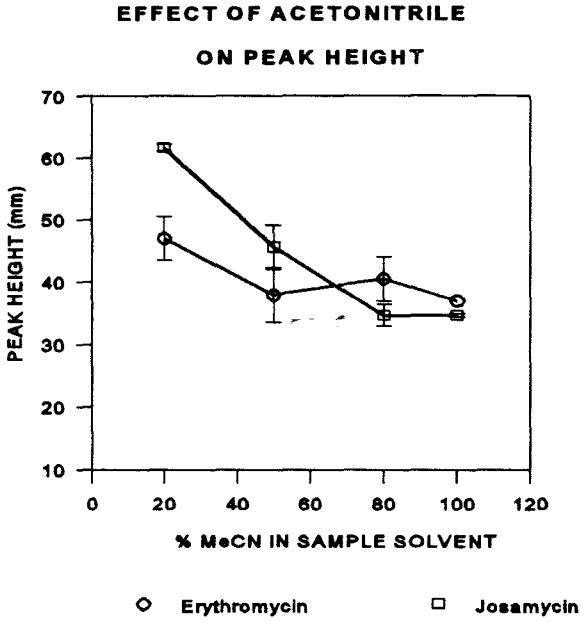


Figure 3.16 Peak height as a function of acetonitrile concentration in sample diluent. Conditions: sample solvent acetonitrile 20 - 100 % (v/v). Other conditions as for Fig. 3.15.

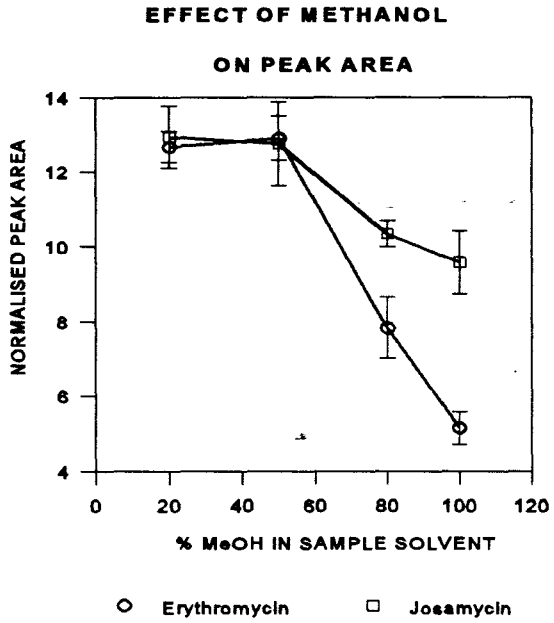


Figure 3.17 Peak area as a function of methanol concentration in sample diluent. Conditions as for Fig. 3.15.

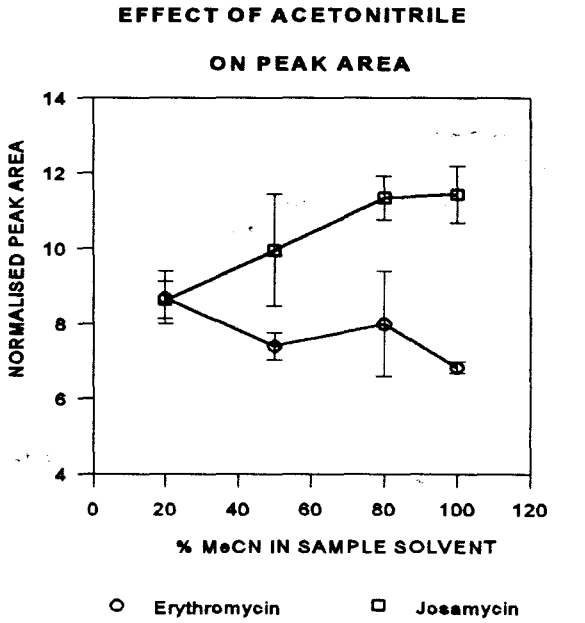


Figure 3.18 Peak area as a function of acetonitrile concentration in sample diluent. Conditions as for Fig. 3.16.

3.7.3 Discussion

One of the challenges in CE is to improve detection sensitivity to compensate for the low sample capacity of the capillary [88]. The minimum detection sample concentration using UV absorption in CE is approximately 10^{-5} M [142]. This concentration limit is several orders of magnitude higher than the detectability of HPLC. Field amplified sample injection is an advantageous technique in that it enhances detector sensitivity by facilitating on-capillary concentration. In addition, FASI allows the introduction of larger amounts of ions into the capillary. In conventional CE, the injection solvent is the background electrolyte. In FASI, the samples are prepared in a highly diluted buffer, water or an organic-aqueous solvent.

The sample solvents selected for this study were either a mixture of acetonitrile and water or methanol and water. The electric field strength at the injection point was significantly amplified because of the low conductivity of the sample solution. Subsequently, the peak height and area should have effectively increased with the use of injection solvents of lower conductivity. A maximum detection sensitivity was obtained with the inclusion of either 20 % (v/v) methanol or acetonitrile in the sample solvent (Figures 3.15 and 3.16). The peak height deteriorated in the presence of organic solvent concentrations of greater than 20 % (v/v) in the sample diluent. The observed peak broadening arose from the mismatch in electro-osmotic mobility between the sample plug and electrolyte as they differed in conductivity [146]. This resulted in the superimposition of a parabolic flow profile onto the plug flow, which was accountable for the deterioration in peak height.

The reduction in peak area with incremental percentages of methanol in the sample diluent implied that peak broadening alone was not accountable for the decrease in peak height (Figure 3.17). The quantity of sample injected onto the capillary, when applying the electrokinetic mode of injection, is dependent on solute electrophoretic mobility, EOF, solute concentration and capillary dimensions. It can only be postulated that a decrease in the electrophoretic mobility of the macrolides in the presence of methanol was responsible for the reduction in peak area.

The reduction of the dielectric constant of the sample solvent with increasing proportions of organic solvent decreased the degree of ionisation of the macrolides as it favoured the more neutral side of the equilibrium between the protonated macrolides and their neutral bases. This led to a decrease in the surface charge and thus electrophoretic mobility of the macrolides. The organic solvents affected the solvation of the macrolide ions and consequently changed their hydration radii. This resulted in an alteration in the mobility of the macrolide ions.

The electro-osmotic mobility also contributes to the amount of sample injected into the capillary. In the presence of organic modifiers, the electro-osmotic mobility in the sample solvent is lower than that in the bulk electrolyte. The increase in the viscosity and decrease of the dielectric constant of the sample diluent with incremental percentages of organic modifiers reduced the electro-osmotic mobility. Therefore it is possible that the reduction in peak area with diluents composed of large percentages of organic solvents was attributed to a decrease in both the electro-osmotic and electrophoretic mobilities.

The discrepancies in peak area and height that were apparent when different sample diluents were used are attributed to the different nature of the organic solvents. Acetonitrile is a protic solvent. It is both a very weak base as well as a very weak acid, and therefore is a good differentiating solvent for acids and bases. Acetonitrile has a negligible effect on the viscosity and dielectric constant of the sample solvent [69]. Subsequently, it changed the electro-osmotic mobility minimally. Acetonitrile is known to compete with hydrophobic intra-molecular interactions [130] and thus it altered the stability of the molecular conformations and the hydration radii of the macrolide ions.

Methanol had a significantly greater effect on the electro-osmotic mobility than acetonitrile. The decrease in the dielectric constant of binary methanol-water mixtures is greater than the corresponding acetonitrile-water systems [69]. In addition, the viscosity of the methanol-water system increases with incremental percentages of methanol (up to 45 % v/v) then decreases [69]. These effects resulted in a decrease in the electro-osmotic mobility. The dramatic decrease in peak height (70 %) observed in the presence of large concentrations of methanol (Figure 3.15) probably arose from the higher viscosity of the sample diluent. This induced a laminar flow which resulted in peak broadening.

The variation in the amount of sample loaded onto the capillary when substituting methanol for acetonitrile as the injection solvent was due to different extent to which the organic solvents affected the pK_a values of the solute. This altered the degree of ionisation of the macrolide ions and hence the ionic charge. In addition, the organic solvents selectively altered the degree of solvation of the macrolide ions and consequently the size of the ions. As a result, the electrophoretic mobility of the ions, which is dependent on its charge and mass, and thus the amount injected differed, depending on the sample solvent used.

Detection sensitivity was optimal with the sample diluent methanol:water (20:80 v/v) as observed from the peak height and normalised peak area.

The deterioration in the signal enhancement attained during FASI resulted from a reduction of the electric field at the injection point. This reduction was due to high concentrations of ions within the sample zone. This effect is simply overcome by injecting a plug of water into the capillary prior to the sample.

3.8 Water Plug Effect

3.8.1 Experimental Procedure

The amplification of the enhanced field strength achieved in FASI by hydrodynamically injecting a plug of water prior to the sample was investigated. The experimental sequence as specified in Section 3.7.1 was followed. The sample comprised a mixture of erythromycin and josamycin (1 mg/ml each) prepared in either methanol:water (20:80 v/v) or acetonitrile:water (20:80 v/v). Water plugs of various lengths were injected onto the capillary by modifying the injection pressure between 10 mbar and 70 mbar for a duration of 1 second.

3.8.2 Results

Graphical representations of the effect of a water plug on the peak height and area for the injection solvent, acetonitrile:water, are illustrated in Figures 3.19 and 3.20.

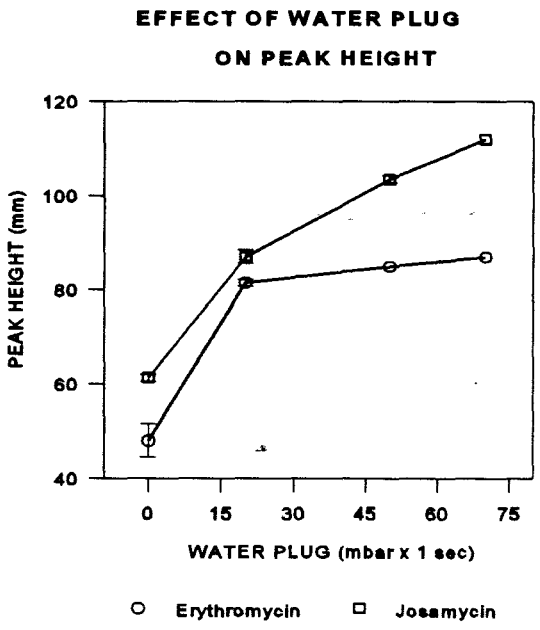


Figure 3.19 Effect of water plug on peak height. Conditions: sample solvent acetonitrile:water (20:80 v/v). Other conditions as for Fig. 3.15.

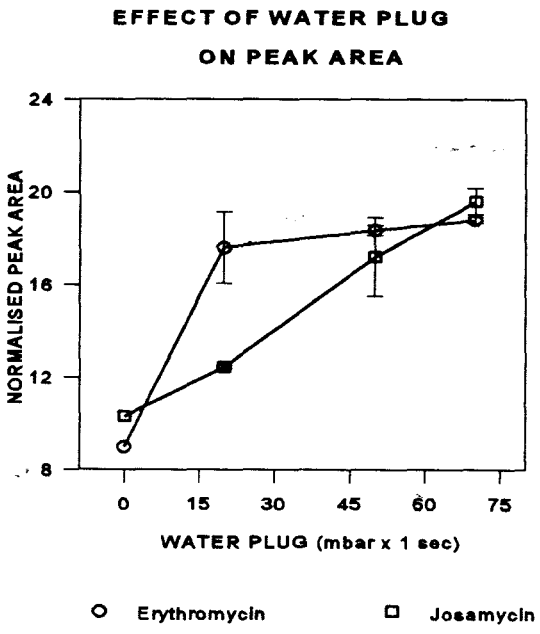


Figure 3.20 Effect of water plug on peak area. Conditions as for Fig. 3.19.

Figures 3.21 and 3.22 illustrate the effect of a water plug on the peak height and area for the sample dissolved in methanol:water (20:80 v/v). The general trend observed is an increase in both peak area and height with water plugs of extended lengths, irrespective of the sample diluent.

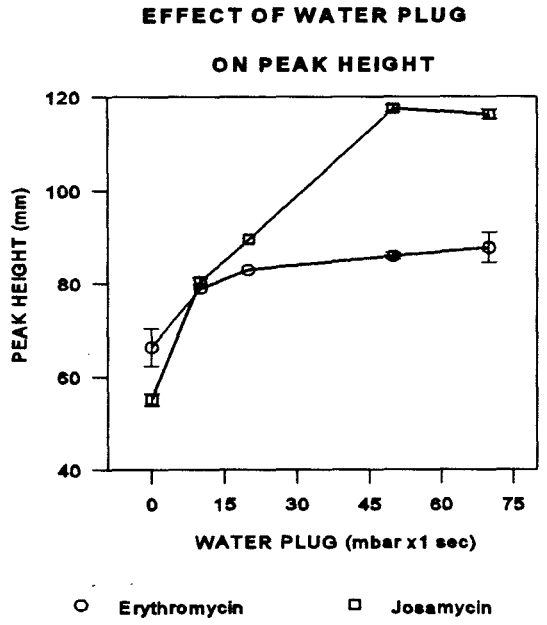


Figure 3.21 Effect of water plug on peak height. Conditions: sample diluent methanol:water (20:80 v/v). Other conditions as for Fig. 3.19.

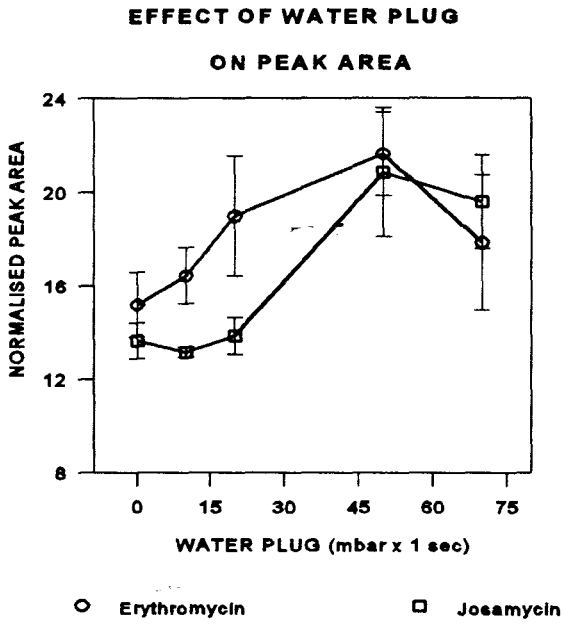


Figure 3.22 Effect of water plug on peak area. Conditions as for Fig. 3.21.

A 45 % increase in the peak height of both erythromycin and josamycin with increasing lengths of water plugs was observed when the injection solvent was acetonitrile:water (20:80 v/v). The percentage increase in the peak height of erythromycin and josamycin with water plugs of extended lengths was slightly less (40 %) when the samples were dissolved in methanol:water (20:80 v/v).

3.8.3 Discussion

The injection of a plug of water may be a prerequisite to fully appreciating the benefits gained from FASI. The electric field at the injection point was maximally enhanced in the presence of a water plug, which provided a void region into which the sample could be introduced. Consequently, the amount of sample injected onto the capillary was significantly larger, as shown by the increase in peak area and height.

It is possible to increase the sensitivity of detection in CE by employing FASI with or without a water plug. The limit of detection may be improved to such an extent that sensitivity is comparable to that obtained in HPLC. This extends the applicability of CE to the detection of low levels of impurities in pharmaceuticals and drugs in plasma and urine.

3.9 Effect of Capillary Diameter

3.9.1 Experimental Procedure

To evaluate the effect of capillary diameter on the sensitivity of detection, three capillaries of inner diameter 20 µm, 50 µm and 100 µm were studied. The instrumentation used was as reported in Section 3.2.1, except that the Z-shaped flow cell was replaced with a straight flow cell in which the pathlength is determined by the inner diameter of the capillary. The total length of the capillary was 114.5 cm with an effective length of 75 cm. An attenuation of 0.01 AUFS was selected for this study. The method as described in Section 3.2.3 was followed. The electrolyte selected was a 50 mM phosphate buffer (pH 7.5), prepared as specified in Section 3.2.3.2. The injection sample consisted of a mixture of josamycin and erythromycin, each at a concentration of 1 mg/ml in methanol:water (20:80 v/v), prepared as described in Section 3.2.3.3.

3.9.2 Results

The relationship between the normalised peak area and height and capillary diameter is represented in Figures 3.23 and 3.24. The improvement in peak sensitivity with capillary diameter, as illustrated by the increase in peak area and height, is a result of the increased pathlength.

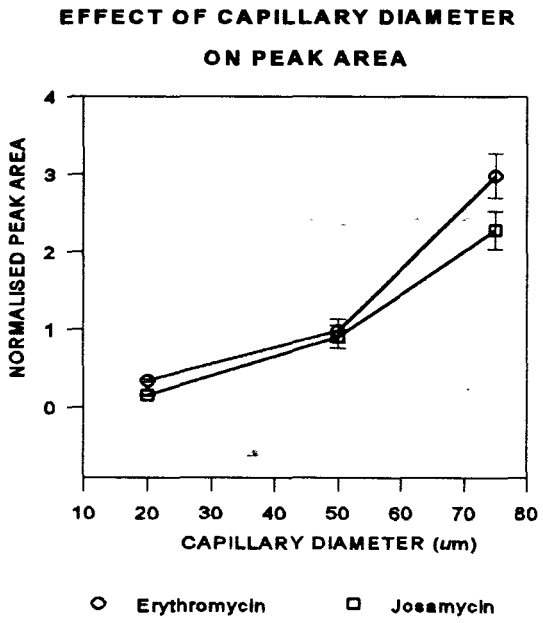


Figure 3.23 Peak area as a function of capillary diameter. Conditions: capillary diameter 20 - 100 µm, total length 114.5 cm, effective length 75 cm. 50 mM phosphate buffer, pH 7.5, sample solvent methanol:water (20:80 v/v) applied voltage 17.5 kV.

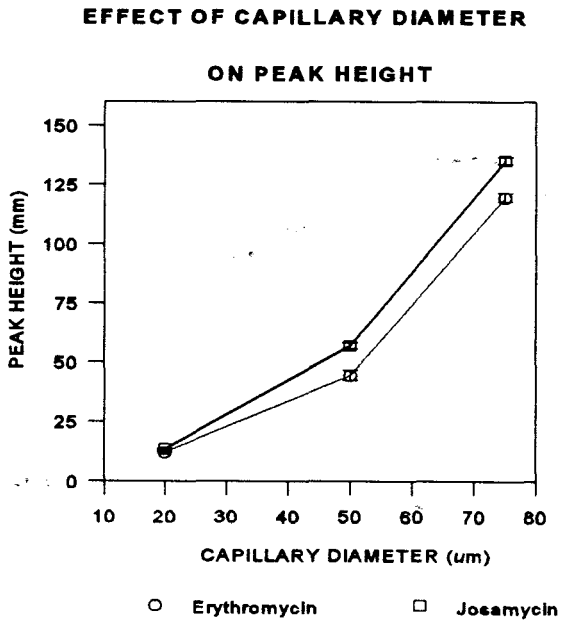


Figure 3.24 Peak height as a function of capillary diameter. Conditions as for Fig. 3.23.

3.9.3 Discussion

The capillary diameter is a critical parameter that requires careful consideration as it regulates the quantity of joule heat that is generated and consequently detection sensitivity. Improvement in detection sensitivity was primarily due to an increase in the volume of the capillary that was employed as the on-capillary detection cell. In addition, detection sensitivity was enhanced by an increase in the volume of sample solution injected into the capillary. The internal volume of the capillary is a square function of the radius. Thus sensitivity is limited by the fact that a small diameter capillary cannot be loaded with the same volume of sample [103].

The inverse relationship between the surface area to volume ratio and inner diameter of the capillary indicates the use of narrow bore capillaries that are capable of effective heat dissipation. Narrow bore capillaries permit the application of larger electric potentials across the capillary, thus reducing the analysis time and improving the separation efficiency significantly. In addition, the resolution is enhanced as narrow bore capillaries facilitate the employment of high ionic strength electrolytes, which induce a reduction in the EOF rate.

Capillaries of inner diameters of between 50 µm and 75 µm are typically employed in CE as was found in this study. High efficiency and selectivity was achieved without the generation of detrimental amounts of joule heat. The increase in the EOF with capillary bore necessitates an adjustment of the injection times. The laminar flow profile and consequent peak broadening that is observed with wide bore capillaries limits their use. It is advisable to eliminate the EOF by adding polymeric additives to the electrolyte or by using coated capillaries when selecting wide bore capillaries. Doubling the capillary bore increases the current by a factor of four, thus the operating voltage must be reduced. Wide bore capillaries have been successfully used to significantly improve sensitivity [85] despite the drawbacks. However, the applied voltage and buffer molarity must be reduced to contain heat production. A compromise between detection sensitivity and the level of joule heat generated is required in selecting the capillary diameter for an analysis.

3.10 Effect of Capillary Length

3.10.1 Experimental Procedure

The importance of capillary length to the detector (effective capillary length) was investigated within a range of 50 cm to 100 cm using a Z-shaped flow through cell, as described previously in Section 3.2.1. Various lengths of capillary, of fixed inner diameter 75 µm, were joined to the

original capillary in the region between the anode and detector, thus resulting in effective lengths of 50, 75 and 100 cm and corresponding total lengths of 83.2, 108.2 and 133.2 cm respectively. The experimental sequence as reported in Section 3.2.3, was followed. A phosphate buffer of 50 mM (pH 7.5) was prepared as described in Section 3.2.3.2. The sample consisted of a mixture of josamycin and erythromycin at a concentration of 1 mg/ml each, dissolved in methanol:water (20:80 v/v) as described in Section 3.9.1. The mode of injection was electrokinetic: 5 kV was applied for a duration of 5 seconds. The separation was effected using an applied voltage of 17.5 kV.

3.10.2 Results

A three-fold increase in the migration time was observed with doubling of the capillary length. Resolution improved with an increase in the capillary length. However, additional peak broadening was observed with capillaries of effective length 100 cm, thus limiting the use of long capillaries. For the analysis of the macrolides a capillary of total length 108.2 cm and effective length 75 cm demonstrated optimal resolution at moderate analysis time.

3.10.3 Discussion

Standard capillary lengths employed in CE are in the range 50 - 100 cm. The length of the capillary employed is limited by the joule heat generated and the physical constraints of the instrument. The peak broadening apparent in long capillaries arose from the prolonged exposure of the analytes to the silica surface and extended interactions with the electrolyte. The higher electrical resistance that is provided by the increased capillary length permits the use of large separation voltages. Large voltages prevent excessive analysis time and subsequently improve resolution by limiting zone broadening.

It is imperative to select a capillary of such dimensions that the temperature gradients are minimised and a high separation efficiency and resolution are attained.

3.11 Effect of Solute Concentration

3.11.1 Experimental Procedure

The effect of erythromycin and josamycin concentration on peak shape, height and area was investigated in the range 0.5 mg/ml to 1.5 mg/ml. The methodology reported in Section 3.2.3 was followed. The analysis was performed in a 50 mM phosphate buffer (pH 7.5), prepared as

specified in Section 3.2.3.2. The sample was electrokinetically introduced into the capillary applying 5 kV for 5 seconds and an applied voltage of 17.5 kV was selected to effect the separation.

Sample Preparation

A stock solution was prepared by accurately weighing approximately 20 mg of each of erythromycin and josamycin into a 10 ml volumetric flask, dissolving in 2 ml methanol and making up to volume with water to yield a concentration of 2 mg/ml. Standards ranging from 0.5 - 1.5 mg/ml were prepared by serial dilution of the stock solution with a methanol:water (20:80 v/v) mixture.

3.11.2 Results

A non-linear increase in the peak height and normalised peak area of erythromycin and josamycin with solute concentration was observed. Deterioration in peak shape was not observed at any of the solute concentrations examined.

3.11.3 Discussion

Theoretically, a linear relationship should exist between the peak area and height and solute concentration. However, in CE, the volume of sample injected into the capillary is not a known, constant quantity. Consequently, injection volume fluctuations are apparent, thus necessitating the incorporation of an appropriate internal standard to minimise this source of error. Peak area and height should be reported relative to that of the internal standard.

Solutes, due to their ionic nature, alter the conductivity of the electrophoretic medium by either enhancing or reducing the local potential gradient. This distortion of the electric field leads to inconsistent migration times and peak tailing or fronting. The extent to which these effects are prominent is dependent on the nature of the solute and the relative electrolyte to sample concentration. Typically, the electrolyte concentration should be at least ten times greater than the sample concentration to minimise these effects [132]. In this study the sample concentration was lower than the electrolyte concentration, even at a sample concentration of 2 mg/ml. Consequently, the electric field was not perturbed and thus no peak tailing or fronting was apparent.

3.12 Effect of an Internal Standard

3.12.1 Experimental Procedure

The significance of including an internal standard to standardise the fluctuations in the volume of sample injected was investigated. The experimental conditions as described in Section 3.11.1 were followed, except that the sample consisted of erythromycin at various concentrations and josamycin as the internal standard.

Sample Preparation

A stock solution of erythromycin of concentration 2 mg/ml, prepared as in Section 3.11.1.1, was serially diluted to obtain concentrations ranging from 0.5 - 0.9 mg/ml. An internal standard solution was prepared by dissolving 10 mg of josamycin in 2 ml of methanol in a 10 ml volumetric flask and making up to volume with water. Sufficient internal standard was added to each sample vial to attain a final concentration of 0.2 mg/ml.

3.12.2 Results

The peak height and normalised peak area of erythromycin relative to that of the internal standard, josamycin, was plotted as a function of erythromycin concentration. A linear increase in the peak height and normalised peak area was observed.

3.12.3 Discussion

In CE it is imperative that an appropriate internal standard be incorporated into the sample for quantitative analysis to compensate for the fluctuations in the volume of sample injected. The dependence of the quantity of sample injected onto the capillary when using the electrokinetic mode of injection on analyte charge and the sample matrix precludes its use for quantitative analysis, even in the presence of an internal standard. Hydrodynamic injections are preferable for quantitative analysis as no inherent sample discrimination is apparent and consequently greater precision is achieved.

3.13 Micellar Electrokinetic Chromatography

3.13.1 Effect of Anionic Surfactants

3.13.1.1 Experimental Procedure

Micellar electrokinetic chromatography (MEKC), employing the anionic surfactant sodium dodecyl sulphate (SDS), was investigated under the following conditions:

- SDS 20 mM, incorporated into a 20 mM phosphate buffer, pH 7.5
- SDS at various concentrations of 20, 50 and 100 mM added into a 20 mM phosphate buffer, pH 7.5
- SDS 20 mM, dissolved in a 20 mM borate buffer, pH 8.5

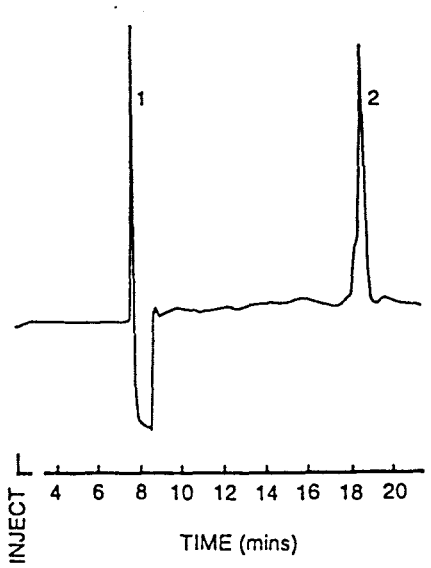
A phosphate buffer of 20 mM was selected to prevent the generation of excess joule heat, as the incorporation of surfactants into the electrolyte increases the ionic strength of the electrolyte.

The phosphate buffer was prepared as specified in Section 3.2.3.1. The borate buffer was prepared by dissolving approximately 0.19 g of sodium tetraborate in 100 ml water and adjusting the pH with a 0.1 M HCl solution to give a pH of 8.5. Two samples were prepared as reported in Section 3.2.3.3. The first was a mixture of erythromycin, troleandomycin and josamycin, each at a concentration of 0.5 mg/ml, dissolved in a methanol:water (20:80 v/v) mixture. In the second sample, the only difference was that the troleandomycin was replaced with oleandomycin. Individual samples of each of the macrolides at the same concentration and in the same sample diluent were also prepared. The mode of injection was hydrodynamic, applying 50 mbar for 5 seconds and the separation was effected using an applied voltage of 15 kV. The instrumental and experimental parameters, as reported in Section 3.2.1 and 3.2.3 respectively, were followed.

3.13.1.2 Results

Electropherograms of each of the macrolides obtained with the phosphate buffer (20 mM, 20 mM SDS, pH 7.5) are illustrated in Figures 3.25 a - d.

Similar electropherograms were obtained when the background electrolyte was borate.



