PHARMACEUTICAL ANALYSIS BY CAPILLARY ELECTROPHORESIS

by Catherine Meehan, BSc,

submitted for the degree of Master in Science,

Institute of Technology Sligo,
Ballinode,
Sligo,

supervised by Dr. Fiona Mc Ardle

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For my husband Brian and son Conal, thankyou for your continuous, unconditional love.

ABSTRACT

This project focused on developing and validating analytical methods for a range of pharmaceutical compounds using Capillary Electrophoresis.

Analytical methods for the determination of tromethamine, oxytetracycline and polypyrrolidone contained in pharmaceutical products were developed. Approaches to method development included consideration of appropriate mode of capillary electrophoresis, detection technique, suitability of electrolyte, effect of electrolyte pH, effect of voltage, temperature and electrolyte ionic strength variation.

Validation of the methods developed for tromethamine and oxytetracycline included conducting investigations into linearity, linear range and limit of detection. Repeatability, precision and accuracy were also examined as well as the specifity and recovery of each method developed.

CONTENTS

CHAPTER 1

INTRODUCTION

1.1 Introduction to Capillary Electrophoresis

Capillary Electrophoresis (CE), is an analytical technique, which permits rapid and efficient separation of charged components. Separations are based on the differences in electrophoretic mobilities of charged species that take place inside small capillary tubes.

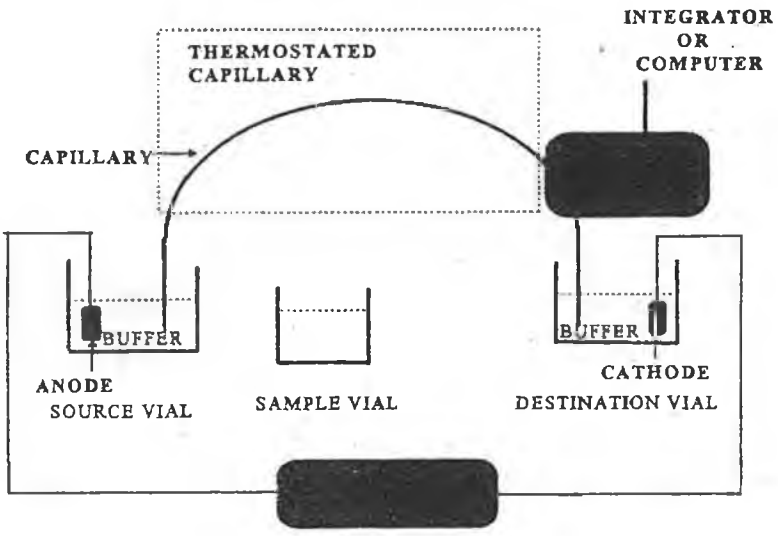


Diagram 1.1 Schematic Diagram of CE Instrumentation

1.2 Principles of Separation by CE

To perform a CE separation, the capillary is filled with an electrolyte solution and sample is introduced at the source vial. The capillary ends are dipped into source and destination vials containing the electrolyte and a potential is applied across the capillary. The bulk electrolyte travels by electroosmotic flow (EOF) and ionic species in the sample migrate with an electrophoretic mobility (EPM) determined by their mass:charge ratio. The analyte ions eventually pass the cell window and a response from the UV detector is generated and passed onto an integrator. Signals generated follow Beer-Lambert Law.

$$A = \varepsilon c l \qquad \text{(Eqn. 1.1)}$$

A = Absorbance (nm), ε = Molar absorptivity coefficient, c = Concentration (mg/L) and l = path cell length (cm)

Normally the system is configured with the source vial anodic and the destination vial cathodic. As a potential is applied across the two electrodes, ions in the buffer travel through the capillary to the electrode of opposite charge. Positively charged ions migrate towards the negatively charged electrode and negatively charged ions migrate towards the positively charged electrode. Neutral species in the solution travel at the same rate as the bulk electrolyte.