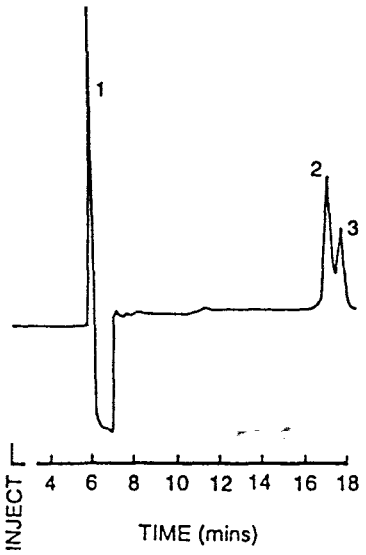
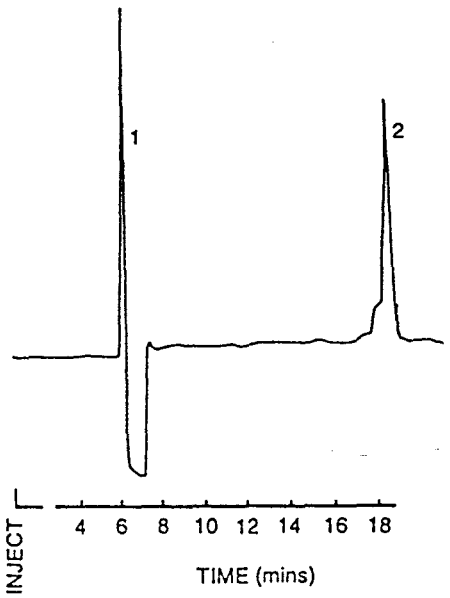


Figure 3.25 a Erythromycin
1. EOF marker
2. Erythromycin

Figure 3.25 b Oleandomycin
1. EOF marker
2/3. Oleandomycin



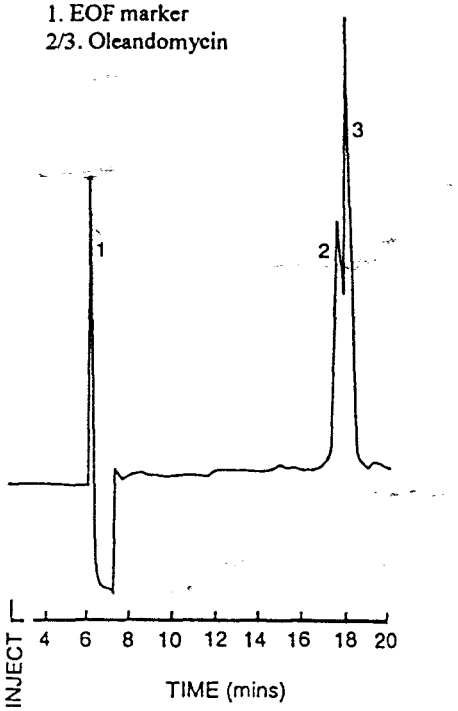


Figure 3.25 c Troleandomycin
1. EOF marker
2. Troleandomycin

Figure 3.25 d Josamycin
1. EOF marker
2/3 Josamycin

Figure 3.25 a, b, c, d Electropherograms of individual macrolides. Conditions: phosphate buffer, 20 mM, 20 mM SDS, pH 7.5, applied voltage 15 kV, injection 50 mbar for 5 s. Capillary 75 µm I.D. x 81.6 cm (effective length 41.4 cm), pathlength 3 mm. Concentration of macrolides 0.5 mg/ml in methanol:water (20:80 v/v).

It was not possible to resolve any of the macrolides from the mixture. The electropherograms of the individual components all exhibited peaks that migrated at the same time. The peaks were broad as all the sample zones co-migrated. There was no notable difference in the migration time or resolution with the use of different buffers at different pH values. The split peaks observed in Figures 3.25 b and d are possibly due to the presence of impurities in the sample as neither oleandomycin nor josamycin were pure samples.

The migration time increased slightly with increasing proportions of SDS. However, there was no improvement in resolution.

3.13.2 Effect of Cationic Surfactants

3.13.2.1 Experimental Procedure

The influence of the cationic surfactants, cetyltrimethylammonium bromide (CTAB) and dodecyltrimethylammonium chloride (DoTAC), on the separation of the macrolides was investigated. A phosphate-borate buffer was prepared by mixing 100 ml of a 100 mM sodium dihydrogen phosphate solution with 100 ml of 25 mM sodium tetraborate solution and making up to 500 ml with water such that the final electrolyte contained 20 mM of each salt. A pH of 8.5 was selected. The sodium dihydrogen phosphate solution was prepared by weighing out approximately 1.38 g of the salt and making it up to volume with water in a 100 ml volumetric flask. The sodium tetraborate solution was prepared by dissolving approximately 0.95 g of the salt in 100 ml of water. The samples that were injected were prepared as described in Section 3.13.1.1 and the injection conditions used were as reported in Section 3.13.1.1. The polarity of the power supply was reversed such that the cathode was on the injection side.

3.13.2.2 Results

Both cationic surfactants reversed the direction of the EOF, thus necessitating the reversal of the power supply, since the bulk flow must move in the direction of the detector. The macrolides all co-migrated, thus no improvement in the resolution was noted with the use of these surfactants.

3.13.3 Discussion

The migration of ionic components in MEKC is determined by three factors:
1) the electrophoretic mobility of the charged solute
2) the distribution ratio of the solute between the micellar phase and the aqueous phase
3) ion-pair formation between the solute and the micelle

The anionic surfactant, SDS, which is extensively used for MEKC, was added to the electrolyte to effect the separation of the macrolides, based on the above three factors. The separation mechanism of the macrolides, which are cationic hydrophilic compounds, in the presence of SDS involved a combination of ion-pairing at the Stern layer of the anionic micelle and migration according to their charge-mass ratio. The absence of any peaks migrating before the EOF marker, methanol, and the presence of a large peak after the marker (Figure 3.25), implied that the macrolides were either all incorporated into the micelles or formed ion-pairs with the micelles and migrated with them. The ionic interaction between the amine group of the macrolide and the anionic surfactant was substantially reduced when electrolytes of alkaline pH were selected, as the macrolides are minimally charged.

In the presence of cationic surfactants, ionic repulsion occurred between the surfactant and the amine group of the macrolide. Thus separation was based on either the electrophoretic mobility of the ions or the distribution of the solutes into the micelle, depending on the degree to which the solute was ionised. In experiment 3.13.2, a pH of 8.5 was selected at which the ionic charge of the macrolides is significantly reduced. Consequently, it is possible that the macrolides were incorporated into the micelle and migrated with it, despite their hydrophilic nature.

The observed increase in migration time, with incremental concentrations of surfactant, was possibly due to enhanced solubilisation of the macrolides into the micelle or greater interactions between the micelle and the macrolides. In addition, the migration velocity of the micelles and the electro-osmotic velocity gradually decreased with increasing concentrations of surfactant in the electrolyte due to an increase in the electrolyte ionic strength.

Micellar electrokinetic chromatography was unsuccessful in resolving the macrolides possibly because they were either all incorporated into the micelle or interacted with the surface of the micelle to the same extent. Therefore, there was no differentiation between their effective electrophoretic mobilities and they consequently all co-migrated.

3.14 Inclusion Complexes - Cyclodextrins

3.14.1 Effect of Heptakis (2,6-DI-O-Methyl)-β-cyclodextrin

3.14.1.1 Experimental Procedure

The effect of the inclusion of heptakis-β-CD on the separation of the macrolides was investigated in the range of 5 mM to 30 mM. A 20 mM phosphate buffer (pH 7.5), to which 30% (v/v) methanol was added, was prepared as described in Section 3.2.3.1. Heptakis-β -CD, at various concentrations (5, 7.5, 10 and 30 mM), was incorporated into the above electrolyte. The samples injected were as specified in Section 3.13.1.1. Injections were performed hydrodynamically by applying 50 mbar for 5 seconds and a separation voltage of 15 kV was used to effect the separation.

3.14.1.2 Results

The migration time decreased slightly with the incorporation of increasing concentrations of heptakis-β-CD in the electrolyte. However, no improvement in resolution was noted. The peak shape deteriorated with the incorporation of 30 mM heptakis-β-CD, which caused a decrease in height and broadening.

3.14.2 Effect of Gamma (γ) Cyclodextrin

3.14.2.1 Experimental Procedure

The influence of γ CD on the resolution of the macrolides was investigated in the range of 5 mM to 20 mM. The analysis was performed in a 20 mM phosphate buffer (pH 7.5), to which 30 % (v/v) methanol was incorporated. The buffer was prepared as specified in Section 3.2.3.1. Various concentrations of γ CD (5, 10 and 20 mM) were incorporated into the above buffer. The sample and injection conditions were as reported in Section 3.14.1.1.

3.14.2.2 Results

No improvement in resolution was observed with increasing concentrations of γ CD. The migration time increased very slightly with increasing concentrations of the CD in the electrolyte.

3.14.3 Effect of Methanol and Cyclodextrins

3.14.3.1 Experimental Procedure

An investigation was performed to determine the influence of methanol and γ CD individually, and in combination, on the resolution of the macrolides. A 20 mM phosphate buffer (pH 7.5) was prepared as reported in Section 3.2.3.1. The resultant buffer was modified in several ways such that it either remained unchanged, or contained 5 mM γ CD, 30 % (v/v) methanol or a combination of 5 mM γ CD and 30 % (v/v) methanol. The sample and injection conditions were as reported in Section 3.14.1.1.

3.14.3.2 Results

The electropherograms of the macrolide in the various electrolytes are shown in Figures 3.26 a - d.

Resolution was found to be identical, irrespective of whether the CD was present in the electrolyte, or not (Figure 3.26 a and 3.26 b). A marked improvement in resolution was noted with the inclusion of 30 % (v/v) methanol in the electrolyte (Figure 3.26 c). Hence, it can be deduced that the organic modifier was responsible for the enhanced resolution and that the CD played a minimal role, if any, in the separation of the components.

3.14.4 Discussion

Cyclodextrins selectively form inclusion complexes with molecules, thus modifying their migration velocity. Resolution in the presence of CD's, is dependent on the difference in stability of the inclusion complex. The stability, in turn, is dependent on hydrophobic interactions, hydrogen-bonding and the size and shape of the molecule. Macrolides consist of a large 14 or 16 membered ring structure to which 2 sugars are attached. Gamma CD's were selected as they possess a large cavity.

The CD's were unsuccessful in resolving the macrolides, possibly due to their size and steric configuration, either of which may not be conducive to inclusion. Furthermore, the macrolides are hydrophilic and are thus unlikely to be incorporated into the hydrophobic CD cavity. Hydrogen - bonding between the macrolide and the exterior of the CD is possible, but was not apparent as the migration time and resolution observed with and without the incorporation of CD into the electrolyte were identical (Figure 3.26 a and 3.26 b).

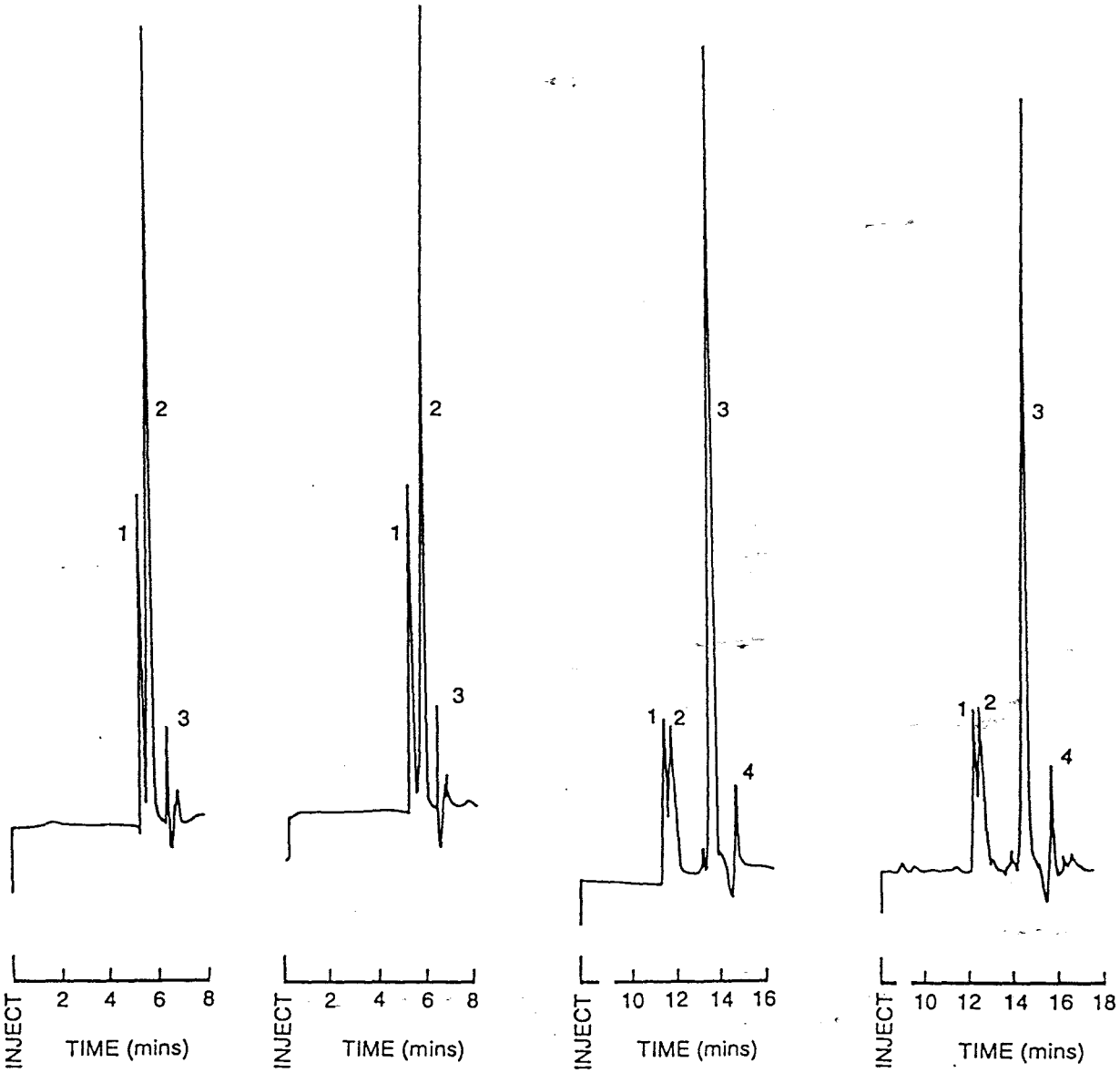


Figure 3.26 a
1. Erythromycin and oleandomycin unresolved
2. Josamycin
3. EOF marker

Figure 3.26 b
1. Erythromycin and oleandomycin unresolved.
2. Josamycin
3. EOF marker

Figure 3.26 c
1. Erythromycin
2. Oleandomycin
3. Josamycin
4. EOF marker

Figure 3.26 d
1. Erythromycin
2. Oleandomycin
3. Josamycin
4. EOF marker

Figure 3.26 a, b, c, d Electropherograms of the mixture of macrolides in various electrolytes. Conditions: 20 mM phosphate buffer, pH 7.5, applied voltage 15 kV, injection 50 mbar for 5 s.
a) phosphate buffer 20 mM, pH 7.5
b) phosphate buffer 20 mM, pH 7.5, 5 mM γ CD
c) phosphate buffer 20 mM, pH 7.5, 30 % (v/v) methanol
d) phosphate buffer 20 mM, pH 7.5, 30 % (v/v) methanol, 5 mM γ CD

3.15 Effect of Organic Additives

3.15.1 Effect of Methanol

3.15.1.1 Experimental Procedure

The methanol content in the electrolyte was investigated within a 0 to 80 % (v/v) range. A 75 mM phosphate buffer (pH 7.5) was prepared as described in Section 3.2.3.1. A high molarity electrolyte was selected as the ionic strength of the electrolyte is reduced with the incorporation of organic solvents into the electrolyte. Aqueous-organic electrolytes were prepared by mixing the above buffer with appropriate volumes of methanol to yield electrolytes with 5, 10, 30, 40, 50, 60 and 80 % (v/v) methanol.

The injected samples were prepared as specified in Section 3.13.1.1. The mode of injection was hydrodynamic (50 mbar) and the duration was 5 seconds. The methodology described in Section 3.2.3 was followed. To avoid excessively long migration times, the separation voltage was increased with increasing percentages of methanol in the electrolyte as follows: 15 kV for 5 % - 10 % (v/v) methanol, 20 kV for 30 % (v/v) methanol and 30 kV for 40 % - 80 % (v/v) methanol.

3.15.1.2 Results

A series of electropherograms of the macrolide mixture containing erythromycin, oleandomycin and josamycin are shown in Figures 3.27 a - c. Figures 3.28 a - c illustrate electropherograms of erythromycin, troleandomycin and josamycin in the presence of varying concentrations of methanol.

Resolution was enhanced in the presence of incremental concentrations of methanol, up to 60% (v/v). Thereafter, resolution deteriorated drastically. Additional peaks were resolved with concentrations of methanol in excess of 30 % (v/v). These peaks are unknown compounds. The migration time escalated significantly with increasing proportions of methanol. Peak shape was not adversely affected with large volumes of methanol as the separation voltage was increased to minimise the time analytes spent in the capillary. Optimal resolution of the macrolide mixture of josamycin, troleandomycin and erythromycin was observed with the inclusion of 30% (v/v) methanol in the electrolyte. A concentration of 50% (v/v) methanol was required to resolve oleandomycin from erythromycin. Further increase in the methanol concentration lengthened the analysis time, without significantly improving the resolution.

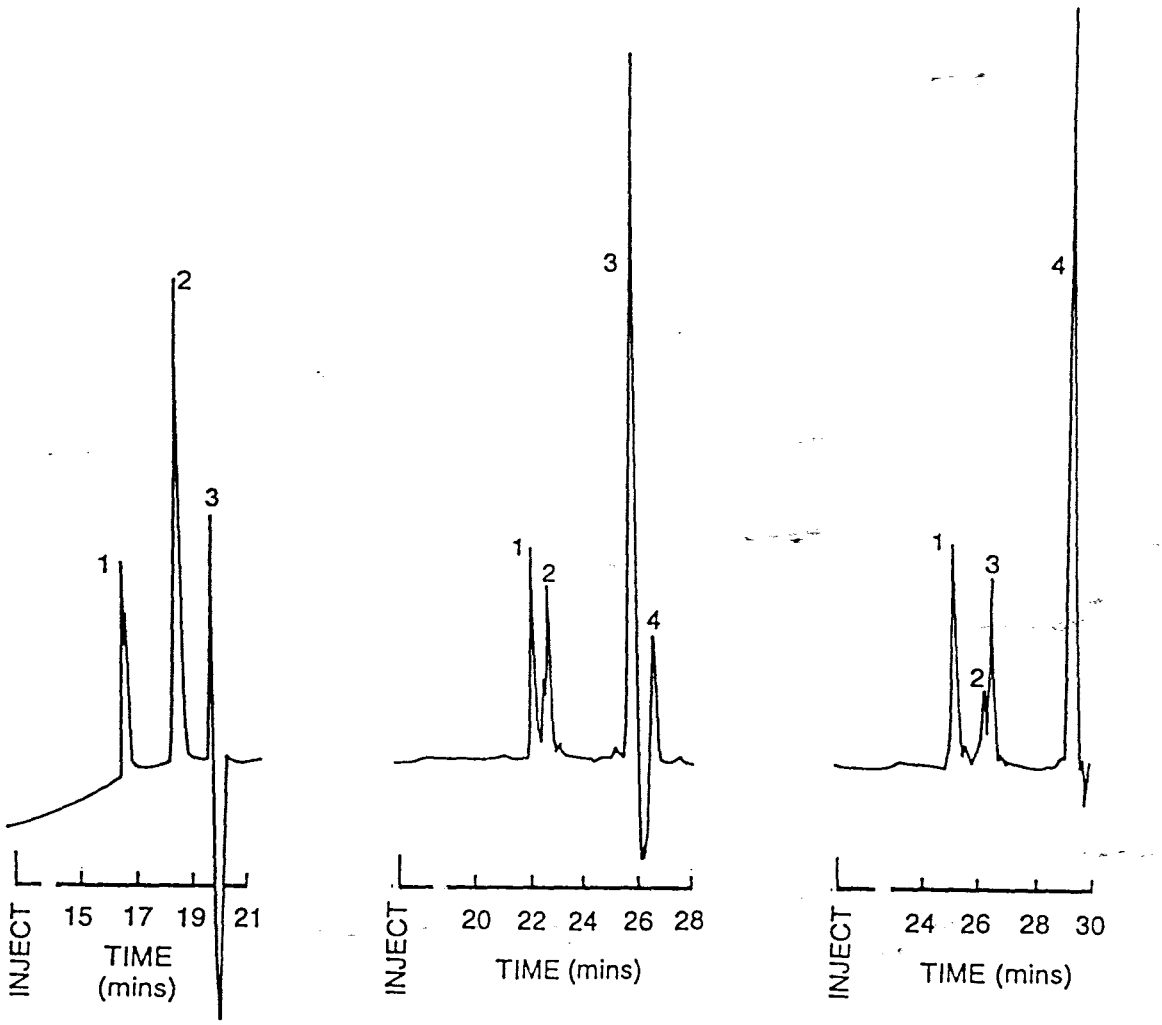


Figure 3.27 a
1. Erythromycin and Oleandomycin unresolved
2. Josamycin
3. EOF marker

Figure 3.27 b
1. Erythromycin
2. Oleandomycin
3. Josamycin
4. EOF marker

Figure 3.27 c
1. Erythromycin
2. Unknown
3. Oleandomycin
4. Josamycin

Figure 3.27 a, b, c Electropherograms of the mixture of macrolides containing erythromycin, oleandomycin and josamycin, analysed in electrolyte with various proportions of methanol. Conditions: phosphate buffer 75 mM, pH 7.5, injection 50 mbar for 5 s, concentration of macrolide 0.5 mg/ml in methanol:water (20:80 v/v).
a) 0 % (v/v) methanol in electrolyte, applied voltage 15 kV
b) 30 % (v/v) methanol in electrolyte, applied voltage 20 kV
c) 50 % (v/v) methanol in electrolyte, applied voltage 25 kV

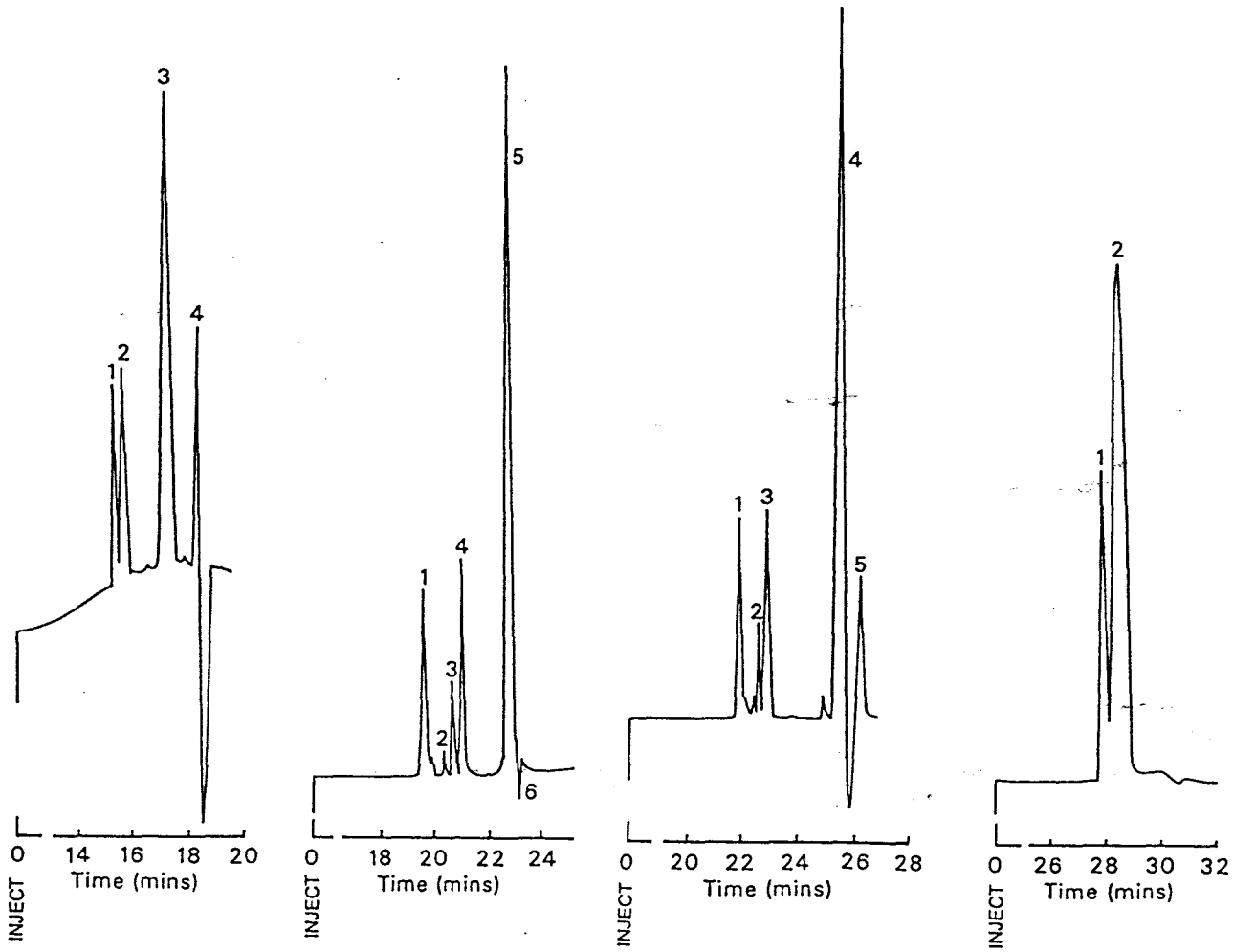


Figure 3.28 a
1. Erythromycin
2. Troleandomycin
3. Josamycin
4. EOF marker

Figure 3.28 b
1. Erythromycin
2. Unknown
3. Unknown
4. Troleandomycin
5. Josamycin
6. EOF marker

Figure 3.28 c
1. Erythromycin
2. Unknown
3. Troleandomycin
4. Josamycin
5. EOF marker

Figure 3.28 d
1. Erythromycin and Troleandomycin unresolved
2. Josamycin

Figure 3.28 a, b, c Electropherograms of the mixture of the macrolides: erythromycin, troleandomycin and josamycin, analysed in electrolyte with various proportions of methanol. Conditions: phosphate buffer 75 mM, pH 7.5, injection 50 mbar for 5 s, concentration of macrolide 0.5 mg/ml in methanol:water (20:80 v/v).
a) 0 % (v/v) methanol in electrolyte, applied voltage 15 kV
b) 30 % (v/v) methanol in electrolyte, applied voltage 20 kV
c) 50 % (v/v) methanol in electrolyte, applied voltage 30 kV
d) 80 % (v/v) methanol in electrolyte, applied voltage 30 kV

The electro-osmotic mobility decreased substantially with increasing percentages of methanol in the electrolyte solution, while minimal changes in the electrophoretic mobility of each component were observed (Figure 3.29). An explanation for the observed changes in the electro-osmotic and electrophoretic mobility with the inclusion of methanol is provided in Section 3.15.5.

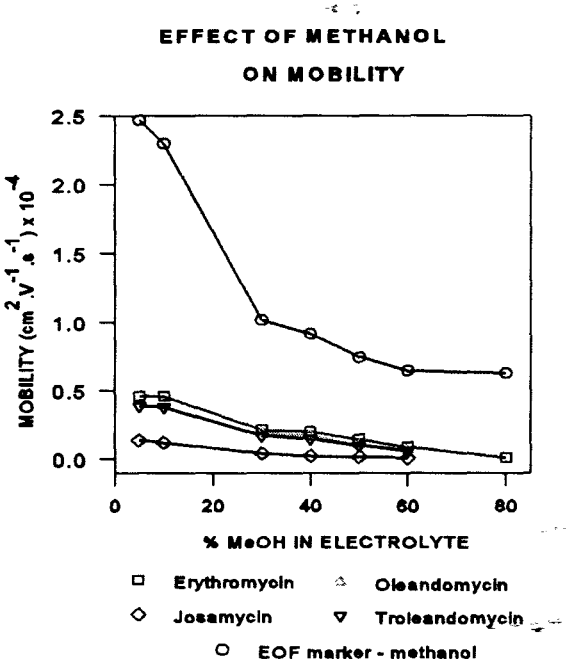


Figure 3.29. Variation in electro-osmotic and electrophoretic mobility with methanol concentration in electrolyte. Conditions: phosphate buffer, 75 mM, pH 7.5, 5 - 80 % (v/v) methanol in electrolyte, Injection 50 mbar for 5 s. Other conditions as for Fig. 3.1.

3.15.2 Effect of Methanol - Constant Ionic Strength

3.15.2.1 Experimental Procedure

The objective of this study was to investigate the influence of methanol on the resolution, while maintaining the ionic strength of the organic-buffer solution constant, during the addition of the organic modifier. Several phosphate buffers of pH 7.5 were prepared. The phosphate buffer concentrations were 55.5, 62.5, 71.43 and 100 mM and corresponded to the incorporation of 10, 20, 30 and 50% (v/v) methanol, such that the final electrolyte solutions all possessed a constant molarity of 50 mM irrespective of the proportions of methanol. The injected sample was prepared as reported in Section 3.15.1.1. The injections were performed hydrodynamically, as specified in Section 3.15.1.1, and the separation was effected using an applied voltage of 20 kV.

3.15.2.2 Results

The plot of electro-osmotic and electrophoretic mobility as a function of methanol concentration in the electrolyte, as depicted in Figure 3.30, showed similar trends to those observed in Figure 3.29, except that the mobility was approximately double.

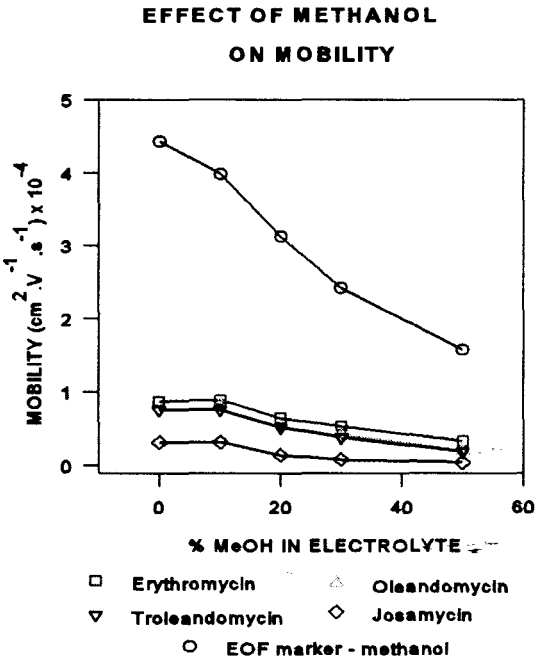


Figure 3.30 Variation in electrophoretic and electro-osmotic mobility with methanol concentration in electrolyte. Conditions: phosphate buffer 50 - 100 mM, pH 7.5, methanol in electrolyte 10 - 50 % (v/v). Other conditions as for Fig. 3.29.

Similar trends in resolution, migration time and peak shape were observed as in Section 3.15.1.2.

3.15.3 Effect of Ethanol

3.15.3.1 Experimental Procedure

The effect of ethanol on the separation of the macrolides was investigated within a 10 - 50% (v/v) range for a phosphate buffer, (75 mM, pH 7.5), that was prepared as described in Section 3.2.3.1. Appropriate volumes of ethanol were added to the above buffer, thus yielding electrolytes containing 10, 30, 40 and 50 % (v/v) organic solvent. The composition of the samples were as reported in Section 3.15.1.1. The methodology described in Section 3.2.3 was followed, except that the sample was hydrodynamically injected by applying 50 mbar for 5 seconds and a separation voltage ranging from 20 kV to 30 kV was used to effect the separation.

3.15.3.2 Results

Figures 3.31 a - c illustrate electropherograms of the macrolides, erythromycin, oleandomycin and josamycin in the presence of varying percentages of ethanol.

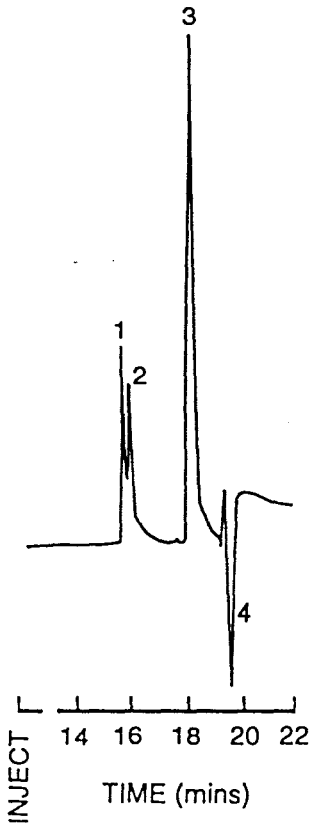


Figure 3.31 a
1. Erythromycin
2. Oleandomycin
3. Josamycin
4. EOF marker

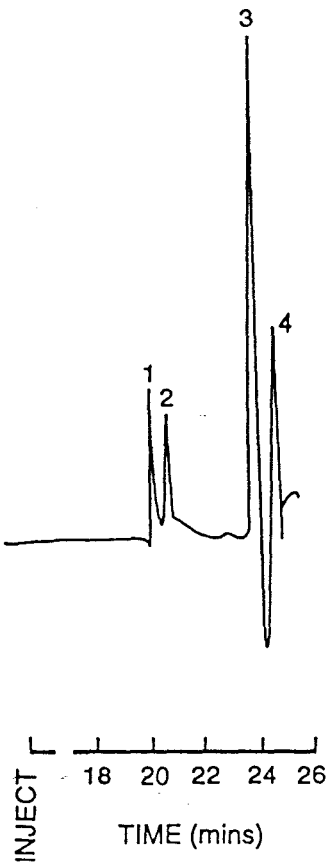


Figure 3.31 b
1. Erythromycin
2. Oleandomycin
3. Josamycin
4. EOF marker

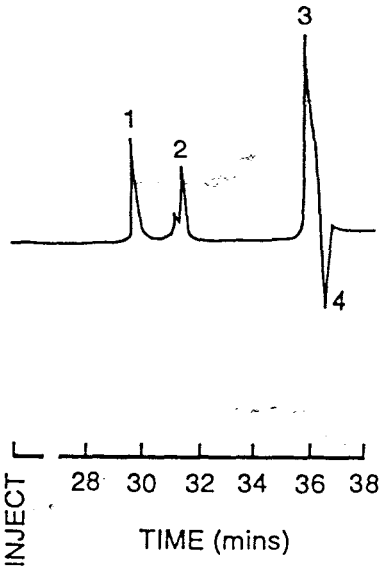


Figure 3.31 c
1. Erythromycin
2. Oleandomycin
3. Josamycin
4. EOF marker

Figure 3.31 a, b, c Electropherograms of the mixture of the macrolides: erythromycin, oleandomycin, josamycin, analysed in electrolyte with various proportions of ethanol. Conditions as for Fig. 3.27.
a) 10 % (v/v) ethanol, applied voltage 20 kV
b) 30 % (v/v) ethanol, applied voltage 25 kV
c) 50 % (v/v) ethanol, applied voltage 30 kV

Resolution improved with increasing proportions of ethanol in the electrolyte, with the inclusion of 30 % (v/v) ethanol yielding the optimal conditions. The analysis time increased four-fold with the addition of 50 % (v/v) ethanol. Distortion in peak shape with peak height deterioration and peak width broadening was observed with the incorporation of ethanol in excess of 30 % (v/v).

The electro-osmotic and electrophoretic mobility exhibited similar trends to those observed in Figure 3.29, with increasing concentrations of ethanol as shown in Figure 3.32.

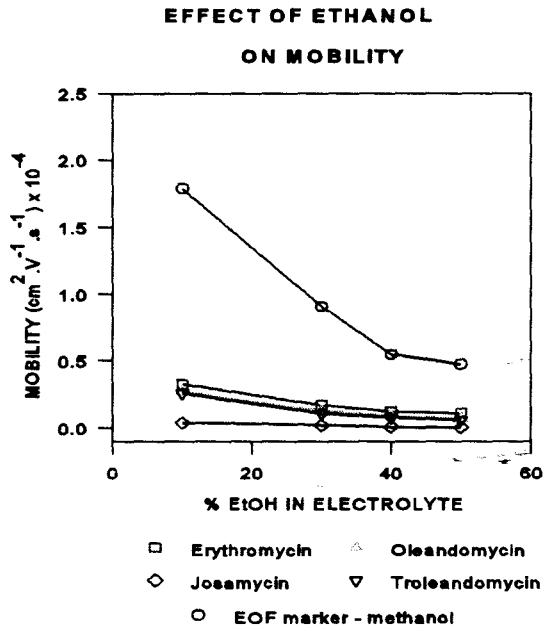


Figure 3.32 Variation in electrophoretic and electro-osmotic mobility with ethanol concentration in electrolyte. Conditions: 75 mM phosphate buffer, pH 7.5, ethanol in electrolyte 10 - 50 % (v/v). Other conditions as for Fig. 3.29.

3.15.4 Effect of Acetonitrile

3.15.4.1 Experimental Procedure

The effect of the acetonitrile content in the electrolyte solution on the resolution of the macrolides was investigated within a 25 to 60 % (v/v) range in a phosphate buffer (75 mM, pH 7.5). The buffer was prepared as described in Section 3.2.3.1. The aqueous organic electrolytes were prepared by mixing the above buffer with appropriate volumes of acetonitrile, to yield electrolytes solutions with 25, 30, 40, 50 and 60 % (v/v) acetonitrile. The injected sample was prepared as in Section 3.13.1.1 and the injection was performed hydrodynamically by applying 50 mbar for 5 seconds. A separation voltage of 15 kV was used.

3.15.4.2 Results

Electropherograms of the separation of the macrolides with increasing proportions of acetonitrile are illustrated in Figures 3.33 a - c. A minimal change in the analysis time with incremental concentrations of acetonitrile was noted. Resolution improved with increasing concentrations of acetonitrile. However, co-migration of josamycin with the EOF marker was apparent at concentrations of 60 % (v/v) acetonitrile in the electrolyte. The peak shapes were not adversely affected at high concentrations of acetonitrile as the EOF is not substantially reduced.

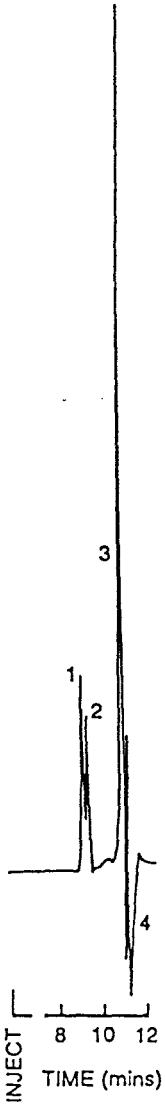


Figure 3.33 a
1. Erythromycin
2. Oleandomycin
3. Josamycin
4. EOF marker

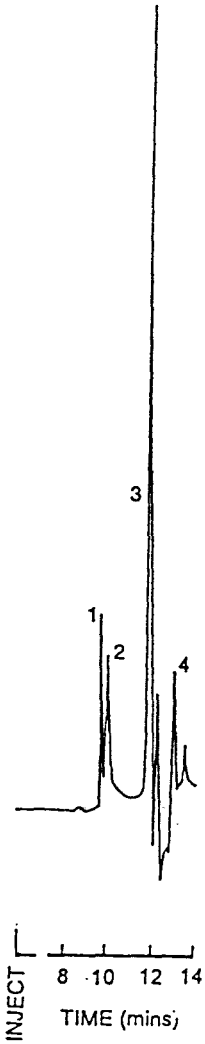


Figure 3.33 b
1. Erythromycin
2. Oleandomycin
3. Josamycin
4. EOF marker

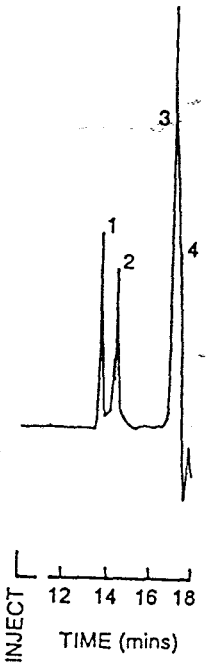


Figure 3.33 c
1. Erythromycin
2. Oleandomycin
3. Josamycin
4. EOF marker

Figure 3.33 a, b, c Electropherograms of the mixture of the macrolides erythromycin, oleandomycin, josamycin, analysed in electrolyte containing various proportions of acetonitrile. Conditions: phosphate buffer 75 mM, pH 7.5. Other conditions as for Fig. 3.27.
a) 25 % (v/v) acetonitrile, applied voltage 15 kV
b) 30 % (v/v) acetonitrile, applied voltage 15 kV
c) 60 % (v/v) acetonitrile, applied voltage 15 kV

The effect of acetonitrile on electro-osmotic and electrophoretic mobility is illustrated in Figure 3.34. The electro-osmotic mobility decreased with increasing concentrations of acetonitrile, while the electrophoretic mobility did not change significantly. Optimal resolution, between the closely migrating peaks, was attained with the inclusion of 30 % (v/v) acetonitrile in the electrolyte.

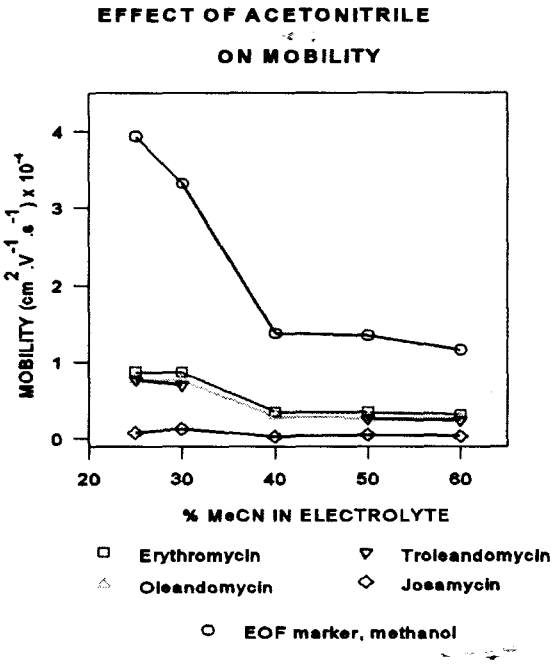


Figure 3.34 Variation of electrophoretic and electro-osmotic mobility with acetonitrile concentration in electrolyte. Conditions: phosphate buffer 75 mM, pH 7.5, acetonitrile in electrolyte 25 - 60% (v/v). Other conditions as for Fig. 3.29.

3.15.5 Discussion

Multiple changes result from the addition of organic modifiers to the electrolyte. These include electrolyte viscosity, pH, ionic strength and dielectric constant, *inter alia*. The pH value of the electrolyte was altered in the presence of organic solvents. However, as pH is only measurable in dilute aqueous solutions, the value obtained for the aqueous electrolyte only was noted. The fluctuations in pH with the inclusion of organic modifiers influenced the degree of ionisation of the macrolides, and may be partly responsible for the observed changes in resolution.

The ionic strength of the electrolyte was reduced with increasing proportions of organic modifiers. This altered the mobility of the analytes. Therefore, experiment 3.15.2 was conducted in which the ionic strength was kept constant to simplify the interpretation of the observed results. The change in the electro-osmotic mobility that was induced by the presence of organic solvents was primarily due to the change in the electrolyte viscosity and net charge of the capillary wall. The capillary wall charge was altered as a result of interactions between the organic additive and the silanol groups on the capillary wall [116].

The graphs depicting the relationship between the organic solvent concentration and mobility (Figures 3.29, 3.30, 3.32, 3.34) all exhibited similar trends. The electro-osmotic mobility was reduced substantially with increasing concentrations of organic solvent. In the presence of 50 % (v/v) of each organic solvent in the electrolyte the electro-osmotic mobilities were as follows: methanol 0.7 x $10^4 cm^2.V^{-1}.s^{-1}$, ethanol 0.5 x $10^4 cm^2.V^{-1}.s^{-1}$ and acetonitrile 1.4 x$10^4 cm^2.V^{-1}.s^{-1}$.

Acetonitrile behaves as an inert solvent in combination with water [69] and thus only nominally altered the electro-osmotic mobility in comparison to methanol and ethanol. Acetonitrile reduces the viscosity of the electrolyte minimally as it has a weaker tendency than water to form hydrogen bonds [116, 69]. Conversely, alcohols increase the buffer viscosity substantially due to extensive hydrogen-bonding [69, 134].

The aprotic nature of acetonitrile has a negligible effect on the adsorption of water molecules onto the capillary surface. The dielectric constants of binary acetonitrile-water systems decrease to a lesser extent than the corresponding alcohol-water systems [124]. Electro-osmotic mobilities are not solely dependent on changes of the viscosity and dielectric constants of the electrolyte. Changes in the zeta potential, with the addition of organic modifiers, also contributed to the decrease in the electro-osmotic mobility. The weak interaction between acetonitrile and the silica surface resulted in a one unit increase in the pK_a of the silanol groups only [69]. Consequently, the zeta potential and EOF were affected to a minor extent.

A substantial reduction in the silica surface charge and consequently the EOF was apparent with the inclusion of protic solvents such as methanol and ethanol. The reduction of the surface charge was due to strong hydrogen-bonding and dipole-dipole interactions between the protic solvents and the silica groups. The inflection point of the graph pH versus electro-osmotic mobility, which corresponds to the pK_a of the silanol groups, is shifted towards higher pH values with increasing amounts of organic solvent [69]. This results in a decrease in the electro-osmotic mobility.

The observed changes in resolution, migration time and peak shape with the inclusion of ethanol were similar to those noted in the presence of methanol. However, ethanol increased the electrolyte viscosity and reduced the dielectric constant to a greater extent than methanol, thus affecting the mobility and migration time significantly. Optimal resolution was achieved in the presence of methanol with respect to analysis time and peak shape.

Appreciable changes in selectivity are apparent in non-aqueous CE with the inclusion of organic solvents in the electrolyte. Marked differences in the solvating power of the organic solvents [142] augmented the variation in mobility amongst the macrolides by altering the hydration radii of the ions. In addition, the pK_a values of macrolides in the presence of organic modifiers, are

significantly different from those in water, thus altering the ionic charge of the macrolides. As a result, the organic solvents shifted the mobility and relative migration order of analytes and augmented the selectivity further.

The electrophoretic mobility of the ions is affected by the nature and percentage of organic modifier and is most sensitive when the pH of the electrolyte is close to the analyte pK_a. Protic solvents are capable of intra-molecular bonding. This suppresses the degree of hydration of the solute, thus reducing the weight of the ions and subsequently increasing their mobility [21]. The ionic charge of the macrolides was reduced in the presence of organic solvents as the lowered dielectric constant of the medium favoured neutralisation. This decreased the fraction of solute in the protonated form and consequently decreased the electrophoretic mobility.

Resolution deteriorated with the inclusion of excessive concentrations of organic modifiers as the reduction in the EOF resulted in exceptionally long analysis times and subsequent zone broadening.

The macrolides all exhibited exceptionally similar electrophoretic mobilities as they possess very similar molecular weights and charge. In addition, their pK_a values are all within a limited narrow range. Consequently, it is extremely difficult, if at all possible, to resolve them using basic CZE. In a study conducted by Flurer [178] on the analysis of macrolide antibiotics by CE, two buffer systems were developed for the separation of the macrolides. One system utilised the bile salt, sodium cholate, and the other, a high concentration of acetonitrile (65 % v/v) in the electrolyte. Each system offers different selectivity, thus providing an additional method of solute identification. Baseline resolution of the macrolides, spiramycin, oleandomycin, erythromycin, clarithromycin, erythromycin ethyl succinate and troleandomycin was attained with the cholate system. With the acetonitrile system complete resolution between oleandomycin, clarithromycin and erythromycin was not achieved.

In this study, it was found that baseline resolution of the macrolides erythromycin, oleandomycin, josamycin was achieved with the incorporation of 50 % (v/v) methanol in a 75 mM phosphate buffer of pH 7.5. Resolution of the macrolides and erythromycin, troleandomycin and josamycin was demonstrated in the presence of 30 % (v/v) methanol in a 75 mM phosphate buffer of pH 7.5.

Organic solvents altered the charge-mass ratio of the macrolides and thus facilitated the separation by enhancing the differential electrophoretic mobilities.

The low ionic mobility in aqueous-organic electrolytes resulted in the production of minimal current levels and subsequently reduced joule heat [124, 122]. This permitted the use of both higher ionic strength electrolytes and applied voltages, thus improving the efficiency of the system

by reducing the migration time and producing narrow, symmetrical peaks. The interaction between the organic solvent and the capillary wall may lead to the formation of a gel layer over time. This will alter the zeta potential and consequently the EOF. Ion adsorption and ion exchange reactions may also influence the zeta potential over time [69]. A uniform zeta potential can be maintained by regularly flushing the capillary with NaOH. To prevent irreproducibility due to pH variation, it is advisable to replace the electrolyte solution after every few experiments (typically after every three runs).

The potential for the separation of closely related compounds which co-migrate in aqueous CE is greater in non-aqueous solvents because of the high separation selectivity that is attainable. Capillary electrophoresis, using organic-aqueous electrolytes, may therefore be used for the determination of the purity content of pharmaceuticals as impurities very often are structurally similar to the main component and exhibit very close electrophoretic mobilities in aqueous electrolytes.

3.1.6 CONCLUSION

The influence of the experimental variables in CE on the analysis of the macrolide antibiotics was systematically examined. The versatile nature of CE can be realised by manipulating the composition of the electrolyte, by modifying the inner surface of the capillary wall or by altering the nature of the analyte. Numerous factors need to be taken into consideration when selecting the optimal conditions for the separation. Of significance, is the influence of the experimental parameters on the production of joule heat. Excess joule heat is detrimental to the quality of the separation and should be minimised. In CE several factors have a combined effect. High molarity electrolytes and high applied voltages can increase the heat generated within the capillary independently. If both factors are increased simultaneously, then the increase in the amount of joule heat will be cumulative and may impair separation performance significantly. Therefore, the effect of each variable on the separation must be considered in conjunction with other variables.

To attain the desired sensitivity the composition of the injection solvent needs to be optimised. Moderate stacking conditions are preferable as peak broadening is limited and thus the resolution and efficiency of the separation are not adversely affected. The mode of injection employed in CE is dependent on the objective of the analysis. If quantitative information is required hydrodynamic injection is preferable. The electrokinetic mode of injection is capable of enhancing detection sensitivity by facilitating on-capillary concentration (FASI). Therefore for the detection of low concentrations of analytes, this injection mode is superior.

The optimal values for each experimental parameter was determined under a specific set of conditions. Manipulation of any one of the variables, changes the overall conditions and consequently the optimal value determined for a parameter may change.

In conclusion, the impact of each experimental variable on the separation should be assessed in determining the ideal separation conditions. All the experimental parameters need to be balanced against each other such that optimal resolution, selectivity and efficiency are achieved within a reasonable analysis time.

CHAPTER FOUR

ASSAY METHOD FOR ERYTHROMYCIN

4.1 INTRODUCTION

Analytical method validation is widely regarded as a crucial aspect in the development of analytical procedures. Validation is performed to provide an assurance of the capabilities of the method under investigation. The key criteria for evaluation of method reliability and overall performance are generally agreed upon. However, differences of opinion within the analytical community world-wide still exist over definitions of terminology. Several regulatory bodies such as the United States Pharmacopoeial Convention (USP) [179], the European Pharmacopoeial Convention (EPC) and recently the International Conference on Harmonisation (ICH) [180] have published recommended validation guidelines. Validation of an analytical method should be considered on the basis of individual experience, despite the availability of numerous official guidelines. It is advisable to establish the boundaries of the validation study, in a manner that is meaningful for a specific analytical situation, rather than to rely on a single set of guidelines.

Validation of any instrumental analysis demands validation of the instrumentation, the method and their suitability for the intended analysis. The majority of manufacturers are accredited with ISO 9000 certification. It is crucial that maintenance and calibration of all instrumentation utilised during the study is performed prior to initiating the study. Instrumentation functions that should be validated in CE include temperature accuracy and stability, voltage accuracy and stability, injection reproducibility and detector and integrator functionality. In addition, any other specialised features that may affect the performance of the assay, should also be validated.

In CE, the criteria for validation are based on those employed by other quantitative separation techniques such as HPLC [181 - 184]. However, instrumentation and technique differences between HPLC and CE should be taken into consideration, prior to selecting the validation parameters. These differences include electrolyte stability and subsequent replenishment requirements, capillary variations and conditioning, method transfer between instruments, reagent purity and data handling procedures [185]. Parameters that are generally considered in method validation are specificity, accuracy, migration time and peak area precision, sensitivity, linearity,

range, stability and robustness. These parameters are dependent on instrumentation features. Voltage, temperature and capillary history and preconditioning affect migration time precision, while peak area precision is dependent on the injection mechanism and temperature. Capillary alignment and detection noise influence the limit of detection.

The advent of highly automated, PC-controlled CE instrumentation and improved knowledge of the technique have assisted in the transfer of CE from qualitative to quantitative determinations [186]. Consequently, CE has gained acceptance as a routine analytical technique in the pharmaceutical industry. A recent survey of major pharmaceutical companies conducted by Altria and Kersey [187] has confirmed that several methods have been successfully accepted by regulatory authorities and that acceptable method validation can be achieved. The Food and Drug Administration (FDA) is actively studying the application of CE to pharmaceutical analysis [188, 189]. The adoption of CE by the pharmaceutical industry is primarily attributed to the numerous benefits afforded by this technique in comparison to HPLC. These include high separation efficiency, speed of analysis, minimal method development, simplicity, low cost of operation, reduced use of organic solvents and ease of operation [190].

4.2 VALIDATION PARAMETERS

4.2.1 Specificity

Specificity is defined as the ability of the assay to unequivocally assess the analyte of interest in the presence of other compounds which may be expected to be in the same sample [179, 180]. These compounds include synthetic impurities, degradants, inactive excipients and analytical artifacts [179, 180, 191]. Specificity, therefore, is a measure of a method's sensitivity to potential sample-related interferents and reflects the ability of the system to resolve these compounds from the peak of interest.

Specificity and selectivity are often used interchangeably, but are distinguishable. A selective method provides accurate results for all analytes of interest, while a specific method provides accurate results for one analyte but the other analytes may interfere with each other [192].

Specificity is determined by demonstrating baseline resolution between the analyte and impurities, excipients or samples from stress testing. It is performed by spiking pure analytes with the appropriate levels of impurities or excipients [191]. If impurities or degradation products are unavailable, a second procedure is required to compare the sample containing the likely interferents [192].

In order to detect co-migration in CE, the purity of the peak and confirmation of its identity needs to be ascertained. Spectral analysis using a diode-array detector facilitates the determination of peak purity. Peak homogeneity can be verified by several methods:

1. by plotting the absorbance ratio of two signals, acquired at two different wavelengths
2. by normalising and comparing UV spectra from various peak sections
3. by calculating the area ratio of the two peaks acquired at two different wavelengths
4. by normalising the signals acquired at two different wavelengths and comparing their migration times.

Furthermore, the analysis of micropreparation fraction collections from the CE separation using an alterative separation technique, such as HPLC, can be used to confirm peak purity.

Peak identity is confirmed by determining UV spectra of a peak and comparing it to that of known standards. Poor correlation between the spectra is indicative of co-migration. In addition, corresponding migration times of an impurity and the standard of the impurity can be used to identify peaks [193]. However, this method is seldomly conclusive, especially for complex samples of several impurities. On-line spiking, performed by co-injecting a standard solution of the specific impurity, is a preferable method of confirming peak identity [193].

4.2.2 Precision

The precision of an analytical procedure is a measure of the closeness of agreement of the data values from multiple sampling of the same homogenous sample under the prescribed conditions [179, 180, 191]. The ICH has defined precision to consist of three components: repeatability, intermediate precision and reproducibility [180]. Precision is expressed as the variance, standard deviation or coefficient of variation of a series of measurements. The smaller the value, the more precise or sensitive the results are to fluctuations [191].

Precision is further categorised into system precision and method precision. System precision is related to the operation of the instrument or performance of the analytical test. Method precision involves all aspects of the procedure, such as sample preparation and sampling techniques [194].

4.2.2.1 Repeatability

Repeatability reflects fluctuations in replicate procedures and is performed by multiple injections of a homogenous sample, within a short time period under the same operational conditions and within the same operational run [194]. It is assessed by performing a minimum of six

determinations at 100 % of the test concentration or a minimum of nine determinations over the prescribed range for the procedure (three concentrations in triplicate) [180].

The repeatability of preparation of sample and standard solutions should also be demonstrated. This is performed by preparing ten individual standard and sample solutions and analysing each in duplicate [195]. The precision of the pooled assay values should be within acceptable limits. The migration time, relative migration time, peak area and peak area ratio should all be consistent.

4.2.2.2 Intermediate Precision

Intermediate precision expresses intra-laboratory variations. The analysis is either performed on different days, by various operators, on different instruments or using reagents provided by an alternate supplier [180, 194]. Intermediate precision evaluates the reliability of the method in an unconventional environment [191]. The objective is to ensure that the same results are obtained when applying the method, irrespective of manipulating certain parameters. Generally, the separation is repeated on a minimum of three separate days and the overall percent relative standard deviation (% RSD) calculated should be less than 3 % [180].

4.2.2.3 Reproducibility

Reproducibility, as defined by the ICH, expresses the precision of inter-laboratory analysis. It is not normally expected to cite this parameter if data on intermediate precision is provided, as multiple laboratory analyses are not always feasible [191].

Quantitative Precision

Quantitative precision in CE depends, to a large extent, on the reproducibility of sample introduction. It is determined from the peak area or peak height [196]. Rose and Jorgenson [132] and Moring et al. [144] each compared the precision of automated hydrodynamic and electrokinetic injections. Electrokinetic injections were found to yield peak area precision values of approximately 3 %, while precision values of less than 1 % were reported for hydrodynamic injections [144]. In a study conducted by Taylor et al. [197], no significant difference in peak area precision was reported when the sample was dissolved in either methanol or water and hydrodynamically injected onto the capillary. However, with electrokinetic injection, peak area precision for the sample dissolved in water was marginally inferior to that when the sample was dissolved in methanol. Generally, analytes dissolved in electrolytes of low conductivity yield poor precision. This can be attributed to variations in the quantity of sample loaded. Erratic sample

loading arises from the progressive change in the ionic strength and pH of the sample solution during electrokinetic injection [144].

The volume of sample that is hydrodynamically injected is inversely-dependent on the sample solution viscosity. Viscosity is a function of temperature, therefore accurate temperature control is crucial for the attainment of reproducible injection volumes [144]. Generally, the hydrodynamic mode of injection is preferable for quantitative analysis. However, this injection method precludes the utilisation of FASI, in which maximum sensitivity of response can be achieved [197].