1.3 Origin of Capillary Electrophoresis

Capillary Electrophoresis represents a merging of technologies derived from slab gel electrophoresis and high performance liquid chromatography (HPLC). The development of capillary electrophoresis has been aided by the investigations of many workers over the past century[1].

Table 1.1 Historical Development of CE

Year	Researcher	Development
1886	Lodge	H^+ migration of phenolphthalein jelly
1892	Smirnow	Electro-fractionation of diphtheria toxin solution
1905	Hardy	Globulin movement in U-tubes with electric current
1930	Tiselius	Moving boundary studies of proteins in solution
1930	Coolidge	Electrophoretic separation of serum proteins in tubes of glass wool
1965	Tiselius	'Free Zone Electrophoresis' of virus in 3 mm i.d. rotating capillary
1981	Jorgenson& Lukacs	Theoretical and experimental approaches to high resolution Electrophoresis in glass capillaries
1984	Terabe	Micellar Electrokinetic Capillary Chromatography for separation of neutral compounds
1989		Availability of commercial CE instruments.

At present, CE is considered a complimentary separation technique to traditional separation techniques. In some cases it has been shown to be the superior technique[2-5] however, due to sensitivity limitations the method may be considered more suitable for analysis of bulk chemicals than trace analysis.

1.4 Modes of Capillary Electrophoresis

Five distinct separation techniques are available with CE. These are Capillary Zone Electrophoresis (CZE), Micellar Electrokinetic Capillary Chromatography (MECC), Capillary Gel Electrophoresis (CGE),

Capillary Isoelectric Focusing (CIEF) and Capillary Isotachophoresis (CITP). The origin of these various CE modes may be attributed to the fact that CE has developed from a combination of many electrophoretic and chromatographic techniques. In Capillary Zone Electrophoresis (CZE), separations are based on the difference in the electrophoretic mobilities of solutes. Velocities of ionic species are based on a mass:charge ratio. In Capillary Gel Electrophoresis (CGE), solutes are separated due to differences in electrophoretic mobility and solute size, as analytes migrate through the pores of a gel-filled capillary. The use of gel allows separations based on molecular-sieving as different substances migrate with different velocities. In MECC, the solute partitions between a micellar phase and a solution phase. An ionic surfactant solution is used to provide a micellar phase to allow chromatographic separation. Organic analytes distribute themselves between the micellar phase and the aqueous electrolyte. CIEF utilises differences in isoelectric points of proteins and peptides to effect separation. Migration zones are focused until a steady state is reached and are migrated from the capillary by a pressurised flow. CITP is performed by sandwiching a sample between a leading and terminating buffer in a capillary and by applying an electric field in a constant current mode. All buffer and solute ions migrate through the capillary and the detector at the same velocity. The combination of capillary electrophoresis and chromatography has lead to the development of a new technique called Capillary Electrochromatography, (CEC). The separating capillary is partially packed with a chromatographic packing which can retain solutes by normal distribution equilibrium upon which chromatography depends.

1.5 Comparison of Capillary Electrophoresis with other separation techniques[6]

Table 1.2 Comparison of CE with other Separation Techniques

Separation Techniques	Capillary Electrophoresis	High-Performance LiquidChromatography	Gas Chromatography
Efficiency	Hundreds of Thousands	20,000	150,000
Sample Volume	Nl	µL	µL
Sensitivity	ppm to ppb	pptr	pptr
Reagent Requirements	< 10 ml buffer / day	500-1000 ml mobile phase / day	500-1000 ml mobile phase / day

CE is gaining increasing approval as an analytical technique, which may be more suitable for the analysis of certain compounds and can be compared with accepted established techniques as illustrated above. Cost of capillaries and columns depend on the application area. CE capillaries are considerably cheaper than other columns and cost in the region of 30-200 Euro, IC columns range from 1500-2000 Euro while GC and HPLC columns may cost 150-3000 Euro. The use of organic solvents is usually incorporated in the analysis of samples by both GC and HPLC while CE, in cases where organic solvents are employed, minimal volumes are needed. With recognised advantages and disadvantages outlined above, CE can be appreciated as a complimentary technique to HPLC, GC and slab gel electrophoresis.