Peak area precision is also dependent on the velocity of the solute. As analytes migrate past the detector at different velocities an over estimation of the peak area is apparent for slower moving analytes. In addition, fluctuations in migration times can be reflected as an increase in imprecision of peak area. Normalisation of this effect can be achieved by dividing the peak area by the migration time for that peak [144, 198]. This results in an improvement in the level of precision. All peak area data presented in this study have been normalised with respect to the migration time.

Quantitative precision is also dependent on the sample concentration: the higher the concentration the lower the percent relative standard deviation [79, 199]. This is attributed to a reduction in integration errors and minimisation of solute absorption at high sample concentration. The reported peak areas are dependent on the functioning of the integrator software. It is essential to use an integrator with a fast sampling rate, since the error in peak area calculation is inversely proportional to the number of digitalisations performed during the width of the peak [196]. Precision deteriorates at low sample concentration as the integrator has difficulty in determining when the up slope and down slope occurs for peaks that are only slightly above the baseline. At low sample concentration peak height measurements are more precise than peak areas as they are less influenced by integration errors [199]. The employment of an internal standard at low sample concentrations further deteriorates precision as the integration errors are, in effect, doubled.

Fluctuations in the volume of sample injected are responsible for the variance in precision. Unlike HPLC, the volume of sample injected in CE is not a known consistent quantity. The incorporation of an internal standard in the sample solution permits quantitative precision to be obtained by compensating for any variability in the injection volume [137, 186]. The peak area, peak height and migration time are reported relative to that of the internal standard. Precision levels are drastically improved and yield values close to those obtained in HPLC. However, the value of employing an internal standard has been questioned by several researchers [181, 183]. The use of two internal standards has been demonstrated to improve precision and accuracy of

electrokinetic injection [200]. The internal standard selected should be structurally similar to the analyte [201]. It is necessary to ensure that the internal standard does not co-migrate with any related substances. In addition, the purity and stability of the internal standard should be established, so that no internal standard related impurities interfere with the sample component peak.

Qualitative Precision

Migration time precision is critical in attaining reproducible resolution of the compounds and confirming peak identity. Precise migration times can only be achieved if the electric field strength and capillary temperature are constant throughout the study. The analyte velocity is dependent on its electrophoretic mobility and the EOF. Variations in the EOF with time, result in deterioration of migration time precision. A consistent EOF can be achieved by adopting appropriate capillary washing techniques between injections [79, 183]. In electrophoresis, the nature of the analyte dictates the wash sequence. Compounds that possess strong adsorption tendencies require stringent rinsing procedures between consecutive injections. This generally includes an alkaline rinse (0.1 M NaOH), followed by water and the background electrolyte. The NaOH regenerates the capillary wall and thus ensures a uniform EOF.

Typically, flushing the capillary with background electrolyte prior to the next injection is sufficient to maintain a reproducible EOF [186]. The inclusion of a rinsing routine in the experimental sequence can assist in reducing the degree of error in CE to a level that is comparable to HPLC [201].

In CE, qualitative precision is a strong function of temperature, as temperature fluctuations result in migration time and injection volume variability. [79, 144, 196]. Numerous factors are dependent on accurate temperature control. These include the electrophoretic mobility of the ions and the electrolyte viscosity and hence the electro-osmotic mobility. These factors influence the volume of sample injected onto the capillary. The peak area in CE is related to both the sample concentration and migration time. Consequently, temperature fluctuations effect a shift in the migration time and apparent peak area. The temperature at the point of injection should be recorded and controlled.

In addition, the electrolyte pH and sample solvent evaporation due to overheating are dependent on temperature [79]. Precision of analyte mobility is more important than analyte migration time, as mobility provides a measure of the fundamental characteristics of the molecule. Thus analyte mobility is often recorded in the validation documentation [144, 202].

Evaporation losses should be minimised by employing sealed sample vials and sample solvents with a nominal level of organic solvent. Evaporation results in an increase in the sample concentration and a reduction in accuracy [44, 79, 144, 196]. The inclusion of an internal standard in the sample solution eradicates any variations arising from sample evaporation. Sample contamination can occur when the electrokinetic mode of injection is selected, as electrochemical by-products are produced with the flow of an electric current through the sample [132, 201]. This reduces the level of precision. Replenishment of the electrolyte in the anode and cathode reservoirs is essential to achieve a consistent EOF and migration times. Electrolysis of the electrolyte, on the application of voltage, results in the acidification and alkalinization of the anodic and cathodic electrolytes, respectively. This acidification and alkalinization affects the migration time and selectivity [150, 201].

4.2.3 Linearity

The linearity of an analytical method is its ability to produce results that are either directly, or by means of a mathematical transformation, proportional to the concentration of analytes within a depicted range [180]. Least-square linear regression is used to mathematically define the calibration line if it is linear [180, 192]. It may be necessary to subject the test data to a mathematical transformation prior to the regression analysis to obtain proportionality between the assay and sample concentration [180, 192]. The variance of the slope provides a measure of linearity where the slope is an indication of the sensitivity of the method and the y-intercept is an estimation of the potential assay bias [192]. These results should form part of the validation documentation. The unweighted regression line should have a slope that is very close to one with an intercept of approximately zero [203].

The linear range of detectability that obeys the Beer-Lambert Law is dependent on analyte concentration, molar absorptivity and pathlength of the flow cell. The size of the detector cell, which is a narrow capillary, limits the linearity of the range. In addition, the presence of stray light adversely affects the linear range.

Linearity of co-injection

On-capillary standard addition is performed by injecting a sample solution, followed by a second solution, prior to the application of a separation voltage [193]. This co-injection procedure is utilised to confirm the identity [134] and to quantify components of interest in a sample mixture [193]. Quantification is achieved by varying the injection time. Subsequently, it is possible to cover the entire range of spiking levels, without having to manually prepare a range of samples spiked with the appropriate levels of each compound [193]. The normalised peak area is plotted as a function of the injection time and a regression line is calculated by the least squares method.

4.2.4 Range

The range of a method is derived from its linearity and is dependent on the application of the procedure. It is validated by verifying that the analytical procedure provides acceptable accuracy, precision and linearity within the range as well as at extremes of the range [179, 180, 191]. The recommended range for the assay of a drug substance or finished product is 80 - 100 % of the test concentration. The range for the determination of impurities is from +20 % of the target concentration to the limit of quantitation of the drug substance or impurity, or to 50 % of the specification of each impurity, whichever is the greater [180, 191].

4.2.5 Accuracy

Accuracy is the closeness of agreement between the value found by the test method and that which is accepted either as a conventional true value or reference value [179, 180, 191]. It should be established across the prescribed range of the analytical procedure [180]. Accuracy is determined for the assay (drug substance or drug product) and for impurities [180, 192]. The accuracy of a method is generally performed at 80, 100 and 120 % levels of the label claim for the assay.

For drug substances, the accuracy of the method is determined by assaying an analyte of known purity against the drug substance. Alternatively, the results of the proposed procedure can be compared with those of a validated second method of known accuracy [180, 191, 192]. The accuracy of the method for drug products is determined by applying the method to placebos, to which known amounts of drug have been added. The quantity of drug added to the placebo must be within the linear range of detection of the analyte. For impurities, the accuracy of the method is illustrated by spiking the drug substance with known amounts of impurities [180].

4.2.6 Sensitivity

The ability of a method to respond consistently to diminishing amounts of analyte is indicative of its sensitivity [194]. Sensitivity is defined by the slope of the calibration curve. However, it is typically expressed in terms of the signal-to-noise ratio for a specific analyte [194]. The limit of detection (LOD) and limit of quantitation (LOQ) are measures of sensitivity. Noise is defined as the random fluctuations of the detector output which is generated with time. It arises from variations in the lamp output and spurious electronic signals [185].

4.2.6.1 Limit of Detection (LOD)

The LOD is the lowest concentration of analyte in a sample that can be detected above the baseline detector noise. However, it is not necessary that the analyte can be quantitated [179, 180, 191]. The USP suggests that the LOD be used for qualitative limit tests [179]. The LOD can be determined either directly or from other validation data. Direct measurement involves visual evaluation of the peak signal of samples of known concentration, and establishment of the minimum level at which the analyte can be reliably detected [180]. The signal-to-noise ratio, which is determined by comparing peak signals from samples of low concentration to those of the baseline noise from blank samples, can also be used to determine the LOD. A signal to noise ratio of 3:1 is generally acceptable [180, 191].

In addition, the LOD can be estimated from the calibration curve, based on the slope and standard deviation of the response [180]. An estimate of the LOD is obtained by multiplying the standard deviation of the response by a factor of two or three and dividing the result by the slope of the curve. The standard deviation of the slope is obtained by analysing several blank samples and calculating the standard deviation of the background response. In addition, the standard deviation of the regression line or y-intercept of the regression line may be used as the standard deviation of the response [194].

4.2.6.2 Limit of Quantitation (LOQ)

The LOQ is the lowest concentration of analyte in a sample that can be quantitated with acceptable precision and accuracy [179, 180, 191]. According to the USP, the LOQ is specified for quantitative determinations [179]. The LOQ is determined by calculating the coefficient of variation of the peak area or peak height for successively diluted samples, until acceptable levels of precision and accuracy are achieved. A minimum of six determinations is required at each concentration [192, 194]. Replicate analysis at the LOQ should give % RSD values of less than 1 % for drug substances and less than 10 % for impurities [138, 185]. Similarly, the LOQ can be determined based on the analysis of the signal-to-baseline noise ratio. A peak response of ten times the baseline noise is acceptable [185]. Furthermore, standard deviations of the regression line and y-intercept may be used in determining the LOQ [179].

The accuracy and precision of the LOQ should be determined independently from the calibration curve. Limit of quantitation quality control samples should be used as LOQ standards are used in the construction of the calibration curve and thus influence the regression equation [203, 204]. The LOQ is generally the lowest calibration standard in the actual study [203].

4.2.7 System Suitability

System suitability tests are a fundamental component of many analytical procedures. These tests are designed to highlight fluctuations in the operating parameters. They provide an indication that either the method or instrument is unable to perform the analysis optimally. In addition, they provide information that is related to the origin of the problem and to possible means of rectifying it [205].

The performance characteristics that are monitored depend on the type of procedure being validated [180, 205]. Typically, migration time precision, resolution and selectivity are recorded. Several other parameters such as linearity, LOD and peak tailing may be included. However, peak asymmetry is often not recommended as it is highly dependent on the sample concentration and is, thus, not totally indicative of system performance [185, 191, 206]. A resolution value of at least one between the peaks of interest and the closest potentially interfering peak and an injection precision of less than 1 % for the main component are desirable [191, 205].

System suitability tests are conducted prior to initiating an analytical test sequence to assess the performance of the instrument. In addition, they are performed on completion of the analysis and possibly throughout the experimental sequence, to demonstrate the continued suitability of the instrument for the analysis [185]. Fluctuations in the monitored parameters warn that the method is becoming unstable and may indicate the need for revalidation.

4.2.8 Stability

Stability tests should be performed to ensure that compound integrity is maintained throughout the study. In addition, the shelf-life of all CE reagents, calibration solutions and sample solutions should be determined. The stability indicating assay must be capable of detecting and resolving any degradation products. Stability tests must also be sufficiently sensitive to accurately detect changes in the concentration of the analyte of interest [204, 205]. Stability is established by performing the analysis with freshly prepared solutions and solutions stored in the refrigerator at 4°C and at room temperature. The time period over which stability is determined is dictated by the length of the study. Degradation products should be identified and quantitated. The shelf-life and storage conditions should be documented.

4.2.9 Robustness

Analytical methods are expected to perform in an acceptable manner at all times. Robustness establishes the ability of the method to perform reliably with respect to deliberate variations in the

method parameters [179, 180]. Robustness can be assumed by adopting good system suitability specifications [192]. These tests should be performed at different values of each operating parameter, that could affect method performance [205].

A univariate or multivariate approach to method robustness can be adopted [185]. The univariate assessment involves sequential, systematic modification of each parameter [207]. The multivariate approach comprises simultaneous evaluation of several parameters. Resolution, peak area, migration time and peak order are measured and statistically examined to assess the magnitude of the tolerance limits that can be prescribed for each operating parameter [185].

Statistical tests such as the fractional factorial and central composite are utilised to screen the impact of modifying several operating parameters, simultaneously [208 - 211]. The electrolyte pH and concentration, rinse conditions, temperature, detection wavelength, injection conditions and applied voltage are varied by 5 - 10 % above or below the ideal value [185]. Interactions between parameters in CE are more prominent than in HPLC [210, 211], therefore it is preferable to adopt the multivariate approach. Changes in individual parameters may not affect system performance, however simultaneous fluctuations in several parameters are more likely to influence the system performance [185].

4.3 VALIDATION PROCEDURE

4.3.1 Precision

4.3.1.1 Experimental Procedure

4.3.1.1.1 Electrophoretic Conditions

- CE system — see Section 3.2.1.1
- Capillary dimensions — Inner diameter 50 µm, total length 114.5 cm, effective length 75 cm (Polymicro Technologies Incorporated, Phoenix, USA)
- Detector wavelength — 200 nm
- Attenuation — 0.005 AUFS
- Rise Time — 3 seconds
- Sample tray temperature — 30°C
- Injection conditions — 50 mbar for 5 seconds
- Applied voltage — 20 kV

- Chart speed 0.5 mm/min
- Capillary conditioning see Section 3.2.3.5
- Electrolyte replenishment as described in Section 3.2.3.6

4.3.1.1.2 Raw Materials and Reagents

- Erythromycin [1] Cat No 24200
- Josamycin [2] Lot JSML149

[1] United States Pharmacopoeial Convention Inc, Rockville, MD, USA.
[2] Yamanouchi Pharmaceutical Company, Tokyo, Japan.

Reagents used in the analysis were as reported in Section 3.2.2.2.

4.3.1.1.3 Preparation of Electrolyte

A 50 mM phosphate buffer (pH 7.5) was prepared as described in Section 3.2.3.2. The molarity and pH of the electrolyte selected were found to yield optimal resolution under the conditions listed in section 4.3.1.1.1.

4.3.1.1.4 Internal Standard Preparation

Josamycin is a member of the macrolide group of antibiotics. It is structurally similar to erythromycin and was therefore selected as the internal standard. An internal standard stock solution was prepared by accurately weighing approximately 30 mg of josamycin into a 25 ml volumetric flask, dissolving it in 5 ml of acetonitrile and making it up to volume with water, to yield a concentration of 1.26 mg/ml.

4.3.1.1.5 Sample Preparation

A standard stock solution of erythromycin (1.02 mg/ml) was prepared by accurately weighing approximately 100 mg into a volumetric flask, dissolving it in 20 ml acetonitrile and making it up to volume with water.

4.3.1.1.6 Repeatability

Repeatability was assessed by performing nine consecutive injections of a solution of erythromycin at a concentration of approximately 0.8 mg/ml. The solution was prepared by

pipetting 16 ml of the standard stock solution (Section 4.3.1.1.5) into a 20 ml volumetric flask and making it up to volume with the internal standard stock solution. The solution was injected through a 0.45 µm membrane filter into a 4 ml vial using a 10 ml glass syringe. The vials were capped as reported in Section 3.2.3.3. The electrophoretic parameters described in Section 4.3.1.1.1 were followed.

4.3.1.1.7 Rinsing Conditions

The influence of the capillary rinse procedure on migration time precision was assessed by performing seven consecutive injections on the same day. Between injections, the capillary was either flushed with electrolyte for 3 min or with 0.1 M NaOH for 3 min, followed by water for 3 min and then electrolyte for 3 min. A pressure of 2000 mbar was applied to flush the capillary. The sample and electrolyte were prepared as reported in Section 4.3.1.1.6 and 4.3.1.1.3 respectively. The operating parameters specified in Section 4.3.1.1.1 were employed.

4.3.1.1.8 Pressure versus Electrokinetic Rinsing

Nine consecutive injections were performed on the same day to assess the effect of applying pressure or voltage during the wash sequence on the migration time precision. Preconditioning between injections consisted of either flushing the capillary with the background electrolyte using 2000 mbar for 3 min or applying 10 kV for 3 min. All other conditions were as described in Section 4.3.1.1.7.

4.3.1.1.9 Intermediate Precision

To assess the intermediate precision of the system, nine replicate injections were performed on each of three consecutive days. Freshly prepared working solutions of erythromycin, internal standard and electrolyte, as described in Sections 4.3.1.1.5, 4.3.1.1.4 and 4.3.1.1.3 respectively were used.

4.3.1.2 Results

The repeatability of the assay was calculated as the relative percent standard deviation (% RSD) of each series of replicate injections. Table 4.1 shows the percent relative standard deviation for the precision of the migration time, relative migration time of erythromycin to internal standard, normalised peak area, relative normalised peak area, peak height and relative peak height.

Table 4.1 Precision Data for Various Parameters (% RSD)

Day	Migration Time	Relative Migration Time	Normalised Peak Area	Relative Normalised Peak Area	Peak Height	Relative Peak Height
Day 1	0.22	0.05	2.60	2.34	1.01	1.06
Day 3	0.04	0.04	2.10	1.91	0.91	1.40
Day 4	0.10	0.03	1.71	1.40	0.72	0.83
Day 6	0.12	0.06	2.86	2.18	0.87	2.01

Peak area and peak height precision of less than 2 % RSD and migration time precision of less than 1 % RSD are acceptable for hydrodynamic injections. From Table 4.1 it is observed that acceptable precision was routinely obtained for the migration time and peak heights using the method developed for the assay of erythromycin. As variations in sample injection volumes and migration time are internally compensated form, variability is reduced by employing an internal standard. The precision for the relative migration time was significantly better than that for the migration time. In addition, normalised relative peak areas of erythromycin to the internal standard were found to be more precise than for erythromycin alone. The precision data for the peak height and relative peak height were within the acceptable range. Peak area precision was not as good as peak height precision due to the inability of the integrator to detect fast moving peaks.

The migration time and calibration response precision for the series of injections in which the capillary was conditioned with different rinse procedures is summarised in Table 4.2.

Table 4.2 Precision Data (% RSD) for the Capillary Rinsing Procedure

Rinse Conditions	Migration Time	Relative Migration Time	Normalised Peak Area	Relative Normalised Peak Area	Peak Height	Relative Peak Height
NaOH Rinse	1.50	0.46	3.89	3.71	2.26	2.55
Buffer Rinse	0.09	0.04	1.75	1.55	1.09	1.22
Pressure Rinse	0.12	0.07	2.02	2.02	1.30	1.41
Voltage Rinse	0.05	0.04	1.92	1.70	0.97	1.09

Superior precision was obtained when the capillary was conditioned with only the background electrolyte between consecutive injections. In addition, improved precision was obtained when applying a voltage during the rinse procedure rather than flushing the capillary with pressure.

The intermediate precision of the method was assessed by calculating the percent relative standard deviations for the various parameters over four days. Acceptable precision of less than 0.25 % RSD was obtained for the relative migration time and less than 2.7 % RSD for the relative normalised peak area and relative peak height. These data indicate that the assay method for the quantitation of erythromycin is precise when conducted on different days. It was not possible for the method to be analysed by a second analyst.

4.3.1.3 Discussion

Instrument manufacturers typically quote that % RSD values of less than 2 % can be routinely obtained [220]. Migration time precision for the main peak assay in CE is typically within the range of 0.5 % - 1.0 % RSD [183, 208, 212 - 215]. For the assay developed for the quantitation of erythromycin migration time and relative migration time precision of less than 0.25% and 0.07 % were achieved. Migration time is an important parameter that is used to assess reproducibility of resolution and peak identity. In addition, migration time is also used to assess the overall performance of the method and instrumentation.

Quantitative precision in CE as illustrated by peak area reproducibility, is generally poorer than in HPLC. Values ranging from 1% - 2 % RSD are attained in CE [183, 213, 214] as opposed to 0.5 % - 1.0 % RSD in HPLC [133, 208]. The peak area precision values in this study were not within the acceptable range for CE. The precision can be improved by employing an integrator that is capable of accurately detecting the up slope and down slope of the peak and thus correctly integrating the peak area. Excellent precision for peak area and migration time can only be achieved through meticulous capillary preparation, sample introduction and peak integration.

Preconditioning the capillary with the background electrolyte between consecutive injections produces superior migration time and calibration response precision [186, 216]. Rinsing the capillary with sodium hydroxide influences the stability of the capillary surface such that a quasi-stable state and not an equilibrium state is produced. This is significantly more apparent with phosphate buffer, which is known to require longer equilibration times. Elaborate washing techniques, such as the use of voltage, improve precision by facilitating equilibration of the system under electrophoretic conditions.

Precision in CE can be reduced further, with typical % RSD values of below 1 %, by employing an internal standard [200]. The migration time and normalised peak area precision were reduced in the presence of an internal standard, however values of less than 1 % for the peak area were not achieved. A marginal decrease in precision was noted with the relative peak heights as opposed to the peak heights. A possible explanation is that the height of either the internal standard peak or the analyte peak may fluctuate independently to that of the other peak as a result of peak broadening. Consequently, greater imprecision will result if the peak height of the analyte is related to that of the internal standard.

Several factors need to be controlled to attain acceptable levels of precision. In addition to controlling the major factors, sample concentration and temperature, control of the subtle factors can improve precision significantly [182, 183]. These factors include minimising siphoning effects, placing the sample vials and corresponding buffers vials adjacent to each other in the carousel and minimising the number of assays performed in each buffer vial, *inter alia* [183, 186].

4.3.2 Linearity

4.3.2.1 Experimental Procedure

For the establishment of linearity six standards of varying concentrations were analysed. The range selected (0.1 - 1.4 mg/ml) covered 10 - 140 % of the nominal assay concentration. The individual standards were prepared by dilution of the stock solution as described below. Five replicate injections were made at each concentration. The operating parameters, as reported in Section 4.3.1.1, were maintained. A 50 mM phosphate buffer (pH 7.5) was prepared as described in Section 4.3.1.1.3. Linearity was established over each of three days.

Sample Preparation

A stock solution of erythromycin (2.04 mg/ml), was prepared by accurately weighing approximately 200 mg into a 100 ml volumetric flask and dissolving it in 20 ml acetonitrile. The resultant solution was made up to volume with water. The internal standard stock solution was prepared as described in Section 4.3.1.1.4. The stock solution of erythromycin was diluted to produce six different concentrations that ranged from 0.1 to 1.4 mg/ml. The internal standard stock solution (4 ml) was added to each of the standard solutions to yield a concentration of approximately 0.25 mg/ml. The resultant solutions were made up to volume with an acetonitrile:water mixture (20:80 v/v).

4.3.2.2 Results

Calibration curves for each day and a mean calibration curve for the three days were constructed by plotting the relative normalised peak area of erythromycin to that of the internal standard as a function of the analyte concentration. The results were evaluated through calculation of a linear regression line by the method of least squares. Individual calibration curves for the three days, together with a mean curve, are illustrated in Figure 4.1.

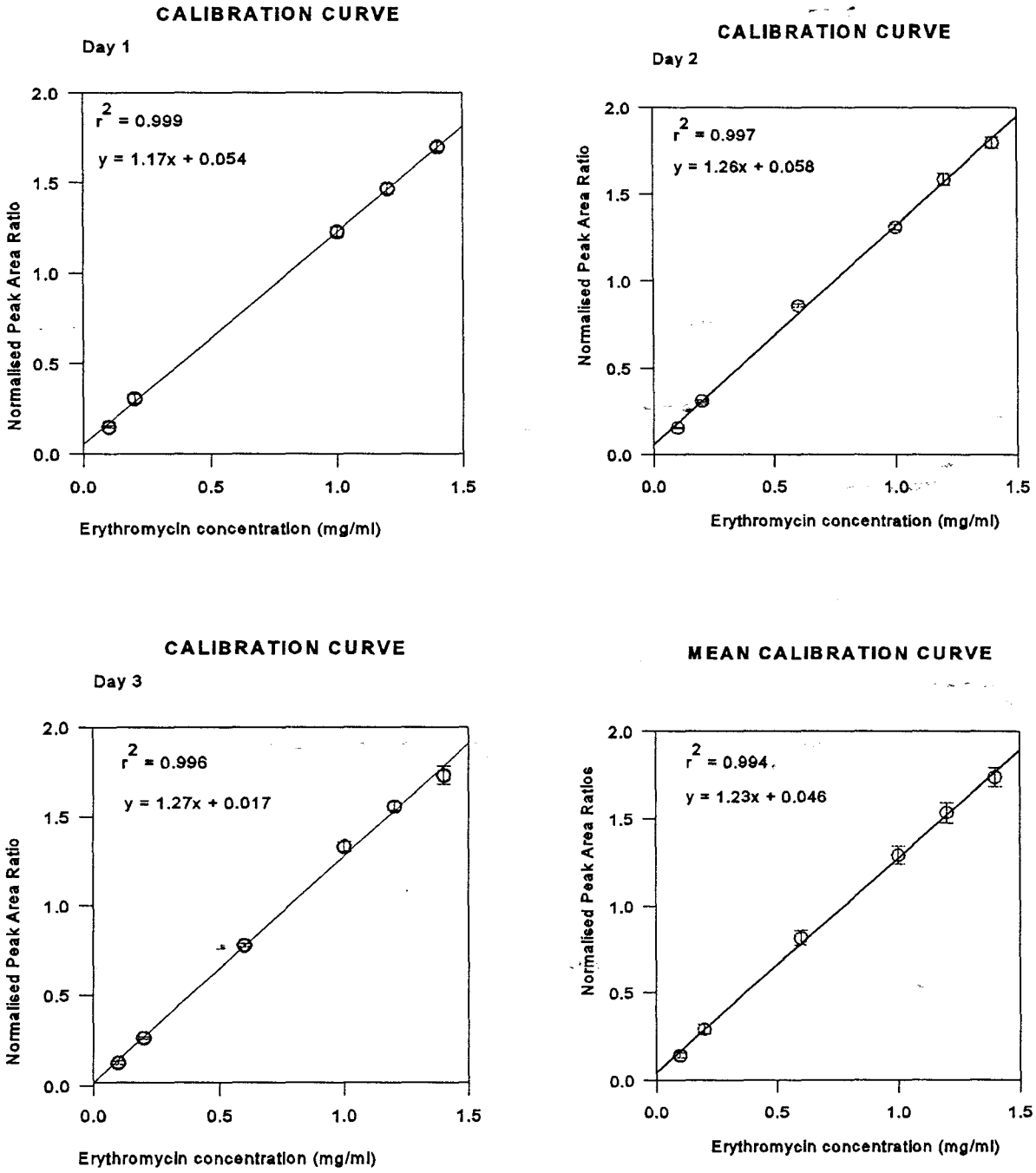


Figure 4.1 Calibration curves constructed on each of three days and mean calibration curve for the three days, by linear regression of mean relative normalised peak area vs concentration.

A parabolic profile was obtained when the relative peak heights of erythromycin to josamycin were plotted as a function of the analyte concentration (Figure 4.2). The regression line was calculated using a second order non-linear regression analysis.

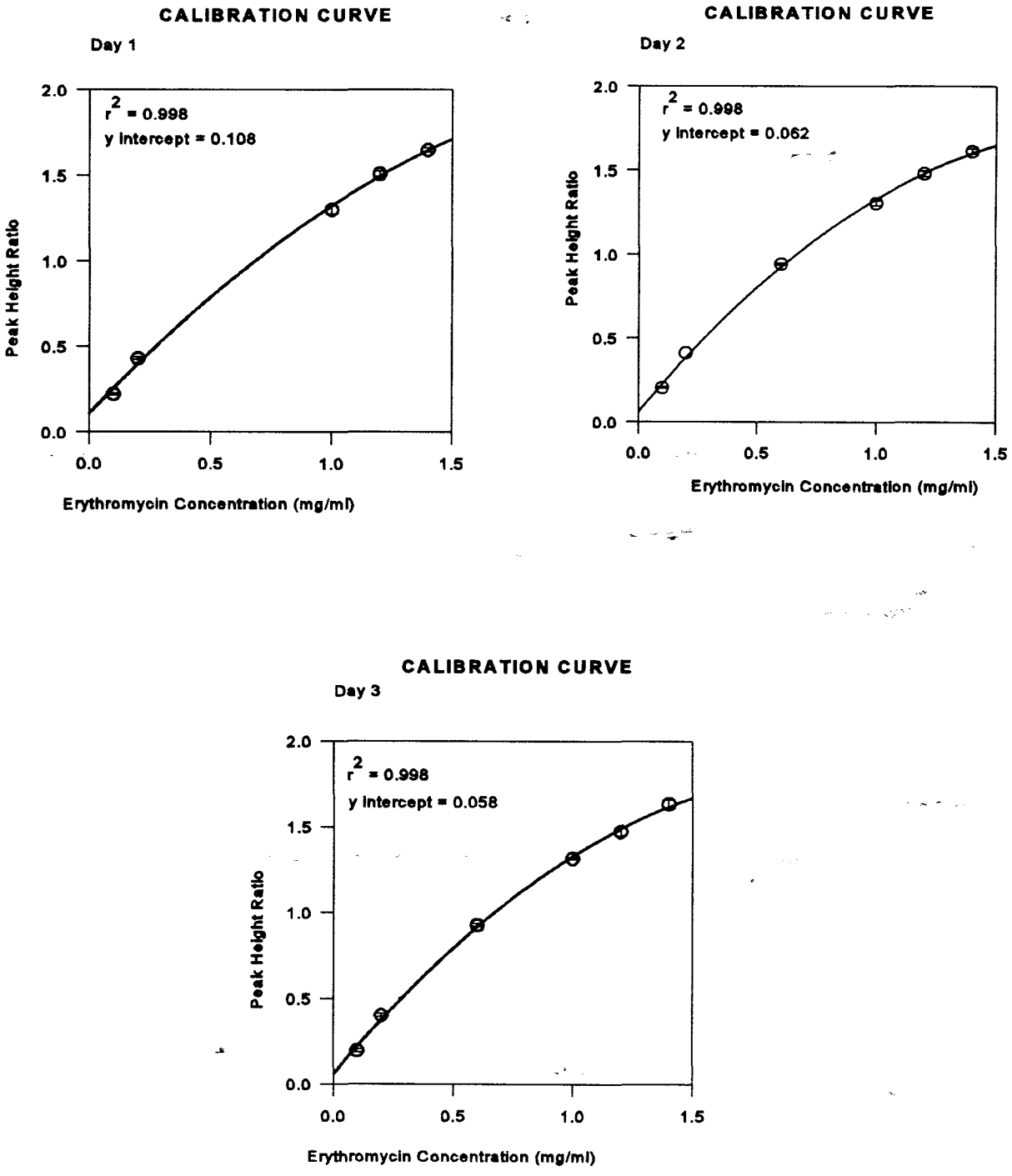


Figure 4.2 Calibration curves constructed on each of the three days by second order regression of mean relative peak height vs concentration.

Acceptable linearity was demonstrated with correlation coefficients of greater than 0.99 for normalised peak area ratio (Figures 4.1). The percentage intercept value for the peak area ratio data was approximately 4 % of the peak area ratio value obtained for the target concentration (1mg/ml). This is above the limit, as values of 2 % are widely regarded as an acceptable deviation [195].

4.3.2.3 Discussion

The method developed for the assay of erythromycin demonstrated acceptable linearity between the detector response and erythromycin concentration in the range 10 - 140 % on all three days. Typically, linearity of response is shown to extend over three orders of magnitude (50 - 150 % of the nominal assay concentration) in CE for UV absorbance detectors [214].

When peak heights were used, the calibration function was curved and approximately parabolic. The non-linearity observed at high sample concentration was due to peak broadening [144, 199]. The slope of the calibration function, and thus the sensitivity, decreased at higher concentrations [144]. Consequently, detector linearity was assessed using normalised peak area ratios as opposed to peak height ratios.

The y-intercept should not be significantly different from zero. Positive deviations of the intercept were attributed to spontaneous injection of sample solution into the capillary on insertion of the capillary into the sample solution. Errors associated with each level were eliminated by performing multiple injections at each concentration.

4.3.3 Accuracy

4.2.3.1 Experimental Procedure

Accuracy was determined concurrently with linearity on each of three days. As recommended by the ICH [180], nine determinations over three concentration levels within the predetermined range, (three concentrations in triplicate) were performed. The electrolyte was prepared as described in Section 4.3.1.1.3 and the operating parameters reported in Section 4.3.1.1.1 were followed.

Sample Preparation

A fresh standard stock solution of erythromycin (2.01 mg/ml), prepared as described in Section 4.3.1.1.5, was diluted to yield analytical controls of concentrations 0.4, 0.7 and 1.25 mg/ml. The appropriate volume of internal standard was added to each solution and the solutions were made up to volume with a mixture of acetonitrile:water (20:80 v/v) to give a final concentration of 0.25 mg/ml.

4.3.3.2 Results

The concentration of erythromycin in each of the analytical controls was calculated by substituting the normalised peak area ratio or peak height ratio of erythromycin to the internal standard obtained from the analysis of the above solutions into the appropriate regression equation. Accuracy was assessed by comparing the mean concentrations of erythromycin that were determined with the actual concentrations and reported as a percentage difference using the equation below:

$$\% \ Bias = \frac{Amount\ determined - Amount\ weighed}{Amount\ determined} X\ 100$$

In addition, accuracy was reported as a percent recovery by assay of a known added amount of erythromycin in the sample, using the equation below:

$$\% \ Recovery = \frac{Amount\ determined}{Amount\ weighed} X\ 100$$

The results are presented in Tables 4.3 and 4.4. The mean value of the measured concentration of erythromycin is an estimation of the accuracy of the method and the average relative percent standard deviation (% RSD) provides an indication of the analysis variation [191].

Table 4.3 Mean Accuracy Data Obtained from the Regression Line of the Relative Normalised Peak Area vs Concentration

Day	Mean Calculated Concentration of Erythromycin (mg/ml)	Mean Actual Concentration of Erythromycin (mg/ml)	Mean % Bias	Mean % Recovery
Day 1	0.41	0.40	2.44	102.5
y = 1.17x + 0.054	0.71	0.70	1.41	101.4
	1.25	1.26	-0.80	99.2
Day 2	0.40	0.40	0.00	100.0
y = 1.26x + 0.058	0.70	0.69	1.42	101.4
	1.25	1.24	0.80	100.8
Day 3	0.42	0.40	4.76	105.0
y = 1.27x + 0.017	0.72	0.70	2.78	103.0
	1.26	1.26	0.00	100.0

Table 4.4 Mean Accuracy Data Obtained from the Second Order Regression Line of the Relative Peak Height vs Concentration

Day	Mean Calculated Concentration of Erythromycin (mg/ml)	Mean Actual Concentration of Erythromycin (mg/ml)	Mean % Bias	Mean % Recovery
Day 1	0.41	0.40	2.44	102.5
	0.71	0.70	1.41	101.4
	1.27	1.26	0.79	100.8
Day 2	0.42	0.40	4.76	105.0
	0.71	0.69	2.82	102.9
	1.26	1.24	1.57	101.6
Day 3	0.42	0.40	4.76	105.0
	0.70	0.70	0.00	100.0
	1.25	1.26	-0.80	99.2

The mean % bias calculated from the regression lines of the relative normalised peak area and from the relative peak height ranged from -0.8 % to 4.76 %. The mean % recovery calculated from both of the regression lines was between 99 % and 105 %. A recovery limit of between 95 - 105 % of the value is acceptable, therefore the accuracy of data in this study was within the acceptable range.

4.3.3.3 Discussion

The accuracy of a CE assay is dependent on precise sample loading, reproducible zone broadening during migration, correct peak signal integration and meticulous migration times [217]. The method developed for the analysis of erythromycin exhibited acceptable accuracy at each of the three concentrations examined. However, at the lower end of the concentration range the mean percentage bias and mean percentage recovery were slightly greater, though acceptable. The assay, therefore, may be more accurate and practical using slightly higher concentrations at the lower end of the range.

4.3.4 Sensitivity

4.3.4.1 Experimental Procedure

The Limit of Detection (LOD) was determined visually, by injecting serially diluted samples. A minimum of six injections were performed at each concentration until the peak area and height were no longer reliably detected. The LOQ was ascertained in a similar manner, until a relative percent standard deviation (% RSD) for the precision of relative peak height or relative normalised peak area of less than 10 % was obtained. The buffer was prepared as previously described in Section 4.3.1.1.3 and the selected operating parameters were as described in Section 4.3.1.1.1.

Sample Preparation

An aliquot of 2.5 ml of a 2 mg/ml stock solution of erythromycin, prepared as described in Section 4.3.1.1.5, was made up to 50 ml with a mixture of acetonitrile:water (20:80 v/v), to yield a concentration of 0.1 mg/ml. This solution was serially diluted to obtain four concentrations ranging from 0.04 to 0.02 mg/ml. The appropriate quantity of internal standard was added to each solution to yield a concentration of 0.25 mg/ml.

4.3.4.2 Results

The % RSD of relative peak height and relative normalised peak area of erythromycin at each concentration are tabulated below (Table 4.5).

Table 4.5 LOQ and LOD Data for Erythromycin

	Concentration of Erythromycin (mg/ml)	Relative Peak Height % RSD	Relative Normalised Peak Area % RSD
Day 1	0.030	4.14	4.10
	0.040	4.57	1.09
Day 2	0.020	1.00	6.80
	0.025	6.40	7.20
	0.030	5.44	7.21
	0.040	1.50	3.90
Day 3	0.025	8.79	11.15
	0.030	6.62	7.27
	0.040	5.01	3.14

A LOD of 0.03 mg/ml, which corresponds to 3 % of the nominal concentration (1 mg/ml), was selected as the lowest concentration of erythromycin at which the peak height and area could be reliably detected. The minimum level at which erythromycin could be quantified, with acceptable precision and accuracy (< 10 % RSD) was determined to be 0.04 mg/ml. This was selected as the LOQ and corresponded to 4 % of the nominal concentration (1 mg/ml). An RSD of less than 10 % for normalised peak area ratios was obtained for five replicate injections.

4.3.4.3 Discussion

Sensitivity in CE is inferior to HPLC. The LOD and LOQ are limited by the small loading capacity of the capillary and the short pathlength of the detector cell. The assay developed for the determination of erythromycin was not found to be very sensitive. Sensitivity can be maximised by enhancing the signal while concurrently reducing the noise level. Several strategies can be implemented to maximise sensitivity in CE to achieve levels of 0.15 % of the nominal concentration or lower.

Detection sensitivity of erythromycin could be improved by increasing the pathlength, either through the employment of wide bore capillaries [183] or modified capillaries such as the Z-shaped flow cell [218] or bubble cell. In addition, on-capillary concentration techniques such as FASI [143, 197], can be used to lower the limit of detection. However, in FASI the sample would have to be electrokinetically injected onto the capillary. This may result in a lower level of precision and thus limit the benefits of this technique in quantitating erythromycin. Detection sensitivity can be enhanced by selecting a low UV wavelength where molar absorptivity coefficients are high [196, 216]. However, in this study a low wavelength of 200 nm was used.

Injecting highly concentrated samples of erythromycin [219] can also be used to improve the detection sensitivity.

Furthermore, detection sensitivity can be optimised by ensuring that the detection window is clean, suitably aligned and that the alignment is stable while the capillary is in the instrument. Therefore, it is feasible to enhance sensitivity in CE to levels that are comparable to HPLC by implementing the above strategies [85].

The gradual loss of sensitivity of detector lamps with age and noise level variations complicate the attainment of good detector precision of low concentrations of compounds. Determination of the sensitivity of the method using the signal-to-noise approach is impractical as the noise level varies with detectors.

4.3.5 Stability

4.3.5.1 Experimental Procedure

Sample Preparation

A stock solution of erythromycin was prepared as described in Section 4.3.1.1.5. Equivalent volumes of this solution were stored in the refrigerator at 4°C and on the bench top at 22°C for four days. On each of three days, the stock solution from the refrigerator and bench top were diluted with the appropriate volume of internal standard solution and made up to volume with an acetonitrile:water (20:80 v/v) mixture to yield a final concentration of approximately 0.8 mg/ml of erythromycin and 0.25 mg/ml of internal standard. The solutions from the refrigerator were allowed to equilibrate to ambient temperature prior to dilution.

Stability was assessed over four days. On each day, fresh electrolyte was prepared as described in Section 4.3.1.1.3. Six consecutive injections of the samples that were stored on the bench and in the refrigerator were performed on each day.

4.3.5.2 Results

Stability data is often presented as a comparison of the mean concentration or response factors found in freshly prepared samples with those found in samples stored under various conditions for various periods of time. Table 4.6 presents the mean and % RSD for the precision of the relative peak height and relative normalised peak area of the samples stored on the bench top and in the refrigerator for several days.

Table 4.6 Stability Data of Erythromycin: Mean and Percent Relative Standard Deviation of Relative Normalised Peak Area and Relative Peak Height

	Day 1	Day 2	Day 4
Relative Peak Height			
Refrigerator	1.14 (0.52%)	1.10 (0.87%)	1.08 (0.58%)
Bench	1.05 (0.81%)	1.05 (0.81%)	1.07 (0.58%)
Relative Normalised Peak Area			
Refrigerator	0.99 (6.26%)	0.84 (2.37%)	0.95 (1.88%)
Bench	0.87 (3.77%)	0.96 (2.43%)	0.89 (3.22%)

Consistent peak height and peak area ratios for the samples stored in the refrigerator and on the bench over 4 days, and the absence of any additional peaks indicated good solution stability. Acceptable precision was obtained as indicated in parentheses in the above table. Erythromycin solutions were found to be relatively stable and the conditions of storage were not found to impair stability. Consequently, solutions of erythromycin in methanol and acetonitrile can be stored for up to 4 days in either the refrigerator or on the bench top.

4.3.5.3 Discussion

In a study conducted by Terespolsky and Kanfer [171] on the stability of erythromycin and some of its esters in methanol and acetonitrile, it was found that erythromycin was relatively stable in both methanol and acetonitrile. Of the two solvents examined, acetonitrile was found to be the preferred solvent as a 5 % degradation of erythromycin in the presence of methanol was noted.

4.4 CONCLUSION

A rapid, uncomplicated assay for the quantitation of erythromycin, with acceptable precision and accuracy has been developed. Successful validation included an assessment of precision, accuracy, linearity, sensitivity and stability.

Migration time precision in CE is comparable to HPLC (less than 1 % RSD), especially when relative migration times are used. The assay developed for the quantitation of erythromycin demonstrated acceptable migration time precision and peak height precision, however peak area precision was marginally above the acceptable limits. The use of CE for quantitative analysis has

been limited by poor peak area precision. Precision of injection, as measured by peak area reproducibility, is poorer in CE than HPLC, with conventional values of 1 - 2 % RSD as opposed to 0.5 - 1 % RSD. Several factors affect peak area precision. These include low sample loadability, injection volume variability, solute adsorption onto the capillary surface and integration errors. Knowledge and understanding of these factors facilitates the design of experiments to overcome these problems. The use of automated instrumentation and averaging of multiple injections reduces random errors [217] and subsequently improves migration time, peak area and peak height precision. In addition, systematic errors can be minimised with the incorporation of an appropriate internal standard.

Sensitivity in HPLC is generally an order of magnitude lower than in CE. Typically, LOD values of 0.1 % are attained in CE as compared to 0.01 % in HPLC. The erythromycin assay developed exhibited poor sensitivity. There are several techniques that can be implemented to improve the sensitivity of detection. These include increasing the pathlength, on-capillary concentration techniques such as FASI, employing low detection wavelengths and injecting sample of high concentration, to name a few.

A combination of the analytical techniques, HPLC, TLC and CE, gives widely differing selectivities and facilitates good overall assessment of product purity. Several reports of cross correlation between CE and HPLC have been published [213, 214, 216, 220, 221]. Ackerman et al. [214] conducted a comparison of three analytical techniques: isotachophoresis, CZE and HPLC for the determination of several drugs. The authors concluded that CZE is capable of providing comparable quantitative information. In addition good cross correlation for related impurities was obtained between HPLC, TLC and CE for ranitidine [222], fluparoxan [223], sumatriptan [210], cefotaxime [221] and salbutamol [216]. However, HPLC results and CE results did not agree for the analysis of domperidone [224] and tetracyclines [225]. The additional information obtained from a second analytical technique necessitates the development and validation of a CE method for all drugs of interest.

The CE method developed for the analysis of erythromycin is rapid, precise, accurate and stability indicating. In addition, if offers distinct advantages over the current official microbiological methods, which lack specificity, are time consuming and tedious to perform.

CHAPTER FIVE

METHOD DEVELOPMENT FOR THE SEPARATION OF ERYTHROMYCIN AND RELATED SUBSTANCES

5.1 INTRODUCTION

Analytical techniques for the assay of erythromycin and its related substances in raw materials and in pharmaceutical dosage forms must be capable of determining the purity and stability of the compound before manufacture and during storage. The USP protocols require IR spectra and or TLC patterns for compound identification and microbial assay for potency determinations. The inability of these methods to differentiate between erythromycin and its related substances has led to the development of alternative analytical procedures for the analysis of erythromycin. These include pyrolysis (GC) [226] and agarose gel electrophoresis [227], which provide qualitative differentiation among the compounds, and LC and HPLC which provide quantitative information.

The HPLC and LC methods developed for the analysis of erythromycin and its related substances lack the resolution required for the separation of several related substances. However, Cachet et al. [228] were successful in separating most of the related substances and impurities by using a column-switching technique. The method adopted by the European Pharmacopoeia for the analysis of erythromycin provides improved selectivity, but is complex and exhibits poor ruggedness. Nasr at al. [229] developed a simple, sensitive, rugged method for the analysis of erythromycin and some of its related substances. The samples injected were at a concentration of between 5 and 10 mg/ml and the analysis time was less than 60 minutes.

An investigation of CE as a separation technique for the analysis of erythromycin and its related substances was conducted in light of the numerous advantages afforded by this technique. High peak efficiencies that are attained in CE facilitate discrimination of structurally-similar impurities and related substances. Sensitive detection of erythromycin and related substances presents a challenge as the compounds lack significant chromophores. In CE, the use of low wavelengths (190 - 210 nm), where most substances have enhanced UV absorbances, permits effective analysis of these compounds.

5.2 EXPERIMENTAL PROCEDURE

5.2.1 Electrophoretic Conditions

• CE system	as described in Section 3.2.1.1
• Uncoated silica capillary	75 µm I.D., total length 132 cm, effective length 80 cm (Polymicro Technologies, Phoenix, AZ, USA).
• CElect P150 coated capillary	75 µm I.D., total length 100 cm, effective length 65 cm (Supelco Inc, Supelco Park, Bellefonte, PA, USA).
• CElect H150 coated capillary	75 µm I.D., total length 100 cm, effective length 65 cm (Supelco Inc, Supelco Park, Bellefonte, PA, USA)
• UV wavelength	200 nm
• Attenuation	0.005 AUFS
• Rise time	3 seconds
• Injection pressure	100 mbar
• Injection time	5 seconds
• Co-injection conditions	50 mbar for 5 seconds
• Separation voltage	30 kV
• Temperature	30°C
• Capillary conditioning	see Section 3.2.3.5
• Electrolyte replenishment	as described in Section 3.2.3.7

5.2.2 Raw materials and Reagents

5.2.2.1 Raw Materials

Erythromycin A (EA) [1]	Lot 1
Erythromycin B (EB) [1]	Lot 1
Erythromycin C (EC) [1]	Lot 1
Erythromycin enol ether (EEE) [2]	Lot RS45232-139
Anhydroerythromycin (AE) [2]	Lot 66-473-BD
N-Demethylerythromycin A (NDEA) [1]	Lot 1

[1] European Pharmacopoeia, Strasbourg, Cedex 1, France.

[2] Abbott Laboratories, North Chicago, IL 60064, USA.

5.2.2.2 Reagents

Phosphoric acid [1]
Sodium Hydroxide pellets [2]
Acetonitrile (distilled in glass, UV grade)[3]
Methanol (HPLC grade) [2]
Ethanol (gradient grade for chromatography) [4]
Hydroxyethylcellulose [2]
Polyvinylalcohol (MM 70,000-100,000)[5]

[1] Holpro Analytics, Johannesburg, SA.
[2] Merck, Darmstadt, Germany.
[3] Burdick & Jackson Division, Muskegon, MI, USA.
[4] British Drug House, Poole, Dorset, UK.
[5] Sigma Chemical Company, St Louis, MO, USA .

5.2.3 Determination of UV Spectra for the Related Substances

UV spectra of erythromycin and its related substances and impurities were determined. The λ_{max} for the compounds are tabulate below.

Table 5.1 λ_{max} of Erythromycin and its Related Substances

Compound	λ_{max}
erythromycin A	200.4
erythromycin B	222.1
erythromycin C	204
erythromycin enol ether	214
demethylerythromycin	205.4
anhydroerythromycin	208

It was decided the select a detection wavelength of 200 nm for the study. Erythromycin A absorbs maximally at this wavelength, however the related substances possess different absorbance spectra.

5.2.4 Preparation of Electrolyte

A phosphate buffer of pH 7.5 and required molarity was prepared by pipetting the appropriate volume of phosphoric acid into a volumetric flask and diluting to volume with HPLC grade water. A 1 M NaOH solution was prepared by weighing 4 g of NaOH pellets into a 100 ml volumetric flask and making up to volume with water. This solution was used to adjust the pH to 7.5. The resultant buffer was deaerated under aspirator vacuum and filtered through a 0.45 µm membrane filter prior to use. The buffer was filled into the anode and cathode reservoirs as reported in Section 3.2.3.2.

The phosphate buffer employed in this study was prepared differently to that in Chapter Three. The high electrolyte molarities investigated and the presence of large concentrations of organic solvent in the electrolyte cause the precipitation of the buffer salts. Consequently, it was decided to decrease the number of ions present in the electrolyte by using phosphoric acid.

5.2.5 Sample Preparation

Individual standard solutions of EA, EB, EC, EEE, AE and NDEA were prepared by accurately weighing approximately 10 mg into a 20 ml volumetric flask, dissolving in 10 ml acetonitrile and diluting to volume with water to yield a concentration of 0.5 mg/ml. A standard solution, comprising all the impurities and related substances, was also prepared, as above, such that the final solution contained each impurity at a concentration of approximately 0.5 mg/ml. An increase in the proportion of acetonitrile in the sample diluent (acetonitrile:water 50:50 v/v) was necessary to maintain the solubility of all the components. The sample injected into the coated capillaries consisted only of the closely migrating compounds, EB, EA, EEE and EC, each at a concentration of 0.5 mg/ml.

5.2.6 Method Development

The initial conditions that were employed for the analysis of the mixture of erythromycin and its related substances were those adopted for the separation of the macrolides erythromycin, troleandomycin and josamycin (Chapter Three). A phosphate buffer (pH 7.5), was selected as the electrolyte. Several parameters were manipulated to achieve resolution of erythromycin and its related substances. Resolution can be enhanced by reducing the EOF and augmenting the differential electrophoretic mobilities of the compounds present in the mixture.

The electrolyte molarity was examined in the range 50 to 190 mM. The EOF is reduced in electrolytes of high molarity. However, the current level and amount of joule heat generated is substantially greater, thus limiting the applied voltages that can be used to effect the separation.

Different organic solvents (methanol and ethanol) at concentrations of between 25 and 50 % (v/v) were incorporated into the electrolyte. Organic modifiers enhance the differences in electrophoretic mobility of the analytes and thus improve the separation selectivity. In addition, organic solvents reduce the current level and thus permit the use of high voltages without generating excessive amounts of joule heat. However, the electrolyte molarity and percentage of organic solvent incorporated into the electrolyte needs to be carefully selected as the tendency for precipitation of the buffer salts in high molarity electrolytes with large concentrations of organic solvents is very likely.

Electro-osmotic modifiers such as hydroxyethylcellulose (HEC) and polyvinyl alcohol (PVOH) were also incorporated into the buffer in various proportions, ranging from 0.05 to 0.1 % (w/v), to reduce the EOF and subsequently improve the resolution between the analytes.

Coated capillaries were also employed in an attempt to improve the resolution between the components. The EOF is sufficiently reduced in coated capillaries, thus the separation space between the analytes increases and resolution should improve. CElect P150 capillaries are coated with a neutral hydrophilic polymer, that is inert and biocompatible. CElect H150 capillaries have a C8 substituted polymer that is bonded and cross-linked to the tubing surface. This coating is moderately hydrophobic. The operating parameters reported in Section 5.2.1, were followed. The sample injected into the coated capillaries consisted only of EB, EA, EC and EEE, as resolution between these four compounds needed to be improved.

The identity of each peak in the mixture was established by co-injecting a sample of pure related substance, applying a pressure of 50 mbar for 5 seconds. An increase in the peak height and area with co-injection confirmed the identity of the peak.

In an attempt to determine the ideal conditions that are capable of providing baseline resolution of erythromycin and its related substances, simultaneous random manipulations of these parameters was performed as depicted in Table 5.2.

5.3 RESULTS

A summary of the effects of various experimental parameters on peak shape, resolution and migration time is provided in Table 5.2.

Table 5.2 Experimental Parameters that were Manipulated to Optimise the Resolution Between Erythromycin and its Related Substances

Table 5.2 a: Analysis performed in uncoated fused-silica capillary

Electrolyte Molarity (mM)	% Organic Modifier	Additive	Analysis Time (min)	Resolution	Peak Shape
100	30 % MeOH	-	28.2	No resolution	
	50% MeOH	-	41.5	Incomplete	
100	30 % EtOH	-	34.8	Incomplete	
	35 % EtOH	-	37.3	Almost baseline	
	40 % EtOH	-	44.0	Almost baseline	Very broad peaks, Decrease in peak height
120	35 % EtOH	-	39.0	Almost baseline	
	35 % EtOH	0.5 % PVOH	38.6	Almost baseline	
	35 % EtOH	0.5 % PVOH + 0.05 % HEC	37.7	Baseline	
	35 % EtOH	0.5 % PVOH + 0.1 % HEC	44.0	Baseline	Very broad peaks, decrease in peak height
140	35 % EtOH		40.0	Baseline	

Electrolyte Molarity (mM)	% Organic Modifier	Additive	Analysis Time (min)	Resolution	Peak Shape
150	30 % EtOH	-	40.4	Almost baseline	
	33 % EtOH	-	40.8	Baseline	
	35 % EtOH	-	44.0	Baseline	good peak shape
180	25 % EtOH	-	37.3	Almost baseline	
	30 % EtOH	-	41.3	Baseline	
	33 % EtOH	-	43.1	Baseline	good peak shape
	35 % EtOH	-	47.1	Baseline	Broad peaks
190	30 % EtOH	-	43.3	Baseline	
	33 % EtOH	-	44.0	Baseline	Broad peaks
	35 % EtOH	-	48.6	Baseline	

Table 5.2 b: Analysis performed in CElect P150 coated capillary

Electrolyte Molarity (mM)	% Organic Modifier	Analysis Time (min)	Resolution	Peak Shape
100	-	17.9	No resolution	
150	30 % EtOH	26.4	Almost baseline	Good peak shape
	35% EtOH	29.8	Almost baseline	Good peak shape

Table 5.2 c: Analysis performed in CElect H150 coated capillary

Electrolyte Molarity (mM)	% Organic modifier	Analysis Time (min)	Resolution	Peak Shape
50	30 % EtOH	26.9	Incomplete	
150	30 % EtOH	35.3	Incomplete	Broad peaks
	35 % EtOH	38.0	Incomplete	Broad peaks

Resolution was enhanced in electrolytes of high molarity and in the presence of increasing percentages of organic solvent for all the capillaries. The EOF was reduced when organic solvents were present in the buffer, consequently resolution improved. The current generated increased with high molarity electrolytes, despite the presence of organic solvents. Peak broadening and long analysis times were apparent with large concentrations of organic solvents even though the maximum separation voltage of 30 kV was selected. This resulted in a decrease in resolution and system efficiency.

There was no apparent improvement in resolution with the inclusion of the electro-osmotic modifiers hydroxyethylcellulose (HEC) and polyvinylalcohol (PVOH) at the suggested concentrations. Superior resolution was attained with the incorporation of ethanol in the electrolyte. In selecting the ideal conditions for the separation of erythromycin from its related substances, a compromise between resolution, peak shape and migration time was necessary. With the use of coated capillaries, resolution deteriorated. The peaks were broader when the CElect H150 coated capillary was used and the analysis time in both coated capillaries was substantially reduced.

Electropherograms illustrating the resolution between erythromycin and its related substances using the uncoated fused-silica capillary and CElect P150 and CElect H150 coated capillaries respectively are illustrated in Figures 5.1 a - c.

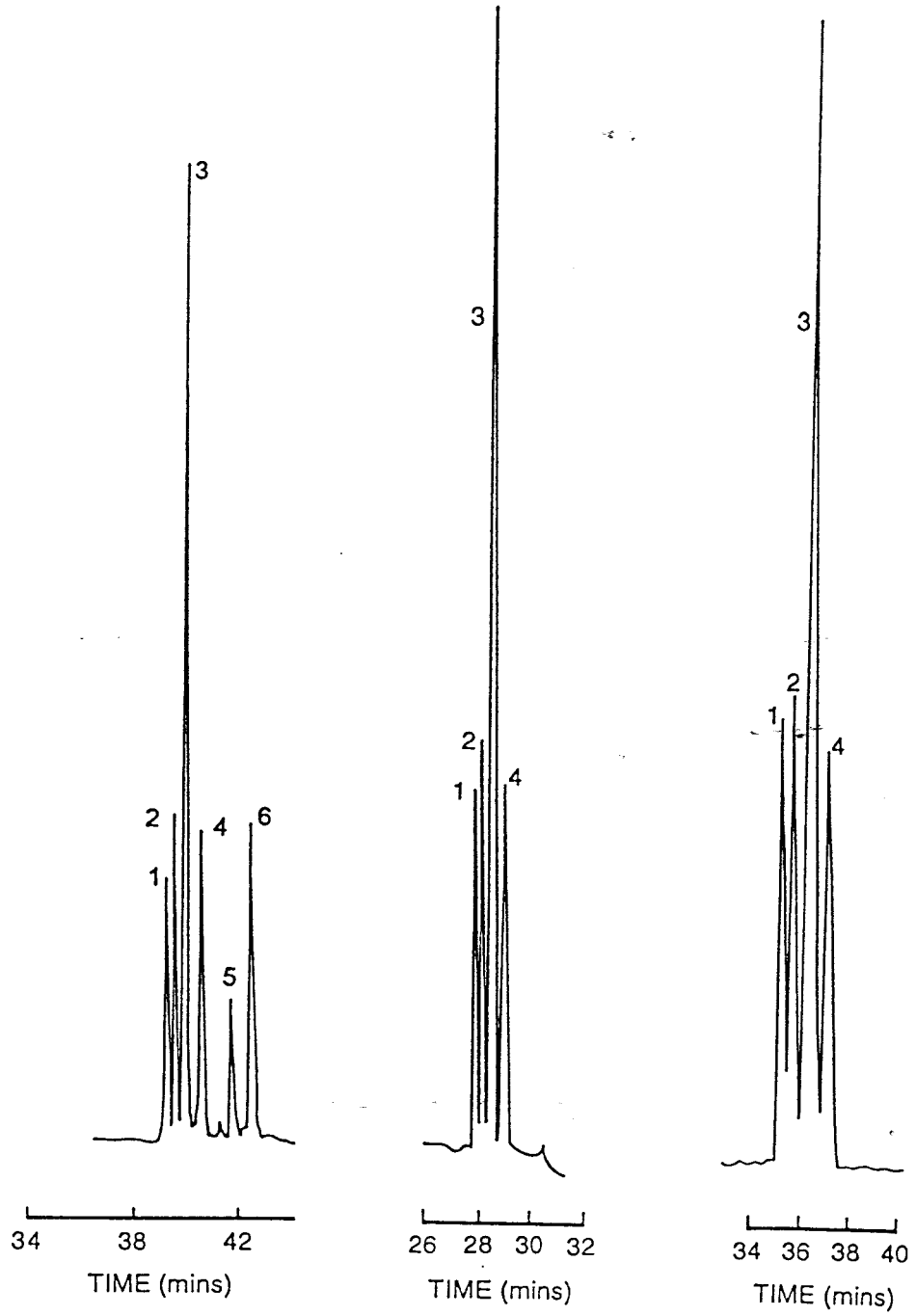


Figure 5.1 a
Uncoated silica capillary
1. Erythromycin B
2. Erythromycin A
3. Erythromycin enol ether
4. Erythromycin C
5. Anhydroerythromycin
6. Demethylerythromycin

Figure 5.1 b
CElect P150 coated capillary
1. Erythromycin B
2. Erythromycin A
3. Erythromycin enol ether
4. Erythromycin C

Figure 5.1 c
CElect H 150 coated capillary
1. Erythromycin B
2. Erythromycin A
3. Erythromycin enol ether
4. Erythromycin C

Figure 5.1 a, b, c Electropherograms of erythromycin and related substances. Conditions: phosphate buffer, 150 mM, pH 7.5, 35 % (v/v) ethanol, injection 100 mbar for 5 s, applied voltage 30 kV. Sample concentration 0.5 mg/ml in acetonitrile:water (50:50 v/v).