1.6 Analytes of Interest in this Study

The objective of this study was to develop capillary electrophoresis methods for compounds used in commercial eye-care products. In discussions with personnel working in the analytical laboratory of an eye-care product production plant[7], a selection of compounds were identified as being difficult to analyse by HPLC and other analytical techniques. These included Tromethamine, Povidone, Sodiumcarboxymethylcellulose, Polyhexamethylene Biguanide, Tris(hydroxyethyl) Tallow Ammonium Chloride and Polyoxyl-(40)-Sterate.

Table 1.3 Current Methods of Analysis[7]

Compound	Current Analytical Method	Detection System	Detection Limit Required	Difficulties
Tromethamine	Ion-Ex	R.I.	1.08%	Interference
Povidone	GPC	UV	0.6%	Interference
Sodiumcarboxy methylcellulose	GPC	R.I.	0.5%	Run Time100 min, Interference
Polyhexamethylene biguanide	RP-HPLC	UV	1 ppm	Run Time 30 min, Interference
Tris(hydroxyethyl) Tallow Ammonium Chloride	RP-HPLC	UV	0.1%	Run Time 30 min, Flow Rate 6 ml/min
Polyoxyl-(40)-Sterate	GPC	R.I.	0.5%	Interference

After examining the structures and carrying out a literature search, it was decided to commence this study with the analyte tromethamine. Hence, the aim of this study was to develop and validate a method of

analysis for tromethamine in an eye-care product. Current analytical methods include High Performance Liquid Chromatography (HPLC), Reversed Phase High Performance Liquid Chromatography (RP-HPLC), Gel Permeation Chromatography (GPC) and Ion-Exchange Liquid Chromatography (IC). These methods suffer from interference and lengthy analysis times.

Initially, it was necessary to become familiar with the technique and for this purpose a range of experiments were carried out using established procedures. These included analysis of benzoic acid by direct UV detection and analysis of inorganic cations by indirect UV detection.

1.7 Applications of Capillary Electrophoresis

CZE and MECC have been used for the analysis of a wide variety of compounds. Literature available for method development and validation is presented for applications mainly in the pharmaceutical industry.

Analyte	Matrix	Sample Preparation	Separation Technique	Electrolyte	Analytical Performance	Reference
Inorganic and Organic Anions	Brine	Solid Phase Extraction, Dilution, Filtration	CZE with Indirect UV Detection	Sodium Chromate Tetrahydrate (5 mM), pH 8.0	R^2=0.9994 1×10^{-5} - 1×10^{-3} mol/L	1
Proteins	-	Dilution, Filtration	CZE-UV	Tris (50 mM)-Phosphoric Acid, pH 3.0	LOD=10^{-2} mg/mL	2
Enantiomers	Neuromuscular blocking drug	Dilution, Filtration	CZE-UV	Phosphate (0.05M), pH 5.0	-	3
Histamine	Fish Tissue	Extraction, Filtration	CZE-UV	Citrate (20mM), pH 2.5	R^2=0.999 LR 0.5-100µg/ml CV<3% Rec. 94.3-109.7%	4
Toxins	Shellfish	Solid Phase extraction	CZE-UV	Acetic Acid (0.1M), pH 2.0	R^2=0.998 LOD 1.5µg/ml	5
Salbutamol etc.	Drugs	Direct Injection	CZE-UV	Phosphate (25mM), pH 2.3	R^2=0.995 LOD 0.4µg/ml RSD=6.6-11.2%	6
Proteins	-	Direct Injection	CZE-UV	Phosphate(50 mM)-PVA, pH 3.0-5.0	RSD 1.2%	7
Estrogen, Warfarin	Steroids	Direct Injection	CZE	Sodium Borate-MeoH (50mM), pH 10	-	8
Paracetamol	Urine	Solid Phase Extraction	CZE-ESMS	Ammonium Hydrogen Carbonate (20 mM), pH 9.0	LR 0.1-100µg/ml R^2=0.9996	9
Denopamine	Tablets	Dilution, Filtration	CD-CZE	Phosphate(25mM)-Urea(2M), pH 2.5	R^2=0.999 RSD 0.1-0.2%	10