5.4 DISCUSSION

Resolution is based on the differential electrophoretic mobilities of solutes in an electric field. Electrophoretic mobility is related to the analyte's charge and hydration radius. The great similarity between the structures of erythromycin and the related substances is responsible for their comparable electrophoretic mobilities. This complicates the attainment of satisfactory separation in the free solution capillary electrophoresis operating mode.

The inclusion of organic modifiers enhanced the differences in the electrophoretic mobilities of the analytes, thereby facilitating resolution. There are several mechanisms by which organic solvents interact to effect the separation. These factors have been discussed in detail in Section 3.24. In summary, the organic solvents altered the pK_a values of the related substances and decreased the dielectric constant of the electrolyte, thus influencing the degree to which the related substances were ionised. In addition, they increased the viscosity of the electrolyte and reduced the charge on the silica capillary wall, thus decreasing the EOF. The combination of all these effects resulted in the improvement in resolution of erythromycin and its related substances.

Careful consideration of the buffer molarity and percentage of organic solvent in the electrolyte was necessary to prevent the precipitation of the buffer salts on the application of voltage. Precipitation results in blockage of the capillary. Therefore, it is advisable to select either a high molarity electrolyte with a low concentration of organic solvent or *vice versa*, depending on the resolution afforded by the different combinations.

The improved resolution in the presence of ethanol, as compared to methanol, was possibly due to the greater decrease in the EOF. Ethanol possesses a higher viscosity and lower dielectric constant than methanol. The EOF modifiers, HEC and PVOH, were incorporated into the electrolyte to reduce the EOF in an attempt to improve resolution. However, they were not very effective at the concentration examined.

Coated capillaries are beneficial as they are designed to provide a reduced but consistent EOF by limiting the degree of ionisation of the charged silanol groups. Consequently, there is an improvement in resolution of sample components that have similar migration times. Coated capillaries also minimise interactions between positively charged samples and the capillary wall, thereby precluding irreproducible EOF and sample loss. In addition, the surface modifications reduce pH hysteresis effects that are apparent in uncoated fused-silica capillaries. The pH range in which these coated capillaries can be utilised extends from 3 to 9. This facilitates the analysis of a wide range of analytes.

The EOF should be slightly reduced in CElect P150 coated capillaries. The profile of EOF versus pH is similar to that of uncoated fused-silica capillaries over the pH range 3 - 10. CElect H150 coated capillaries have been found to reduce the EOF significantly (approximately 42 %). Thus an improvement in resolution of the components would be expected.

Resolution between erythromycin and its related substances did not improve substantially despite the decrease in EOF that was achieved by either employing high molarity electrolytes, incorporating organic modifiers into the electrolyte or using coated capillaries. It can therefore be concluded that the decrease in the EOF was not responsible for the observed resolution. The presence of the organic solvent in the electrolyte was probably responsible for enhancing the differential electrophoretic mobilities of the compounds and thus improving resolution. The deterioration in resolution and decrease in analysis time that was noted with the coated capillaries, under similar conditions to the uncoated silica capillary, was probably due to the shorter capillary length (effective length of coated capillaries, 65 cm; effective length of uncoated capillary 80 cm).

Comparing the relative migration time of an impurity in the sample to that of a standard of the impurity resulted in incorrect peak identification. Consequently, co-injection was used to confirm peak identity as the components possessed very similar mobilities and migrated close to each other.

5.5 CONCLUSION

The CE method developed is capable of separating the impurities of erythromycin and its related substances that are commonly present in commercial samples. In addition, the method allowed for the separation of the most important metabolites of erythromycin. The optimal conditions for the resolution of erythromycin and its related substances were determined viz: uncoated fused-silica capillary 75 µm I.D., total length 132 cm, effective length 75 cm, 150 mM phosphate buffer, pH 7.5, 35 % (v/v) EtOH.

The analysis time was comparable to that attained using HPLC [228, 229], approximately 50 minutes. The resolution between the analytes was approximately one. It would be preferable to achieve resolution values of greater that two to ensure that all the compounds are well resolved from each other. Generally, resolution is improved by enhancing the differential electrophoretic mobilities of the analytes and by reducing the EOF. In this study it was found that resolution could not be further improved by decreasing the EOF, as observed from the use of coated capillaries, organic solvents and high molarity electrolyte. Therefore, enhancing the differential electrophoretic mobilities provides a plausible means for improving resolution. Organic solvents

were incorporated into the electrolyte to alter the mobilities of the analytes and consequently enhance selectivity. However, resolution could not be improved above one, even with the incorporation of various organic solvents at different concentrations.

Flurer [178] demonstrated adequate resolution between several macrolides using a cholate buffer system and an acetonitrile buffer system. In addition, it was demonstrated that the minor components present in erythromycin, oleandomycin and spiramycin and the degradation products of clarithromycin could be resolved from the parent peak when each compound was analysed separately. Flurer concluded that both systems developed for the separation of the macrolides could potentially be used for the determination of impurities, degradation products and metabolites of erythromycin in bulk pharmaceuticals.

The experimental conditions determined in this study provide one method by which erythromycin can be separated from its related substances and impurities. There are possibly several different methods by which resolution between erythromycin and its related substances and impurities can be achieved. Investigation into the various modes of CE, the development of diverse electrolyte systems and the use of different types of capillaries may lead to the development of additional methods for the analysis of erythromycin and its related substances. Each method developed offers different selectivity and provides a second means of solute identification. Therefore, it is preferable to develop several methods to ensure correct identification of the peaks.

CHAPTER SIX

ASSAY OF ERYTHROMYCIN RELATED SUBSTANCES

6.1 INTRODUCTION

Analytical methods for the determination and assay of both the active ingredients and impurities in pharmaceuticals are required for quality control. The determination of drug related impurities is currently considered to be a primary role for CE in pharmaceutical analysis [183, 187, 212, 216, 221, 230]. Analytical methods are described in the various pharmacopoeiae for the determination and characterisation of pharmaceutical raw materials and excipients. However, minimal information has been published by regulatory authorities as a guidance for impurity requirements. Consequently, the ICH has addressed this issue and proposed guidelines for the requirements for quality and safety of raw materials and drug products. In addition the guidelines describe the requirements for identification, qualification and control of impurities, in determining the purity level of raw materials and drug products [231].

Presently, HPLC is the technique of choice for the determination of drug-related impurities. High performance liquid chromatography offers the desired sensitivity for trace level determination, with typical detection limits of 0.1% or lower, and a high degree of automation. In addition, the availability of a wide range of stationary phases and operating modes extend the applicability of HPLC to many classes of drugs [219].

Impurities arise from various sources, such as starting materials and reagents, through interactions of the drug substance with excipients and packaging material and through degradation [231]. As a result, impurities may have widely differing structures and polarities. Identification of all impurities at or above 0.1% area/area of the main component is expected. Therefore, it is necessary to employ a detection system with a suitable linear range and a separation system with good resolution capability.

Verification of HPLC impurity data by employing a secondary support analytical technique, is customary. In the past, thin layer chromatography (TLC) has been employed. However, the

advent of highly automated CE instrumentation presents a novel, viable analytical alternative to the pharmaceutical industry [219]. Capillary electrophoresis offers complementary, orthogonal information to HPLC in the confirmation of purity results. Thus, these two analytical techniques are often used in combination when assessing drug purity. The differences in their separation mechanism, being partitioning in HPLC and mobility in CE, provides comprehensive information on analyte purity. An accurate reflection of the purity characteristics of a sample is desirable [180]. The degradation of the active ingredient requires judicious control.

The criteria for method validation has been discussed in detail in Section 4.2 and will be followed in this study.

6.2 VALIDATION PROCEDURE

6.2.1 Selection of an Internal Standard

6.2.1.1 Experimental Procedure

6.2.1.1.1 Electrophoretic Conditions

• CE system	as described in Section 3.2.1.1
• Capillary	75 µm I.D., 375 µm O.D. fused-silica capillary, (Polymicro Technologies, Phoenix, AZ, USA).
• Effective capillary length	80 cm
• Total capillary length	132 cm
• UV wavelength	200 nm
• Attenuation	0.005 AUFS
• Rise time	3 seconds
• Injection pressure	200 mbar
• Injection time	5 seconds
• Co-injection conditions	50 mbar for 5 seconds
• Separation voltage	30 kV
• Temperature	30°C
• Capillary conditioning	see Section 3.2.3.5
• Electrolyte replenishment	as described in Section 3.2.3.7

6.2.1.1.2 Raw Materials and Reagents

6.2.1.1.2.1 Raw Materials

• Erythromycin base [1]	Cat No 24200
• Tylosin Tartrate [2]	Lot T-6134
• Midecamycin [2]	Lot M-3143
• Spiramycin [2]	Lot S-9132
• Rifamycin [2]	Lot R-8626
• Troleandomycin base [2]	Lot 58F0356
• Oleandomycin phosphate [3]	Lot 406-767001B
• Josamycin base [4]	Lot JSML149
• Phenylpropanolamine HCl [5]	75-432
• Erythromycin base [3]	E-5889
• Erythromycin base [5]	A-7662
• Erythromycin base [6]	980-26E

[1] United States Pharmacopoeial Convention, Rockville. MD, USA.

[2] Sigma Chemical Company, St Louis, MO, USA.

[3] Pfizer Pharmaceuticals Inc, Pietermaritzberg, SA.

[4] Yamanouchi Pharmaceutical Company, Tokyo, Japan.

[5] Lennon Limited, PE, SA.

[6] Abbott Laboratories, North Chicago, USA.

6.2.1.1.2.2 Reagents

- Hydrogen peroxide (6 %) [1]
- Hydrochloric acid (32 %) [1]
- Sodium hydroxide pellets, Batch No 871009 [2]

[1] Holpro Analytics, Johannesburg, SA.

[2] British Drug House, Poole, Dorset, UK.

All other raw materials and reagents used were as specified in Section 5.2.2.

6.2.1.1.3 Internal Standard Preparation

Various macrolides and other drugs were investigated in order to acquire an appropriate internal standard for the assay. Individual samples of each of the macrolides, tylosin tartrate, spiramycin, midecamycin, rifamycin, josamycin, oleandomycin and troleandomycin, and phenylpropanolamine HCl were prepared by accurately dissolving approximately 10 mg of drug in 10 ml of acetonitrile and bringing it up to volume with water to yield a concentration of 0.5 mg/ml.

The analysis was performed in a phosphate buffer (150 mM, 35 % (v/v) EtOH, pH 7.5), prepared as described in Section 5.2.4. The sample consisted of a mixture of Erythromycin A (EA) and its related substances Erythromycin B (EB), Erythromycin C (EC) and the impurities anhydroerythromycin (AE), erythromycin enol ether (EEE) and N-demethylerythromycin (NDEA) and was prepared as described in Section 5.2.5. The sample was hydrodynamically injected by applying a pressure of 200 mbar for 5 seconds, followed by co-injection of each of the internal standard solutions, using 50 mbar for 5 seconds. A voltage of 30 kV was then applied to effect the separation.

6.2.1.2 Results

Midecamycin, rifamycin and tylosin tartrate all migrated with the EOF marker. Two peaks were observed on injecting spiramycin, which were in close proximity to EC. Troleandomycin and oleandomycin co-migrated with EEE. Josamycin, which was selected as the internal standard in the assay for erythromycin, could not be used in this assay as it co-migrated with EC. Phenylpropanolamine HCl migrated before erythromycin and all the related substances. It was distinctly resolved from all the components in the mixture.

Phenylpropanolamine HCl was selected as the internal standard for the study. It possesses strong UV absorbance at 200 nm. Consequently, a concentration of approximately 0.03 mg/ml was selected at which a peak of comparable height and area to that of erythromycin was obtained.

6.2.1.3 Discussion

The importance of incorporating an internal standard in the sample has been addressed in Chapter Four. It is generally advisable to select an internal standard that possesses physicochemical properties and electrophoretic behaviour close to those of the compounds analysed. However, as this study does not involve the extraction of erythromycin from a plasma or urine matrix, the internal standard does not have to possess similar extractability to those of

the analytes. The internal standard should be stable in the sample solvent and electrolyte over the analysis time and should not co-migrate with any of the likely degradation components or related substances. Thus phenylpropanolamine was selected as the internal standard as it meets all these criteria.

The migration time, normalised peak area and peak height were all reported relative to the internal standard. Errors in sample introduction and concentration fluctuations due to sample solvent evaporation were consequently minimised in the presence of the internal standard.

6.2.2 Selectivity

The ability of the assay to discriminate between erythromycin and its related substances and impurities was demonstrated in Chapter Five. The assay developed for the separation of erythromycin and its related substances was further challenged here by analysing stressed samples. Forced degradation studies were performed on erythromycin to verify the stability-indicating nature of the CE method for the assay of erythromycin and its related substances.

A sample of erythromycin was subjected to six stress modes. The stressed samples were then analysed, using the assay developed for the determination of erythromycin and its related substances to detect the degradation products. The identity of the degradation products was confirmed by co-injecting a pure sample of each known impurity and degradation product.

Stress Testing

The method developed for the assay of any compound should be specific. It should be capable of resolving the peak of interest from any likely degradation products and extraneous components. Ideally, the most effective means of assessing whether the assay confirms product expiry-dating, would be the evaluation of the stability of samples retrieved throughout the study duration. However, the duration of most stability studies makes this approach untenable.

An alternative procedure that is adopted by most pharmaceutical companies involves the artificial production of degraded samples via accelerated methods. The degradation mechanism and extent of degradation under normal manufacture, storage and conditions of use should be considered [194]. Typically, compounds are subjected to acid, alkali, oxidation, high temperature and intense light [191]. The accelerated decomposition conditions should be specific for the compound, based on a scientific understanding of the product's decomposition mechanism and use conditions.

The duration that samples are subjected to extreme conditions is gauged from the likely conditions and period of compound storage. Therefore, stress conditions should be designed to encompass the extreme conditions the product is likely to be subjected to.

Representative electropherograms should be used to demonstrate specificity. The identity of the peaks should be determined by co-injection with standards of known degradation products. If impurities and degradation products are unavailable, specificity may be demonstrated by using a second well-characterised procedure and comparing test results against those for the degradation products and impurities.

6.2.2.1 Degradation by Reflux at 90 °C

6.2.2.1.1 Experimental Procedure

6.2.2.1.1.1 Sample Preparation

A stock sample solution of erythromycin (Sigma Chemical Company) was prepared by weighing out approximately 200 mg into a 20 ml volumetric flask, dissolving it in 10 ml of acetonitrile and making it up to volume with water to give a final concentration of approximately 10 mg/ml .

6.2.2.1.1.2 Standard Preparation

Individual standard solutions of EA, EB, EC, EEE, AE and NDEA were prepared by dissolving approximately 10 mg in 10 ml of acetonitrile and diluting it to volume with water to give a concentration of 0.5 mg/ml . Similarly, a standard solution comprising all of the above compounds was also prepared, such that the final solution contained each impurity at a concentration of approximately 0.5 mg/ml.

6.2.2.1.1.3 Internal Standard Preparation

An internal standard stock solution containing 0.1 mg/ml of phenylpropanolamine HCl was prepared by dissolving 5 mg in a mixture of acetonitrile:water (50:50 v/v) and making it up to volume. An appropriate volume of the internal standard solution was added to the sample solutions after they had been subjected to the stress conditions and to the control. The concentration of internal standard in the final solution was 0.03 mg/ml.

Refluxing

An aliquot of 10 ml of the stock sample solution, prepared as described in Section 6.2.2.1.1.1, was refluxed for one hour in a paraffin bath at 90°C. The solution was allowed to equilibrate to room temperature, after which the internal standard solution (6 ml) was pipetted into the solution. The stressed sample solution was then diluted to volume (20 ml) with a mixture of acetonitrile:water (50:50 v/v) to give a concentration of 5 mg/ml. The remaining 10 ml of the stock sample solution served as the control. The control was allowed to stand for one hour, after which the internal standard was added and the solution was diluted to volume, as for the stressed sample.

Analysis was performed in a phosphate buffer (150 mM, 35 % (v/v) EtOH, pH 7.5), prepared as reported in Section 5.2.4. The electrophoretic conditions specified in Section 6.2.1.1.1 were followed. The solutions were injected in triplicate.

The relative normalised peak area of erythromycin in the treated sample was compared to that in the control sample to determine the extent to which the parent compound degraded. Additional peaks were identified by co-injecting standard solutions of each of the impurities. An increase in the peak area or height confirmed the identity of the additional peaks. This procedure for determining parent degradation and peak identification was followed for all successive stress studies.

System suitability was demonstrated for each study by injecting the standard solution of all the impurities in duplicate prior to the analysis and on completion of the analysis. This was performed to ensure that the system operated optimally throughout the study.

6.2.2.1.2 Results

Figures 6.1 a and b illustrate electropherograms of the control and treated samples. No additional peaks were observed. Also the relative normalised peak area and relative peak height of the control and of the sample that was refluxed for one hour, were similar. Erythromycin does not undergo any degradation when refluxed at 90°C for one hour.

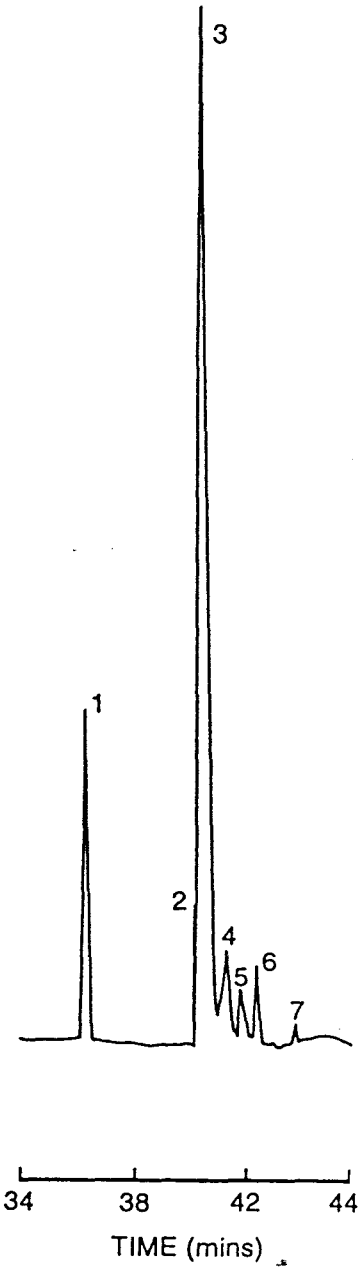


Figure 6.1 a Control
1. Phenylpropanolamine HCl
2. Erythromycin B
3. Erythromycin A
4. Erythromycin enol ether
5. Unknown
6. Unknown
7. N-demethylerythromycin

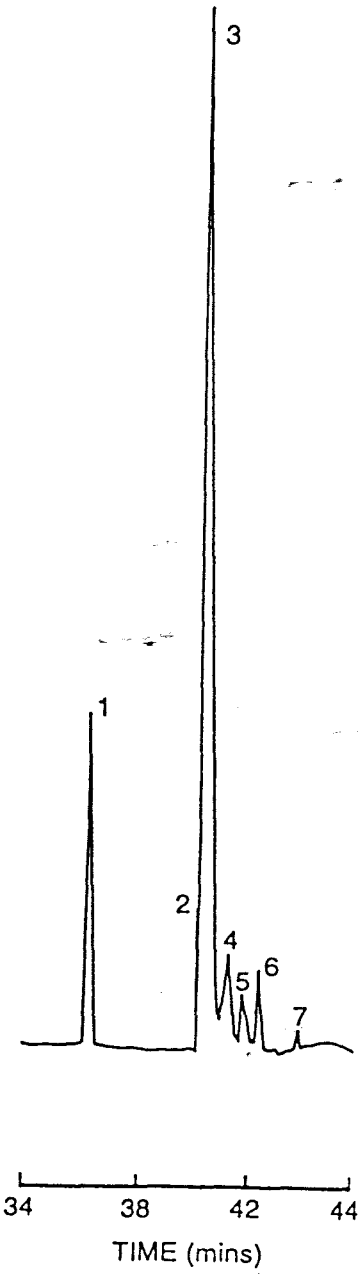


Figure 6.1 b Treated sample
1. Phenylpropanolamine HCl
2. Erythromycin B
3. Erythromycin A
4. Erythromycin enol ether
5. Unknown
6. Unknown
7. N-demethylerythromycin

Figure 6.1 a and b Electropherograms of the control and sample subjected to reflux at 90°C.
Conditions: injection 200 mbar for 5 s, sample concentration 10 mg/ml. Other conditions as for Fig. 5.1.

6.2.2.2 Degradation by Dry Heat Treatment

6.2.2.2.1 Experimental Procedure

Approximately 100 mg of erythromycin was accurately weighed and placed in an oven at 100 °C for 24 hours. The treated sample was then transferred into a 10 ml volumetric flask. The internal standard solution (3 ml) was added and the solution was diluted to volume with a mixture of acetonitrile:water (50:50 v/v). The control (10 mg/ml) was freshly prepared by dissolving 100 mg of erythromycin in 3 ml of internal standard solution and making up to volume with acetonitrile: water (50:50 v/v).

6.2.2.2.2 Results

From the electropherograms of the control and compound that was subjected to dry heat, it was apparent that no additional peaks were present. The relative normalised peak area and relative peak height of all the components in the stressed sample were equivalent to those in the control solution. Erythromycin powder was found to be stable when subjected to a temperature of 100°C for 24 hours.

6.2.2.3 Degradation by UV Light

6.2.2.3.1 Experimental Procedure

a) Powder

Erythromycin powder (approximately 100 mg) was weighed out onto a watch glass and subjected to UV light for 24 hours. The stressed powder and control were prepared similarly to that of the sample subjected to dry heat.

b) Solution

A stock sample solution was prepared as specified in Section 6.2.2.1.1.1. An aliquot of 10 ml was transferred into a glass container with a glass lid. The solution was exposed to UV light as above for 24 hours, after which 6 ml of internal standard solution was incorporated and the solution was made up to volume with a mixture of acetonitrile:water (50:50 v/v). The remaining 10 ml was left to stand in a sealed glass container for an equivalent time period and was used as the control. The internal standard was added and the solution was diluted to volume to yield a concentration of 5 mg/ml.

6.2.2.3.2 Results

The electropherograms of the controls, sample powder and solution that were subjected to UV light were identical. No degradation, in the form of additional peaks or changes in the relative normalised peak area and relative peak height was noted. Erythromycin solution and powder were found to be stable in the presence of UV light, when exposed for 24 hours.

6.2.2.4 Degradation by Oxidation

6.2.2.4.1 Experimental Procedure

a) Reflux with hydrogen peroxide

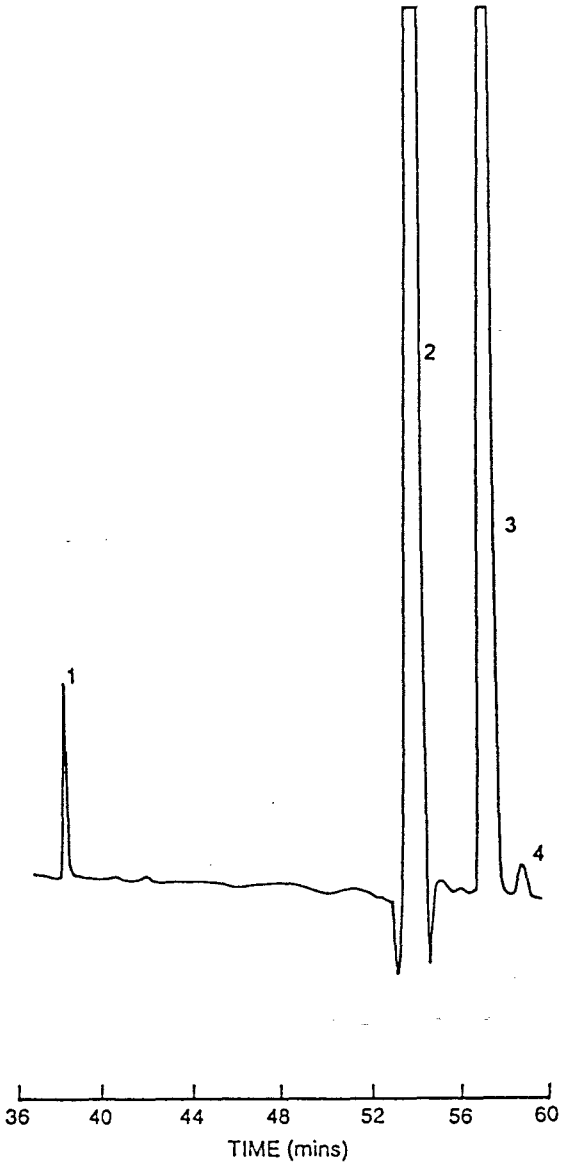
An aliquot of 10 ml of the sample stock solution, prepared as reported in Section 6.2.2.1.1.1, was treated with 1 % (v/v) hydrogen peroxide (H_2O_2) (10 ml of sample solution + 2 ml of 6 % (v/v) H_2O_2). The solution was refluxed for one hour in a paraffin bath at 90°C. After cooling, the appropriate volume of internal standard was added (6 ml) and the solution diluted to volume with a mixture of acetonitrile:water (50:50 v/v) to yield a concentration of 5 mg/ml. The control was prepared in a similar manner from the remaining 10 ml of stock sample solution.

b) Treatment for sixteen hours with hydrogen peroxide

Similarly, 10 ml of the stock sample solution was treated with 1% (v/v) H_2O_2, and was allowed to stand for 16 hours at room temperature. The internal standard solution (6 ml) was then added and the solution was diluted to volume. The remaining 10 ml of the stock sample solution served as the control and was left to stand for 16 hours. It was prepared in the same way as the stressed sample.

6.2.2.4.2 Results

Figures 6.2 a and b illustrate electropherograms of the samples that were subjected to H_2O_2: refluxed for one hour and 16 hours of treatment respectively. The electropherogram of the control (Figure 6.1 a) was compared to that for the sample refluxed and subjected to 16 hour treatment with hydrogen peroxide. Erythromycin disappeared and additional peaks were detected slightly before and after the EOF marker. The identity of these compounds could not be established as no standards were available.



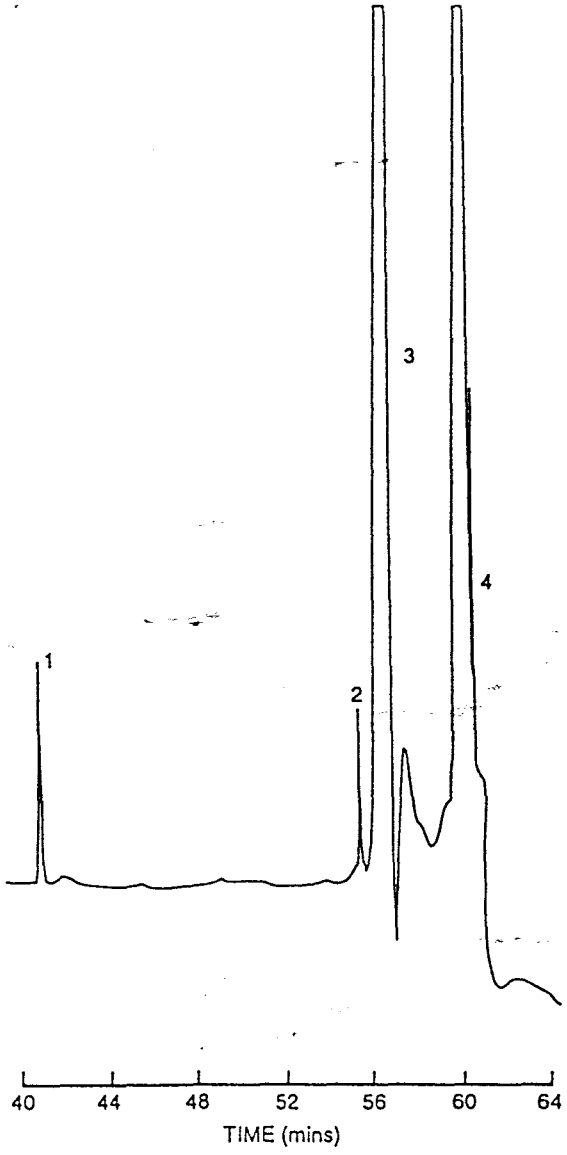


Figure 6.2 a Sample subjected to oxidation treatment, refluxed for 1 hour.
1. Phenylpropanolamine HCl
2. EOF marker
3. Unknown
4. Unknown

Figure 6.2 b Sample subjected to oxidation treatment for 16 hours at room temperature.
1. Phenylpropanolamine HCl
2. Unknown
3. EOF marker
4. Unknown

Figure 6.2 a and b Electropherograms of the sample subjected to oxidation; refluxed for an hour and 16 hour treatment at room temperature.
Conditions as for Fig. 6.1.

6.2.2.4.3 Discussion

Oxidation of erythromycin with hydrogen peroxide results in the production of erythromycin N-Oxide [158]. This compound possesses a pK_a of 6.0 and thus is slightly acidic. It is possible that the peak that migrates before the EOF marker (Figure 6.2 b) is this compound. However, positive identification could not be established as no standards were available. The large peak for the EOF implies that compounds arising from the degradation of erythromycin co-migrated with the marker. Migration with the EOF marker is indicative of neutral compounds and after the marker, of negatively charged compounds.

6.2.2.5 Degradation by Strong Acid Treatment

6.2.2.5.1 Experimental Procedure

a) Reflux with HCl

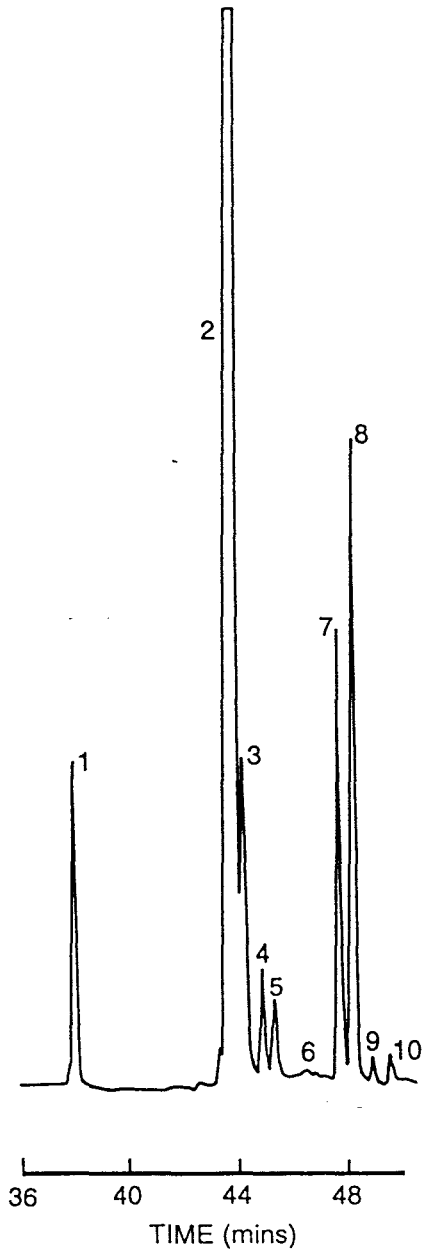
A stock sample solution was prepared as reported in Section 6.2.2.1.1.1. From this solution, 10 ml was transferred into a round bottom flask. The solution was refluxed with 0.5 M hydrochloric acid (HCl) (10 ml of stock sample solution + 2 ml of 3 M HCl) in a paraffin bath at 90°C for one hour. After cooling, the solution was neutralised (pH 7.21) with a 5 M NaOH solution. The NaOH solution was prepared by dissolving 2 g of NaOH pellets in 10 ml of water in a volumetric flask. The internal standard solution was added and the solution was made up to volume with a mixture of acetonitrile:water (50:50 v/v). The control was prepared similarly from the remaining 10 ml of stock sample solution.

b) Treatment for sixteen hours with HCl

To 10 ml of stock sample solution, prepared as described in Section 6.2.2.1.1.1, 2 ml of 3 M HCl was added (0.5 M HCl). The solution was allowed to stand for 16 hours, after which it was neutralised (pH 7.13) with 5 M NaOH. The internal standard was added and the solution was diluted to volume. The residual 10 ml of stock sample solution served as the control and was prepared in a similar manner as the stressed sample.

6.2.2.5.2 Results

The acid degradation products of erythromycin have been discussed in Section 2.2.3. On subjecting erythromycin to acid, several additional peaks were observed. (Figures 6.3 a and b)



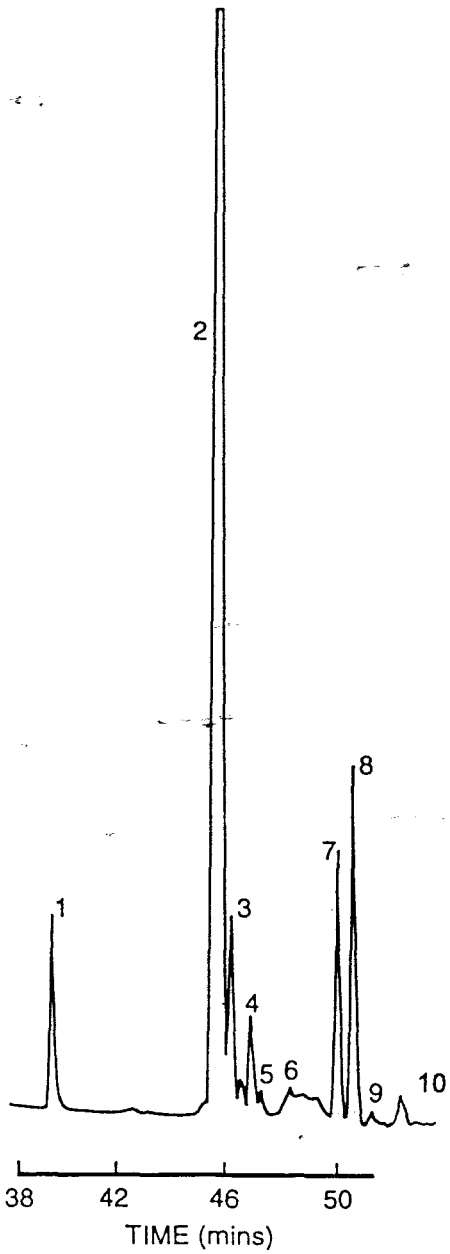
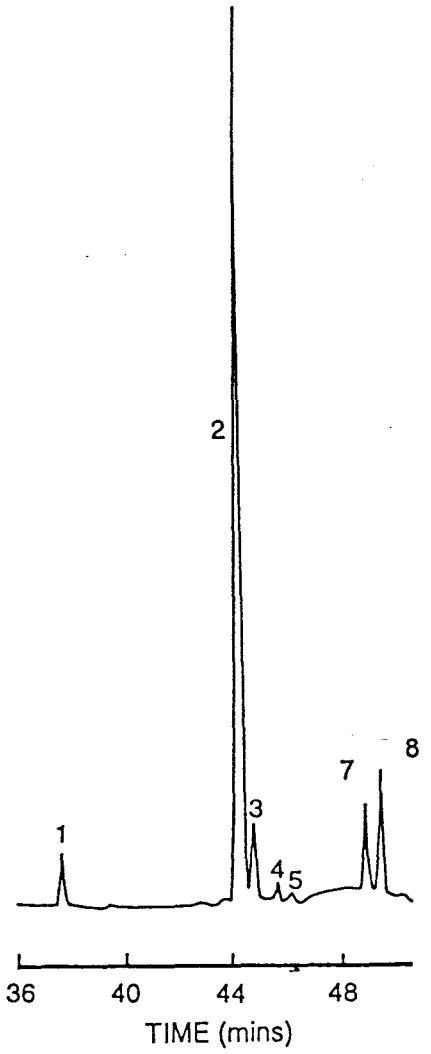


Figure 6.3 a Sample subjected to acid treatment, reflux for 1 hour.
1. Phenylpropanolamine HCl
4. Anhydroerythromycin
2, 3, 5, 6, 7, 8, 9, 10 Unknown

Figure 6.3 b Sample subjected to acid treatment for 16 hours at room temperature.
1. Phenylpropanolamine HCl
4. Anhydroerythromycin
2, 3, 5, 6, 7, 8, 9, 10 Unknown

Figure 6.3 a and b Electropherograms of the sample subjected to acid, refluxed for an hour and 16 hour treatment at room temperature.
Conditions as for Fig. 6.1.

The treated samples were diluted by a factor of five with a mixture of acetonitrile:water (50:50 v/v) (Figure 6.4 a), so that all the peaks were on scale. This was necessary for the identification of peak 2, as an increase in peak height and area is used to confirm the identity of a peak. Pure standards of EEE and AE were co-injected to confirm the identity of the peaks formed on subjecting erythromycin to acid. Co-injection with EEE resulted in a peak that migrated between the internal standard and peak 2. The increase in height and area of peak 4 (Figure 6.4 b) noted when a pure sample of AE was co-injected, indicated that peak 4 was AE.



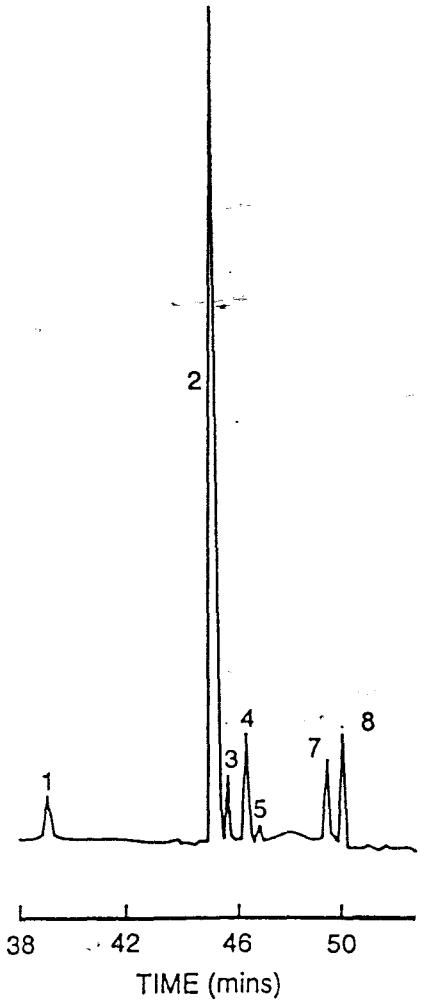


Figure 6.4 a Sample subjected to acid treatment, reflux for 1 hour.
Peaks as for Fig. 6.3 a
Peaks 6, 9, 10 were not detected.

Figure 6.4 b Sample subjected to acid treatment, refluxed for 1 hour and co-injected with AE 0.5 mg/ml applying 50 mbar for 5 s.
Peaks as for Fig. 6.3 a. Peaks 6, 9, 10 were not detected.

Figure 6.4 a and b Electropherograms of sample (5-fold diluted) subjected to acid refluxed for an hour and sample spiked with anhydroerythromycin.
Conditions as for Fig. 6.1.

6.2.2.5.3 Discussion

Erythromycin enol ether is reported to be formed when erythromycin is subjected to mild acid conditions, while AE is formed under strong acid conditions. Cleavage of the glycosidic linkages of erythromycin has not been reported. However, it is possible for these linkages to be broken, especially under harsh acid treatment. This would result in the formation of erythralosamine and the sugar, cladinose.

Erythromycin disappeared completely on treating the sample with acid. Erythromycin enol ether was not detected and small quantities of anhydroerythromycin (peak 4) were found to be present in the treated sample. This is expected as strong acid conditions were used. Erythralosamine is a basic compound and has a lower molecular mass than anydroerythromycin. Consequently, it should have a higher mobility and migrate before anhydroerythromycin. It is possible that peak 2 is erythralosamine. However, positive identification was not possible due to the unavailability of a standard sample. Cladinose in a neutral compound, therefore it would migrate with the EOF marker.

6.2.2.6 Degradation by Strong Alkali Treatment

6.2.2.6.1 Experimental Procedure

a) Reflux with NaOH

An aliquot of 10 ml of the stock sample solution, prepared as described in Section 6.2.2.1.1.1, was refluxed with 0.5 M NaOH (10 ml of stock sample solution + 2 ml of 3 M NaOH) in a paraffin bath at 90°C for one hour. The solution was neutralised (pH 6.98) with a 5 M HCl solution after it reached ambient temperature. The internal standard was incorporated and the solution was made up to volume with a mixture of acetonitrile:water (50:50 v/v) to yields a concentration of 5 mg/ml. The control consisted of the residual volume of the stock sample solution and was prepared similarly.

b) Treatment for sixteen hours with NaOH

Stock sample solution (10 ml) was treated with 0.5 M NaOH and was allowed to stand for 16 hours, before neutralization (pH 7.03) with 5 M HCl solution. The solution was made up to volume after the internal standard was added. The remaining 10 ml of stock sample solution served as the control and was prepared in the same manner as the treated sample.

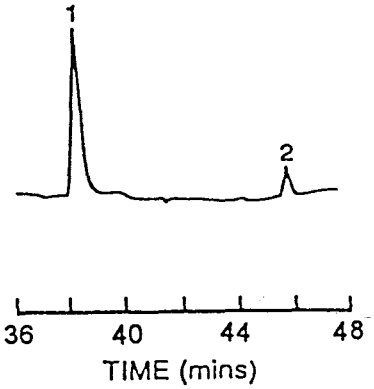


Figure 6.5 a Sample subjected to alkali treatment, refluxed for 1 hour.
1. Phenylpropanolamine HCl
2. Unknown

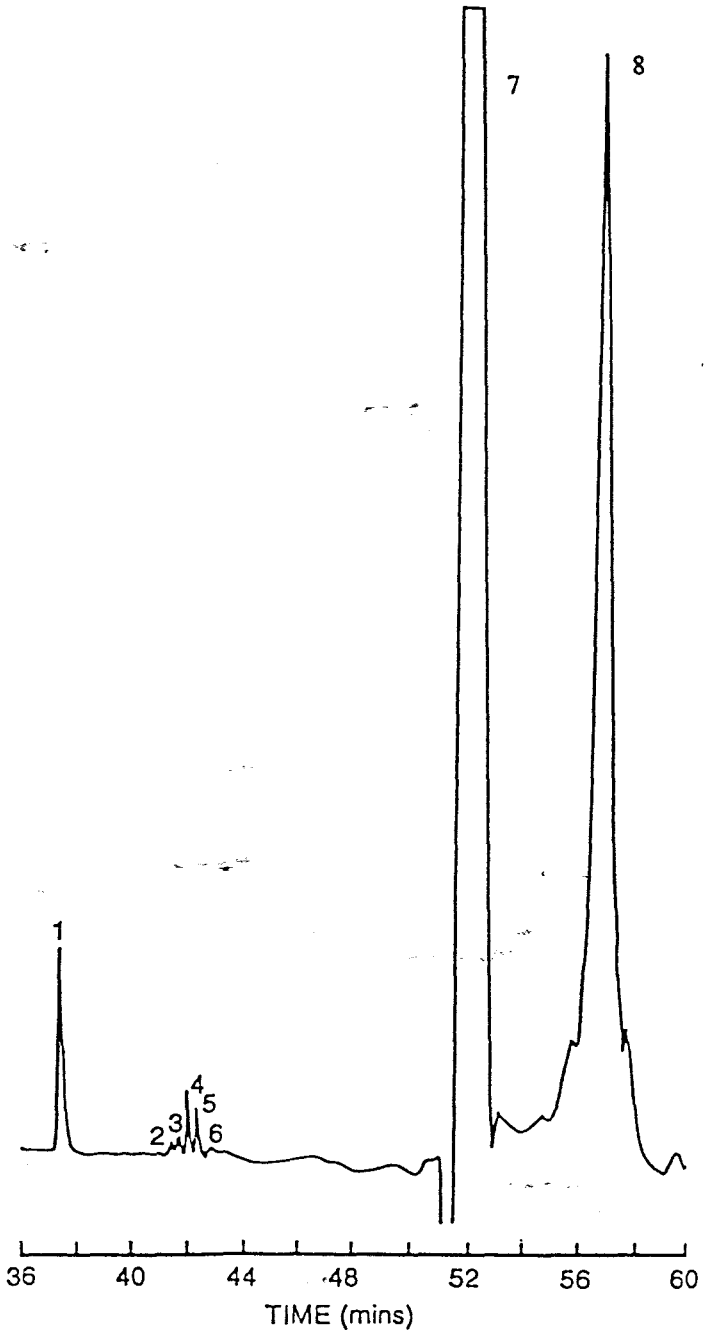


Figure 6.5 b Sample subjected to alkali treatment for 16 hours at room temperature.
1. Phenylpropanolamine HCl
2. Unknown
3. Erythromycin B
4. Erythromycin A
5. Erythromycin enol ether
6. Unknown
7. EOF marker
8. Unknown

Figure 6.5 a and b Electropherogram of sample subjected to alkali, refluxed for an hour and 16 hours treatment at room temperature. Conditions as for Fig. 6.1.

6.2.2.6.2 Results

The stability of erythromycin base in the presence of alkali was reported in Section 2.2.3. Refluxing erythromycin with alkali resulted in the complete degradation of the compound as observed in Figure 6.5 a. A small unidentified peak was apparent and the peak that indicated the EOF marker was substantially larger. Identification of the compounds that were produced on subjecting erythromycin to alkali was not possible by co-injection as no standards were available. Traces of EA, EB, EC and EEE were apparent after subjecting erythromycin base to 16 hours of alkali treatment at room temperature, as depicted Figure 6.5 b.

6.2.2.6.3 Discussion

The large peak observed after the EOF marker suggests that a negatively charged compound was formed in the presence of alkali. The large peak for the EOF marker implies that a neutral compound could have been formed and co-migrated with the marker. In alkali media, erythromycin degrades to form a zwitterionic product, dihydroerythromycin as well as pseudoerythromycin hemiketal and pseudoerythromycin enol ether. Due to the unavailability of standards of the above compounds, it was not possible to identify the additional peaks, unequivocally. It can only be postulated that the very large peak that migrated with the EOF marker is the zwitterionic product, dihydroerythromycin.

6.2.2.7 Conclusion

Erythromycin was subjected to various stress tests. The assay for the determination of erythromycin and its related substances was capable of resolving most of the compounds formed on subjecting erythromycin to extreme conditions. The unavailability of some of the known degradation products of erythromycin such as dehydroerythromycin, pseudoerythromycin hemiketal, pseudoerythromycin enol ether, erythralosamine and erythromycin N-oxide, prevented the confirmation of the peaks formed on stressing erythromycin.

Erythromycin powder was found to be stable in the presence of heat, when treated for 24 hours. Refluxing erythromycin at 90°C for one hour did not result in any degradation. Erythromycin powder and solution did not degrade in the presence of UV light, when subjected for 24 hours. Degradation of erythromycin was apparent when it was subjected to oxidation, acid and alkali. These stress tests were harsh and in most cases resulted in the complete loss of erythromycin and the formation of several additional peaks.

Table 6.1 Precision Data for Various Parameters (% RSD)

Table 6.1 a Day 1 (n = 11)

Compound	Migration Time	Relative Migration Time	Normalised Peak Area	Relative Normalised Peak Area	Peak Height	Relative Peak Height
EB	2.07	0.96	3.50	2.77	2.16	2.35
EA	2.40	1.02	1.24	1.13	1.84	2.23
EEE	2.61	1.08	1.92	0.95	0.84	2.54
EC	3.92	0.95	3.08	2.61	2.70	4.84
AE	4.39	1.16	3.39	3.03	2.98	5.14
NDEA	3.91	0.98	1.66	2.80	2.27	4.36

Table 6.1 b Day 2 (n = 9)

Compound	Migration Time	Relative Migration Time	Normalised Peak Area	Relative Normalised Peak Area	Peak Height	Relative Peak Height
EB	4.23	1.12	3.51	2.25	2.17	2.45
EA	4.37	1.10	1.85	1.58	2.11	2.52
EEE	3.58	1.79	2.80	1.71	1.07	1.90
EC	1.49	1.23	4.88	2.57	2.57	3.04
AE	4.68	1.54	5.49	4.44	3.82	6.15
NDEA	1.01	1.39	2.11	2.68	3.20	4.52

Table 6.1 c Day 3 (n = 5)

Compound	Migration Time	Relative Migration Time	Normalised Peak Area	Relative Normalised Peak Area	Peak Height	Relative Peak Height
EB	0.73	0.29	1.09	2.50	1.95	1.98
EA	0.81	0.30	0.43	1.39	1.29	1.65
EEE	0.75	0.27	2.00	2.90	0.59	1.24
EC	0.76	0.31	2.70	1.77	1.40	1.40
AE	0.82	0.47	2.25	3.72	3.05	3.18
NDEA	0.61	0.32	1.05	2.55	2.32	2.87

Acceptable precision of less than 10 %, was obtained for quantitation of all the related substances and impurities. The % RSD values for AE are slightly larger than for the other components. This is probably due to integration errors in calculating the area of this small peak. As indicated by the lower % RSD values for the relative migration time and relative normalised peak area, variability is reduced by incorporating an internal standard. However, precision for the peak height was greater than for the relative peak height.

6.2.3.2 Intermediate Precision

6.2.3.2.1 Experimental Procedure

Intermediate precision was assessed by performing a number of consecutive injections of the sample solution of the components (prepared as specified in Section 6.2.3.1.1.2) on each of three consecutive days. Freshly prepared stock sample solutions, internal standard solutions and electrolyte were prepared on each day. The analysis was performed in a phosphate buffer (150 mM, 35 % (v/v) EtOH, pH 7.5), prepared as described in Section 5.2.4. The electrophoretic conditions stipulated in Section 6.2.1.1.1 were followed.

6.2.3.2.2 Results

The intermediate precision of the method was assessed by calculating the percent relative standard deviations (% RSD) for the various parameters for each compound over the three day period. The results are illustrated in Table 6.2.

Table 6.2 Intermediate Precision Data for the Various Parameters (%RSD) n = 25

Compound	Migration Time	Relative Migration Time	Normalised Peak Area	Relative Normalised Peak Area	Peak Height	Relative Peak Height
EB	3.20	1.49	6.69	2.97	2.98	5.11
EA	3.68	1.58	4.64	3.03	2.24	5.28
EEE	2.98	1.73	3.83	3.01	1.15	2.06
EC	3.05	1.16	4.45	3.99	2.57	5.47
AE	4.91	1.23	7.43	2.46	3.85	5.39
NDEA	2.98	1.20	3.58	2.80	3.34	5.02

Acceptable intermediate precision of less than 10 % was obtained for all the compounds over the three days. The relative migration time gave better precision than the migration time with % RSD values of less than 2 %. This method is thus precise for the determination of impurities and related substances of erythromycin when conducted on different days.

6.2.3.2.3 Discussion

Peak area precision is closely affiliated to sample concentration. Therefore, large % RSD values for peak area and peak height are expected for trace levels of impurities. Typically, % RSD values of approximately 10 % are acceptable for impurities at low levels [212, 215, 216, 221]. In this study acceptable precision of less than 10 % RSD was obtained for the normalised peak area and peak height. In addition, repeated injections of the same sample solution should produce equivalent numbers of impurities, levels and migration times for each impurity and total level of impurities [185]. Migration time precision of less than 5 % was achieved for all compounds, with relative migration times of less than 2 %. Generally, relative migration times confer improved precision as minor variations are taken into account. Precise migration time is essential to enable confirmation of the exact identity of individual impurities.

The relative normalised peak area exhibited greater precision than the normalised peak area as small fluctuations in the quantity of sample introduced were compensated for by relating the area to that of the internal standard. Relative peak heights were found to be less precise than peak heights. This can be explained by the independent change in peak height of either the internal standard or solute, due to peak broadening. Consequently, a greater error is apparent when the peak heights are reported relative to that of the internal standard. Acceptable precision was achieved with the method developed for the assay of erythromycin related substances.

In CE, several minor factors need to be considered to obtain highly reproducible results. Some of these factors include minimising siphoning effects and evaporation, regular washing of the capillary to ensure a constant EOF, electrolyte replenishment and voltage ramping. Thus, it is possible to obtain levels of precision comparable to HPLC by controlling these subtle factors in addition to the well-documented major factors.

6.2.4 Linearity

6.2.4.1 Experimental Procedure

Detector linearity was assessed by analysing five standards over the range 10 - 200 % of the nominal assay concentration (0.5 mg/ml). Five replicate injections were made at each concentration. A stock sample solution prepared as described below, was diluted to yield concentrations of 0.025, 0.05, 0.25, 0.375 and 0.5 mg/ml. A phosphate buffer (150 mM, 35 % (v/v) EtOH, pH 7.5), was prepared as specified in Section 5.2.4. The operating parameters described in Section 6.2.1.1.1 were followed. Linearity was established over three days.

Sample Preparation

A sample stock solution was prepared by accurately weighing approximately 12.5 mg of EA, EB, EC, EEE, AE and NDEA into a 20 ml volumetric flask and dissolving it in 10 ml of acetonitrile. The resultant solution was diluted to volume with water to yield a solution of concentration 0.626 mg/ml for each component. The internal standard solution (0.2 mg/ml) was prepared as described in Section 6.2.3.1.1.1. Five sample solutions of incremental concentrations were prepared by diluting the appropriate volume of sample stock solution. The correct volume of internal standard solution was added such that each solution contained 0.02 mg/ml and the solutions were made up to volume with a mixture of acetonitrile:water (50:50 v/v).

6.2.4.2 Results

Calibration curves for each day were constructed by plotting the relative normalised peak area of each compound to that of the internal standard as a function of the analyte concentration, as shown in Figure 6.6. A regression line obtained by the method of least squares was calculated to evaluate the results.

It is suggested that relative normalised peak areas as opposed to relative peak heights are used to assess detection linearity, as the latter usually illustrate deterioration in linearity at high sample concentration [144, 199]. In this study calibration curves were constructed for both the relative normalised peak areas and peak heights to determine the concentration at which non-linearity developed. The peak heights were used instead of the peak height ratios as improved precision was generally achieved when the heights were not related to the internal standard. Calibration curves constructed by linear regression of a plot of the peak height of each compound versus analyte concentration are illustrated in Figure 6.7.

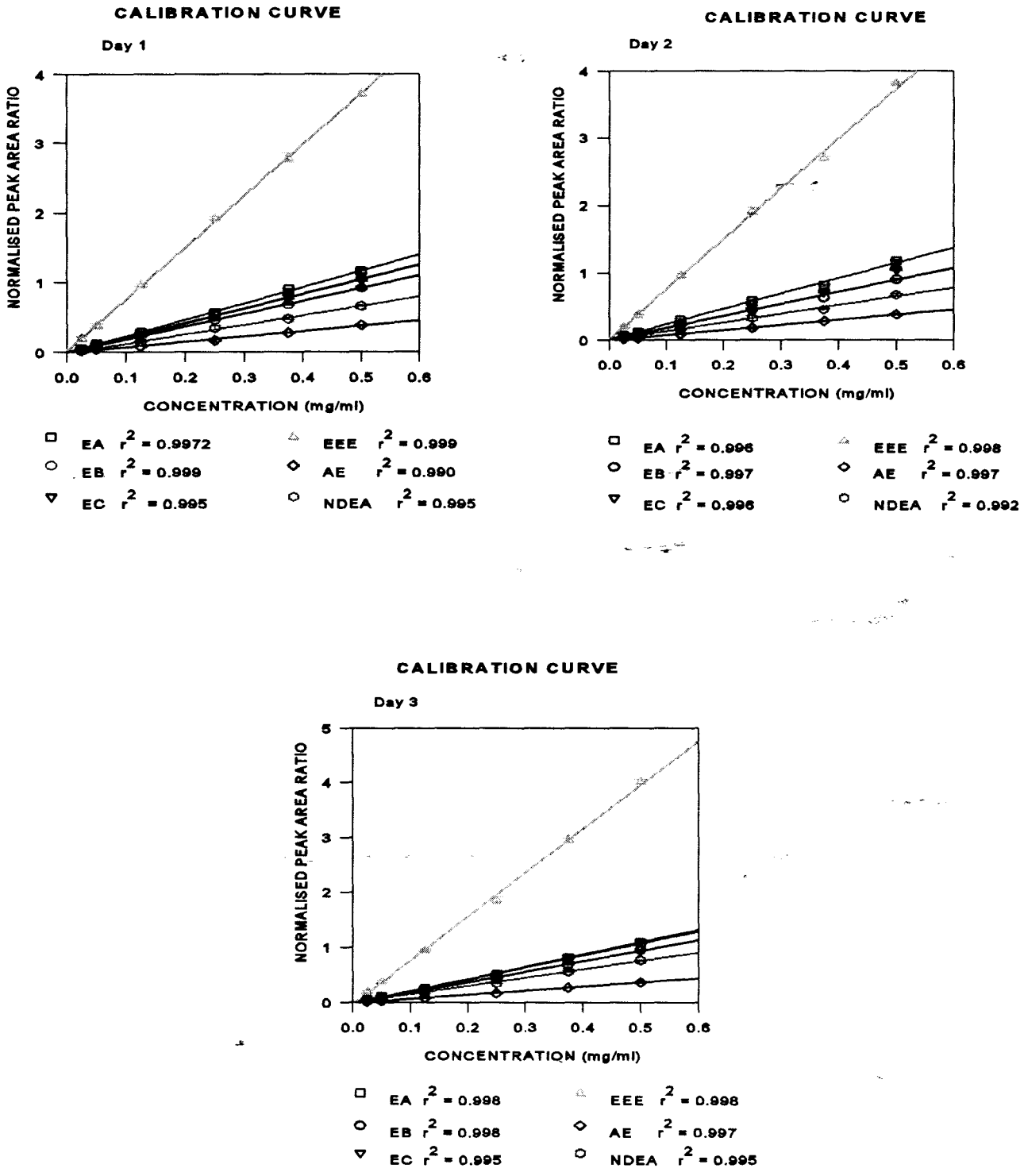


Figure 6.6 Calibration curves constructed on each of three days by linear regression of mean relative normalised peak area vs concentration.