Table 1.4 Literature Review for CZE

Inorganic cations/anions	Food	Solid Phase Extraction	CZE-Indirect UV	Sodium Chromate Tetrahydrate (5 mM), pH 8.0	-	11
Organic Acids	Food	Thawing, Dilution	CZE-Indirect UV	Phthalate (5 mM), pH 5.6	RSD 1.2% LR 10-50µg/ml R^2>0.999	12
Vitamin C	Food	Centrifugation, Filtration, SDS Addition	CZE-UV	Borax (35 mM), pH 9.3	-	13
Codeine, Morphine	Crude opium	Centrifugation, Dilution, Filtration	CZE-UV	Ammonium Acetate(25mM)-Acetic Acid(1M)	-	15
Proteins	Serum	Dilution	CZE-UV	Boric Acid (50mM), pH 9.7	R^2=0.96 CV<11	16
Sulphonamides	Pork Meat	Extraction	CZE-UV	Phosphate(0.02M)-Borate(0.02M), pH 7.0	R^2=0.999 LR 2-9 µg/ml Rec 100%	17
Phenylalanine	Drug	Direct Injection	CZE-UV	Phosphate (0.05M), pH 2.0	-	18
Cleaning Surfactants	Eye-care solutions	Direct Injection	CZE-UV	Copper Sulphate (4 mM)-H_2SO_4(1M), pH 3.0	R^2=0.9995 LR 0.15-0.3µg/ml RSD 1.5% Rec. 100%±1.5%	19

Analyte	Matrix	Sample Preparation	Separation Technique	Electrolyte	Analytical Performance	Ref
Vitamins, Penicillins, Antibiotics	Cold Medicine	None	MECC	Phosphate -Borate (0.02M)-SDS-TAA Salts, pH 9.0	-	25
Antibiotics	Animal Feed	Extraction, Centrifugation	MECC	Sodium Tetraborate Decahedrate (20 mM)-SDS (150mM), pH 9.2	R^2=0.9992 LR=0.01-0.2 µg/ml RSD<4% Rec. 98.4-108%	26
Insecticides	Soil, Vegetation	Extraction, Centrifugation	MECC	Borate(200mM)-Phenylphosphonic Acid (10 mM), DDAOH/ CTAOH	LOD=75µg/L	27
Proteins	Meat	Extraction, Centrifugation	MECC	Borate (100mM)-SDS (75 mM), pH 8.4	LOD=1µg/ml	28
Amino, Thiol Drugs	Direct Injection	Derivatisation	MECC	Borate (0.1M)-SDS(10mM), pH 9.5	-	29
Dyes	Hair	Addition of Sodium Sulphite	MECC	Phosphate-Borate (25mM)-SDS (75mM), pH 5.5	R^2=0.9812-0.9962	30
Cefotaxime	Degradation Products	Direct Injection	MECC	Phosphate (30mM)-SDS (165mM), pH 7.2	R^2=0.9993 LR=0.05-1.50 g / L RSD<2% REC.98.5-98.9%	31

Biological Surfactants	-	Direct Injection	MECC	Phosphate (50mM)-SDS(10mM), pH7.0 Dehydrocholate	-	32
Hydrochlorothiazide Chlorothiazide	Drug Matrix	Direct Injection	MECC	Borate (20mM)-SDS (20mM), pH 9.5	R^2=0.998 LR=0.5-1.5 mg /ml RSD 1-2% LOD= 1µg/ml Rec.=99.5%	33
Lead and Selenium Compounds	Environmental Samples	Extraction, Drying, Filtration	MECC	Phosphate-Borate (25mM)-SDS (50mM), pH 6.0	R^2=0.998-0.999 LR=0.5-1.5 mg /ml RSD 1-2% LOD= 8-20 µg/ml Rec.= 83-104%	34

CHAPTER 2

THEORY

2.1 Capillary Zone Electrophoresis (CZE)

Separations by CZE are performed in a homogenous carrier electrolyte. Ionic components are separated into discrete bands when each solute's individual mobility is sufficiently different from all others.

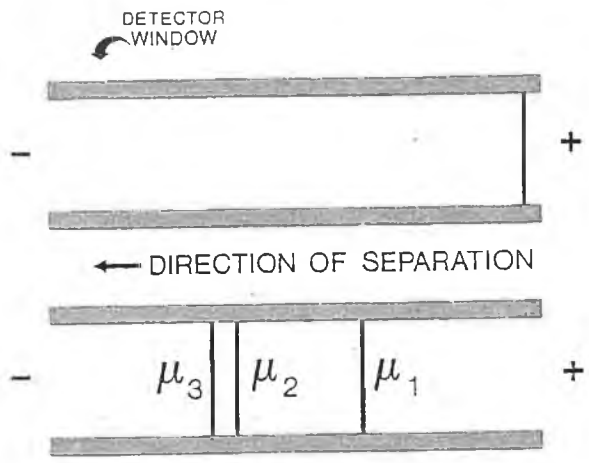


Diagram 2.1 Separation of a Three Component System[35]

2.1.1 Order of Elution

If a sample is introduced into the capillary at the positive end, positively charged ions will travel through the capillary towards the negatively charged electrode at a rate faster than the neutral species. The negatively charged ions will be pulled back toward the positively charged electrode and will therefore travel toward the negatively charged electrode at a slower rate than the neutral species. Hence the order of elution will be (i) positively charged ions, (ii) neutral species and (iii) negatively charged ions. The order of elution will be reversed if the sample is introduced into the capillary at the negative end of the capillary.

2.1.2 Fundamental Features Required for Separation

Four fundamental features required for good separations in capillary electrophoresis are:

(a) individual mobilities of each solute in the sample differ from one and other,

(b) background electrolyte is homogenous and the field strength distribution is uniform throughout the length of the capillary,

(c) solutes nor sample matrix elements interact or bind to the capillary wall,

(d) conductivity of the buffer substantially exceeds the total conductivity of the sample components.

2.1.3 Capillaries

Capillaries used in CZE are fused silica capillaries or capillaries packed with an internal coating. The selection of the capillary depends on the application.

2.1.3.1 Fused Silica Capillaries

The cost of silica capillaries depends on the instrument being used. These capillaries, precut to size, cost from approximately 150-200 Euro and are frequently employed in Beckman and Hewlard Packard instruments. Fused silica capillaries can also be purchased uncut and range from 30-40 Euro per meter. On column detection is facilitated by the creation of a capillary cell window. The fused silica capillary is transparent to UV and visible light and therefore the capillary itself can be used as the detector cell.

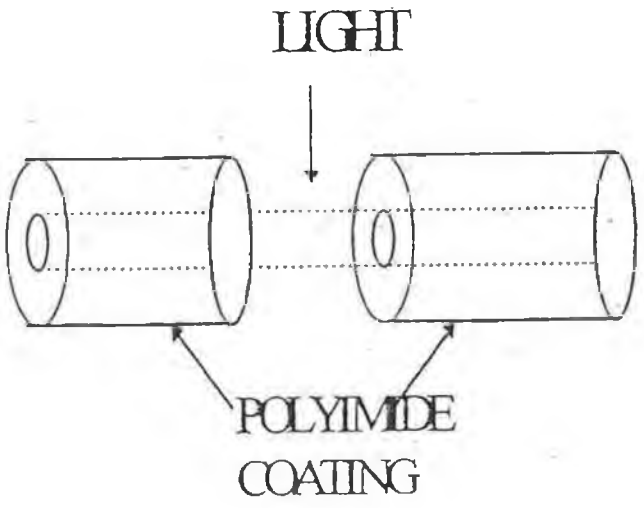


Diagram 2.2 Capillary Cell Window

2.1.3.2 Capillary Dimensions

Capillaries with internal diameters of 50-100 μm, and outer diameters of 375 μm are most frequently used. Migration time is directly proportional to the capillary length therefore longer capillaries will induce longer migration times, however efficiency and resolution will be increased. As the capillary length increases, an associated decrease in the electrical field strength at constant voltage occurs and this enables the use of higher voltages. Short capillaries are useful for fast method development, conducive for the analysis of less