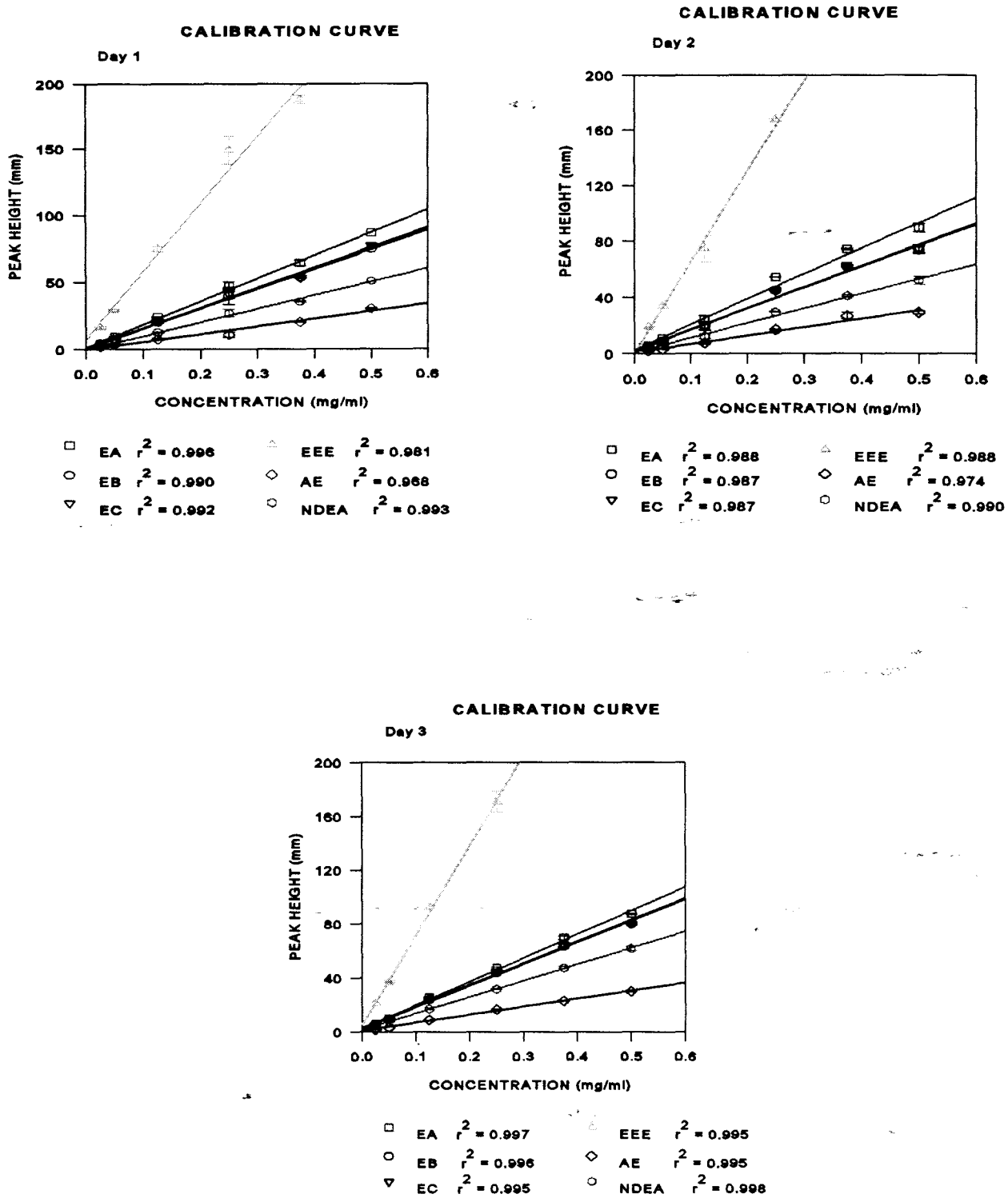


Figure 6.7 Calibration curves constructed on each of three days by linear regression of mean peak height vs concentration.

6.2.4.3 Discussion

This study demonstrates a linear relationship between the detector response and analyte concentration within the range 10 - 200 %. Comparable linearity was achieved with relative normalised peak areas and peak heights. The calibration line constructed with the peak heights does not exhibit non-linearity in the concentration range examined. This implies that the capillary was not overloaded at the top end of the concentration range and consequently no peak broadening was apparent. The peaks for EEE were off-scale at higher concentrations. Thus linearity could only be established in a narrow range (10 - 100 %). Therefore, either peak height or relative normalised peak area can be used to establish linearity of erythromycin and its related substances in the range 10 - 200 %.

Acceptable detector linearity with correlation coefficients greater than 0.99 for the relative normalised peak area and greater than 0.97 for peak height were acquired. The intercept values for the peak height and relative normalised peak area were close to zero. The method developed for the assay of erythromycin related substances exhibited satisfactory linearity over three days.

6.2.5 Accuracy

6.2.5.1 Experimental Procedure

The accuracy determination of each of the related substances of erythromycin in the presence of large quantities of the parent was assessed, by spiking erythromycin base with known amounts of impurities. Accuracy was determined concurrently with linearity on each of three consecutive days. Four solutions, prepared in duplicate, contained the parent spiked with 0, 20, 100 and 200% (v/v) of an impurity solution, prepared as described below. Appropriate volumes of internal standard solution were added to each sample solution such that the final solution contained 0.02 mg/ml. Each of these solutions were analysed in triplicate.

The electrolyte was a phosphate buffer (150 mM, 35 % (v/v) EtOH, pH 7.5), prepared as described in Section 5.24 and the electrophoretic conditions stated in Section 6.2.1.1.1 were followed. Fresh sample solutions and electrolyte were prepared on each day.

Table 6.3 Mean Accuracy Data from the Regression Line for the Relative Normalised Peak Area vs Concentration

Table 6.3 a: Erythromycin C

	Spiking level	Mean Actual Concentration (mg/ml)	Mean Calculated Concentration (mg/ml)	Mean % Bias	Mean % Recovery
Day 1	20%	0.049	0.051	3.92	104.1
y = 2.11x -0.01	100%	0.245	0.240	-2.08	98.0
	200%	0.490	0.490	0.00	100
Day 2	20%	0.052	0.055	5.45	105.8
y = 2.05x - 0.02	100%	0.260	0.270	3.70	103.8
	200%	0.520	0.512	-1.56	98.5
Day 3	20%	0.051	0.055	7.27	107.8
y = 2.23x - 0.02	100%	0.255	0.259	1.54	101.6
	200%	0.510	0.512	0.39	100.4

Table 6.3 b: Anhydroerythromycin

	Spiking level	Mean Actual Concentration (mg/ml)	Mean Calculated Concentration (mg/ml)	Mean % Bias	Mean % Recovery
Day 1	20%	0.050	0.052	3.85	104.0
y = 0.76x - 0.01	100%	0.250	0.250	0.00	100.0
	200%	0.500	0.510	1.96	102.0
Day 2	20%	0.052	0.054	3.70	103.8
y = 0.77x - 0.01	100%	0.260	0.260	0.00	100.0
	200%	0.520	0.530	1.89	101.9
Day 3	20%	0.051	0.053	3.77	103.9
y = 0.75x- 0.01	100%	0.255	0.252	-1.19	98.8
	200%	0.510	0.521	2.11	102.2

Table 6.3 c: N-Demethylerythromycin

	Spiking level	Mean Actual Concentration (mg/ml	Mean Calculated Concentration (mg/ml	Mean % Bias	Mean % Recovery
Day 1	20%	0.052	0.051	-1.96	98.08
y = 1.36x - 0.02	100%	0.260	0.267	2.62	102.7
	200%	0.520	0.518	-0.39	99.62
Day 2	20%	0.050	0.051	1.96	102.0
y = 1.3x + 0.00	100%	0.250	0.257	2.72	102.8
	200%	0.500	0.505	0.99	101.0
Day 3	20%	0.052	0.051	-1.96	98.8
y = 1.5x + 0.00	100%	0.260	0.260	0.00	100.0
	200%	0.520	0.515	-0.97	99.04

The mean concentrations found on spiking the parent compound with various concentrations of impurities were very close to the actual concentrations present in the spiking solution for all three compounds tested. Improved accuracy was generally observed at higher sample concentrations. Typically, a recovery limit of between 90 - 110 % of the theoretical value is acceptable as was observed in this study. The accuracy data for EC, AE and NDEA in this study was within the acceptable range. Thus the assay developed for the analysis of the related substances and impurities of erythromycin, in the presence of the main component, exhibited satisfactory accuracy.

6.2.5.3 Discussion

Resolution between erythromycin and its related substances is difficult to achieve as the compounds have very similar charge/mass ratios and thus electrophoretic mobilities. These compounds therefore migrate close to each other. Consequently, an increase in the concentration of any of the compounds results in the loss of resolution.

6.2.6 Sensitivity

6.2.6.1 Experimental Procedure

The limit of detection (LOD) and limit of quantitation (LOQ) were determined visually and by

calculating the percent relative standard deviations (% RSD) of the peak heights or relative normalised peak areas. Serially-diluted samples of the mixture of all the related substances, prepared as described below, were injected a minimum of six times until % RSD values of less than 15 % were obtained. This concentration was denoted as the LOQ. Concentrations at which the % RSD values were less than 20 % and where the peaks were visible above the baseline (> 2.0 mm), were reported as the LOD. It was possible to detect peaks of such height as the level of background noise was minimal. The analysis was performed in a phosphate buffer (150 mM, 35 % (v/v) EtOH, pH 7.5), prepared as reported in Section 5.2.4 and the electrophoretic conditions listed in Section 6.2.1.1.1 were followed.

Sample Preparation

A 0.025 mg/ml sample solution of EB, EA, EC, EEE, AE and NDEA was prepared by pipetting 1ml of the stock sample solution, prepared as specified in Section 6.2.4.1.1, into a 25 ml volumetric flask and making up to volume with a mixture of acetonitrile:water (50:50 v/v). This solution was serially diluted to yield concentrations of 0.0125 mg/ml and 0.0075 mg/ml. Two further solutions, at concentrations of 0.025 mg/ml and 0.0375 mg/ml, were prepared from the stock sample solution, as reported in Section 6.2.4.1.1. The appropriate volume of internal standard solution was added to each solution. Each solution was injected eight times.

6.2.6.2 Results

The percent relative standard deviation of the peak height and relative normalised peak area were calculated for each impurity at each concentration. The results are tabulated below.

Table 6.4 Limit of Quantitation for Erythromycin Related Substances

Compound	Concentration (mg/ml)	% Nominal Concentration	Relative Normalised Peak Area (%RSD)	Peak Height (% RSD)
EB	0.025	10	6.24	8.60
EA	0.025	10	8.32	7.02
EEE	0.0125	5	6.60	7.14
EC	0.025	10	4.86	6.90
AE	0.0375	15	6.83	8.60
NDEA	0.0375	15	5.05	8.00

Table 6.5 Limit of Detection for Erythromycin Related Substances

Compound	Concentration (mg/ml)	% Nominal Concentration	Relative Normalised Peak Area (% RSD)	Peak Height (% RSD)
EB	0.0125	5	12.45	7.90
EA	0.0125	5	14.10	7.00
EEE	0.0075	3	13.50	11.70
EC	0.0125	5	16.32	9.96
AE	0.025	10	20.10	11.92
NDEA	0.025	10	19.90	9.96

The LOQ and LOD obtained for each impurity were acceptable.

6.2.6.3 Discussion

The maximum wavelength of erythromycin and its related substances are listed in Chapter Five. At a detection wavelength of 200 nm EA, EB and EC have comparable UV activity, while AE and NDEA possess significantly lower UV activity. The specific absorbance of EEE is more than ten times greater than for other related substances. Consequently the LOD and LOQ value for EEE are lower. In CE the detection sensitivity is higher than in HPLC. The LOD for drug related impurities in CE may be set at 0.1% area/area with reference to the main component [125,133, 230]. In HPLC it is possible to determine impurity levels of 0.02 % area/area [232]. There are several mechanisms by which the sensitivity of the assay may be improved. These procedures are discussed in Section 4.3.4.3.

If sensitivity is limited by the narrow linear dynamic range of the detector, high-low injections may be conducted to improve the determination of trace levels of impurity. The low injection is performed by injecting a diluted sample or injecting for a shorter duration, such that the main peak is within the linear range of the detector and, subsequently, its peak area can be recorded. An injection of a more concentrated sample or for a longer duration is then performed. Under these conditions, the main component is off-scale and the impurities can be easily detected. The area of the main component in the high injection is calculated by multiplying the area of the on-scale main component by the dilution factor. The impurities are quantified against this calculated main peak area as a percentage area/area [232].

The differences in UV activity between the parent compound and impurities and related substances will adversely affect the LOD. The LOD may be underestimated or overestimated depending on whether the impurity has a lower or higher UV activity than the parent compound.

An alternate method of determining the LOD, when evaluating purity, is to express the smallest peak that can be detected as a percentage area/area of the electropherogram. However, these values are also dependent on the UV absorptivity of the solutes and thus incorrect LOD values may be determined.

6.2.7 Stability

6.2.7.1 Experimental Procedure

Sample Preparation

A stock solution containing EA, EB, EC, EEE, AE and NDEA was prepared as described in Section 6.2.4.1.1. Equivalent volumes of this solution were stored in the refrigerator at 4°C and on the bench top at 22°C for a period of three weeks. On day 1 and on each of three days over 21 days, stock solutions stored in the refrigerator and on the bench top were diluted with an appropriate volume of freshly prepared internal standard solution and were made up to volume with a mixture of acetonitrile:water (50:50 v/v). The final solution contained 0.25 mg/ml of erythromycin and the related substances and impurities and 0.02 mg/ml of internal standard. Prior to dilution, the samples stored in the fridge were allowed to equilibrate to ambient temperature.

6.2.7.2 Results

In determining the stability of the compounds, the relative normalised peak area of each compound was recorded on each day for the samples stored in the refrigerator and on the bench top. The response factors obtained were compared to those of the freshly prepared sample on day 1. The mean and % RSD of the relative normalised peak areas are reported in Table 6.6.

Table 6.6 Stability Data of Erythromycin Related Substances: Mean (% RSD)

	Day 1	Day 5		Day 19		Day 21	
	Fresh	Fridge	Bench	Fridge	Bench	Fridge	Bench
EB	0.47 (2.25)	0.47 (0.87)	0.48 (3.96)	0.23 (4.56)	0.22 (2.10)	0.21 (2.60)	0.21 (0.00)
EA	0.58 (2.42)	0.58 (1.80)	0.59 (3.91)	0.30 (2.84)	0.29 (3.10)	0.27 (2.00)	0.29 (1.92)
EEE	1.93 (3.64)	1.87 (2.50)	1.96 (5.14)	0.94 (0.95)	0.94 (3.50)	0.87 (2.40)	0.93 (1.92)
EC	0.53 (5.47)	0.53 (5.47)	0.56 (2.90)	0.23 (4.40)	0.22 (3.80)	0.22 (3.70)	0.25 (1.16)
AE	0.24 (11.8)	0.19 (3.30)	0.19 (3.40)	0.09 (4.60)	0.08 (10.2)	0.09 (6.40)	0.09 (9.10)
NDEA	0.38 (15.3)	0.36 (3.90)	0.35 (3.90)	0.16 (6.10)	0.17 (6.52)	0.16 (6.93)	0.16 (13.4)

The peak areas of all the compounds decreased from day 5 to day 19, irrespective of whether they were stored in the fridge or on the bench. No additional peaks were apparent. It is possible that the degradation products formed were not detected due to their concentrations being below their respective LOD.

6.2.8 Analysis Of Commercial Samples

6.2.8.1 Experimental Procedure

Three commercial samples of erythromycin obtained from Lennons Limited, Abbott Laboratories and Pfizer Pharmaceuticals were analysed. Each sample was prepared by accurately weighing approximately 110 mg of the commercial sample into a 10 ml volumetric flask, dissolving it in 5 ml acetonitrile and diluting it to volume with water. An aliquot of 9 ml of this solution was pipetted into a 10 ml volumetric flask. Internal standard solution (1 ml of a 0.2 mg/ml) was added to yield a final solution contained approximately 10 mg/ml of erythromycin and 0.02 mg/ml of internal standard.

The analysis was performed in a phosphate buffer (150 mM, 35 % (v/v) EtOH, pH 7.5), prepared as specified in Section 5.2.4 and the electrophoretic conditions stipulated in Section 6.2.1.1.1 were followed. Each sample was analysed in duplicate. The identity of the peaks was confirmed by co-injecting a solution of each of the pure substances, prepared as described in Section 6.2.2.1.1.2, by applying a pressure of 50 mbar for 5 seconds.

6.2.8.2 Results

Figures 6.8 a, b, c illustrate electropherograms of the commercial samples of erythromycin. Baseline resolution between EB and EA was not achieved for all three samples. It was possible to resolve EEE from EA in the commercial samples as the EEE was present in higher concentrations in the samples. The absence of a peak for erythromycin B in the sample from Lennons Limited is possibly due to co-migration of erythromycin B with erythromycin A.

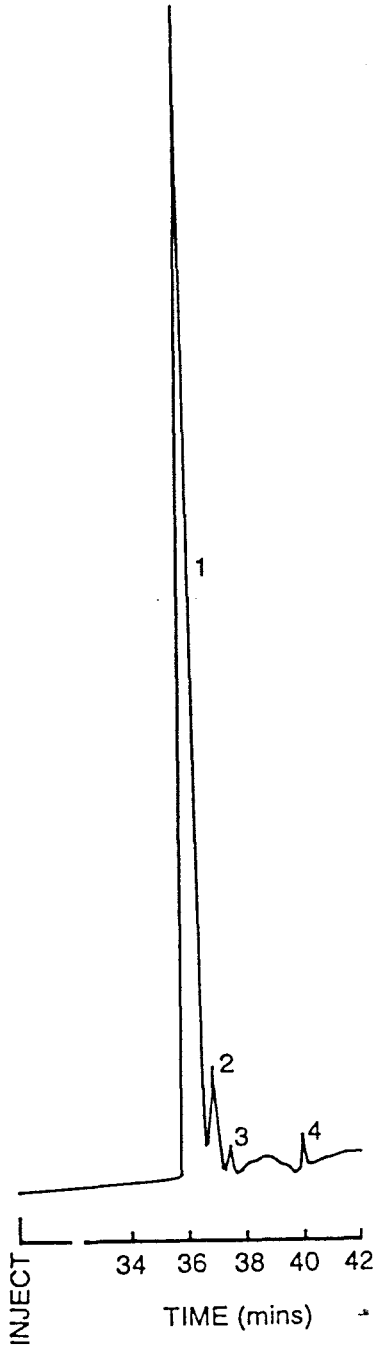


Figure 6.8 a Erythromycin sample from Lennons Limited.
1. Erythromycin A
2. Erythromycin enol ether
3. Unknown
4. N-demethylerythromycin

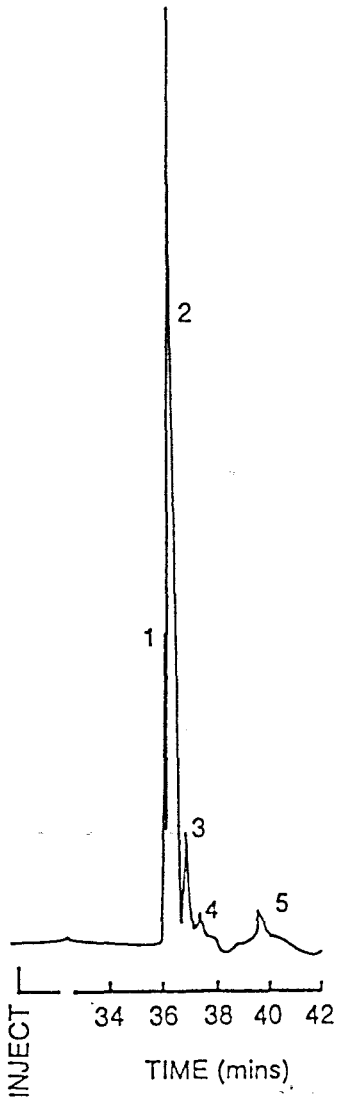


Figure 6.8 b Erythromycin sample from Abbott Laboratories.
1. Erythromycin B
2. Erythromycin A
3. Erythromycin enol ether
4. Unknown
5. N-demethylerythromycin

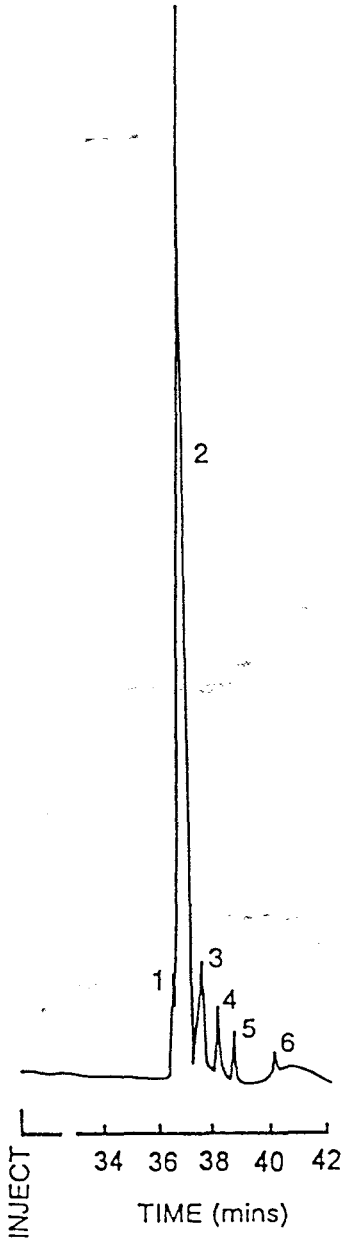


Figure 6.8 c Erythromycin sample from Pfizer Pharmaceuticals.
1. Erythromycin B
2. Erythromycin A
3. Erythromycin enol ether
4. Unknown
5. Unknown
6. N-demethylerythromycin

Figure 6.8 a, b ,c Electropherograms of commercial samples of erythromycin. Conditions as for Fig. 6.1. Electropherogram size reduced (X 0.70)

It is feasible to quantitate the impurities NDEA and EEE using both the regression equations obtained from the calibration curve of the relative normalised peak areas or by quoting the impurity levels of EEE and NDEA as a percentage area/area of the electropherogram. The results of the mean impurity levels are tabulated below (Table 6.7). The quantity of impurity is either determined from the calibration curve and expressed as a percentage of the total amount of erythromycin base (a) or as a percentage area/area of the impurity to the total area of the electropherogram (b). The regression equations used were: EEE, $y = 7.98x - 0.038$ and NDEA, $y = 1.5x + 0.003$.

Table 6.7 Mean Impurity Levels in Commercial Sample of Erythromycin (%)

	EEE		DM	
	a	b	a	b
Lennons	1.25	3.64	1.40	0.94
Pfizer	1.55	7.02	1.76	1.00
Abbott	1.45	4.27	0.70	0.71

From the results, it can be noted that the percentage of DM calculated from the calibration curve is slightly greater than when the impurity levels are quoted as a percentage area/area of the electropherogram. The opposite is observed for EEE. This effect can be explained in terms of the different UV activities of the compounds. Erythromycin enol ether has a significantly higher UV activity at the detection wavelength. Thus the level of impurities are overestimated, while DM possesses lower UV activity at 200 nm. Consequently impurity levels are underestimated.

6.2.8.3 Discussion

Levels of impurities may be determined from the calibration curve of the impurities [216] or alternatively, if standards of all the impurities are unavailable, impurity levels may be quoted as a percentage area/area of the electropherogram [215, 224]. Although, the latter approach is most widely used, it should be noted that the impurity levels determined are dependent on the UV activity of the impurities at the detection wavelength.

If impurities in the commercial samples are present at a low concentration, below the linear dynamic range of detection, high-low injection as described in Section 6.2.6.3 may be performed. This technique lowers the detection limit and improves the precision of quantification and identification of all impurities present [232].

The assay developed for the analysis of erythromycin related substances was capable of quantitating NDEA and EEE present in commercial samples of erythromycin base. It was not possible to resolve EB from erythromycin base when it was present in large concentrations. Therefore, the assay cannot be used for the quantitative determination of EB in commercial samples of erythromycin.

MAIN CONCLUSION

Two methods have been developed and successfully validation of the assay of erythromycin alone and for erythromycin related substances using CE. For the quantitative determination of erythromycin base the assay method developed for erythromycin alone can be used. The assay method developed for erythromycin related substances can be used for the quantitative determination of EC, AE and NDEA only. The inability of this assay to resolve EA from EB and EEE when large concentrations of EA are present, precludes its use for the quantitative determination of EB and EEE. Therefore, for the quantitation of EB and EEE a third system needs to be developed in which EB and EEE are well resolved from EA, even at the expense of unresolved EC, AE and NDEA. In summary, it will be possible to quantitatively determine erythromycin and all its related substances with the use of three methods.

The quantitative analysis of erythromycin by reversed-phase liquid chromatography was performed by Cachet et al. [228]. Superior resolution was achieved with this technique as compared to that developed using CE. The LC method and CE methods both demonstrate acceptable linearity, however the range selected in the LC method was significantly lower (130 - 200 µg). In addition, the LC method demonstrated acceptable precision (< 1 % RSD) and accuracy at low sample concentration levels. The sensitivity of the LC method was significantly greater with LOQ values of between 0.05 % and 0.5 %, as compared to 5 % and 15% in the CE method. The CE methods for the quantitation of erythromycin and its related substances demonstrated acceptable precision and accuracy at higher sample concentration.

The methods developed for the analysis of erythromycin and its related substances are advantageous in that they are simple, require minimal sample and electrolyte preparation and utilise very small quantities of sample and organic solvent. The methods are therefore environmentally friendly and cost effective as smaller quantities of organic solvents are used and the capillaries are cheaper than HPLC columns. Manipulation of the experimental parameters and consequently the development of methods for the assay of erythromycin and related substances using CE was relatively simple and significantly faster than for HPLC. As a result, several methods have been developed using CE for the analysis of pharmaceuticals [233 - 235].

In addition to the above advantages, CE is preferable to HPLC in drug analysis as high separation efficiencies and the resolution of a large number of peaks are attainable in a reasonable analysis time.

The main drawbacks in CE are instrument based. Capillary electrophoresis systems, although equipped with automated features, are still first generation instruments. Improvements in injectors, detectors, capillary technology and capillary dimensions are required. The principle disadvantage of CE relates to precision and detection levels achieved. Regulatory authorities require the identification of all impurities and degradation products that are observed in the stability studies, at or above 0.1 % in batches of commercial raw materials. Precision of injection in CE is 1 - 2 % RSD for the main components compared to RSD's of less than 1 % obtained in HPLC. Precision levels can be improved by incorporating an internal standard.

Detection limits obtained in CE are an order of magnitude less sensitive than HPLC. This is attributed to the very low volume of sample that can be injected onto the capillary. Although this is considered advantageous with respect to the amount of sample required for the analysis, it is a disadvantage with regards to the detection of analytes. It is possible to improve the detection limits in CE by employing low UV wavelengths where significant enhancement in UV absorption activities are possible. In addition, on-capillary concentration techniques such as FASI and stacking are further methods of improving detection sensitivity. Detection sensitivity may also be enhanced by using modified capillaries, such as Z-shaped flow through cells, bubble cells and rectangular capillaries. The pathlength in these capillaries is significantly lengthened and thus sensitivity is improved.

Capillary electrophoresis is probably one of the fastest-growing analytical techniques in the separation sciences. It is an established analytical technique capable of providing additional information to augment that obtained in HPLC. Capillary electrophoresis is capable of generating high quality data with acceptable levels of precision, accuracy and linearity as demonstrated by the assay for erythromycin and for its related substances. Continued efforts aimed at improving the separation capabilities, detection sensitivity and reliable quantitation of commercial instruments will encourage the widespread acceptance of CE in pharmaceutical analysis.

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