complex mixtures, or when selectivity or migration times are excessive[1]. Long or narrow capillaries are useful for high efficiency and resolution needs, analysis of complex matrices, or when analysis times are inadequate[1]. The main advantage resulting from increasing the capillary inner diameter is the enhancement of detector sensitivity, however accompanying an increase in diameter is a decrease in the surface-to-volume ratio. This may lead to less efficient dissipation of Joule heat which then results in a temperature gradient across the capillary and band broadening due to thermal effects.

2.1.4 Injection Techniques

In CZE, sample introduction may occur by hydrodynamic injection or by electrokinetic injection. Hydrodynamic injections may be performed by gravity or pressure. Sample introduction by gravity injection is the most common injection method as it offers good reproducibility[35]. It relies on the siphoning of the sample into the capillary by elevating the injection (inlet) end of the capillary relative to the outlet end. Hydrodynamic injection can be achieved by placing the end of the capillary into a sample solution followed by moving the sample container and column end to a certain height Δh, higher than the opposite end of the capillary for a period of time. The volume injected, q, is given by

$$q = \frac{\rho g r^4 \Delta h t_i}{8 \eta L} \qquad \text{(Eqn. 2.1)}$$

q = injection volume, ρ = density of sample solution, g = constant for gravitational acceleration, r = internal radius of capillary, Δh = height difference between anodic and cathodic capillary ends, t = duration of injection, η = solution viscosity and L = total capillary length.

The amount injected is represented as a function of the sample concentration C as

$$w = \frac{\rho g \pi r^4 \Delta h C t_i}{8 \eta L} \qquad \text{(Eqn. 2.2)}$$

w = the amount of sample injected, ρ = density of the sample solution, r = internal radius of capillary, Δh = height difference between anodic and cathodic capillary ends, C = concentration, ti = injection time, η = solution viscosity and L = capillary length.

Thus, the amount injected is independent of the electrophoretic mobility, and the composition of the sample solution has no effect on the amounts injected by this method. The quantity introduced can be controlled by variations in the injection time, t_i, and the injection height Δh.

2.1.4.1 Pressurised and Vacuum Injection

During pressurised injections, pressure is applied to the vial containing the sample, pushing it into the capillary. During vacuum injection, a sample is placed at the opposite end of the capillary, drawing the sample into the capillary. The amount of analyte injected with pressure can be calculated from the Poiseuille law.[36]

$$W = \frac{\Delta P \, \pi \, r^4 \, \Delta h \, C \, t_i}{8 \, \eta \, L} \qquad \text{(Eqn. 2.3)}$$

where ΔP = pressure difference across the capillary.

Other injection techniques include electric sample splitting methods, split flow syringe systems, rotary injection, freeze plug injection, optical grating and microinjection techniques.

Detection methods used in CE

2.1.5 Detection

Many detection systems have been successfully coupled with CE[35], these include UV / visible fluorescence, mass spectrometry, conductivity, amperometric, radiometric, raman-based and refractive index detectors. Most commercial instruments have, as standard, a UV / visible detector, some have fluorescence and conductivity available and can be purchased separately. The standard UV / vis detector allows detection of organic components which have chromophores and coloured compounds. Charged species which do not have a chromophore must be detected by indirect photometric detection.

2.1.5.1 Direct UV Detection

The principle of UV / vis absorbance detection is based on Beer-Lambert's Law. Direct UV detection is used to detect compounds that have an intrinsic UV absorbing species. The chromophore is the light

absorbing part of the molecule and thus according to the Beer-Lambert Law, the absorbance signal generated by the analyte passing through the capillary depends on (1) the chromophore of the solute, (2) wavelength of incident light and (3) pH and composition of the run buffer. Compounds may be detected at their UV maxima using aqueous electrolyte systems that exhibit very stable baselines at low absorbance values.

2.1.5.2 Indirect Photometric Detection

This is used for detecting charged species which can be separated by CE but which do not have a UV chromophore. It is achieved by incorporating a chromogenic reagent into the electrolyte thus giving a constant high background UV absorbance. The mechanism of indirect UV detection involves the physical displacement by the analyte of interest of some of the chromogenic molecules in the electrolyte, as a result, decrease in absorbance is observed.

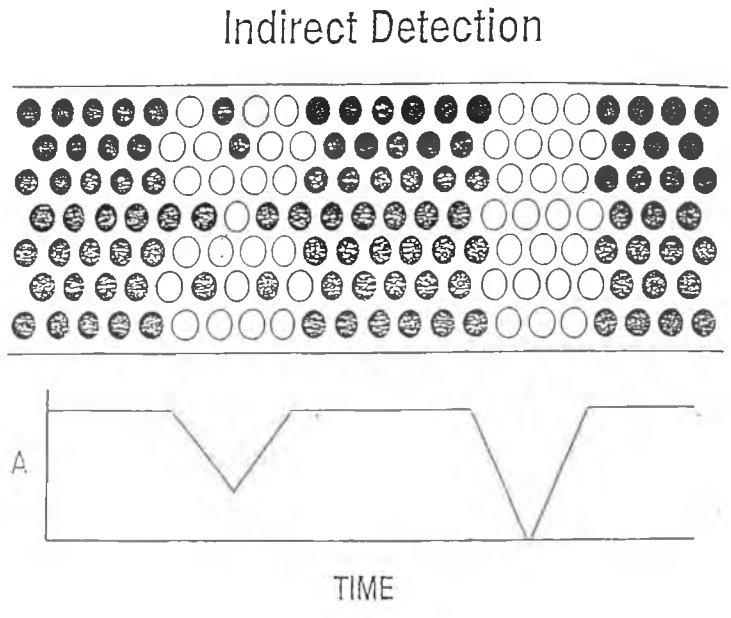


Diagram 2.3 Indirect UV Detection

For lower limits of detection, indirect detection requires a well characterised displacement mechanism such as charge displacement, a large transfer ratio and a very stable background. The transfer ratio refers to the number of chromogenic molecules displaced by an analyte molecule while charge displacement refers to an anionic signal generation being displaced by an anionic analyte molecule. As the analyte concentration

is decreased, smaller differences between the background signal and the chromogenic signal are observed. Sensitivity and limit of detection can be optimised in indirect UV detection by careful selection of the chromogenic species.

2.1.6 Electroosmotic Flow and Electrophoretic Mobility

Bulk electrolyte travels through the capillary by eletroosmotic flow (EOF) and the analyte molecules are separated due to differences in electrophoretic mobility (EPM).

2.1.6.1 Electroosmotic Flow

When using fused silica capillaries, the inner surface of the capillary acquires a charge when a high pH buffer is introduced. This is thought to occur due to the ionisation of the capillary. Using electrolyte at pH > 3, the surface silanol (Si-OH) groups are ionised to negatively charged silanoate ($Si\text{-}O^-$) groups. New capillaries conditioned with NaOH or KOH facilitate this ionisation. The silanoate groups attract positively charged cations that form a positively charged layer on the inner wall of the capillary. These cations are not of sufficient density to totally neutralise the negative charges of the silanoate, so a second layer of cations forms. The $Si\text{-}O^-$ groups attach a fixed layer of positive ions tightly at the capillary surface and a second layer of positive cations are held less tightly because they are further from the $Si\text{-}O^-$ charges.

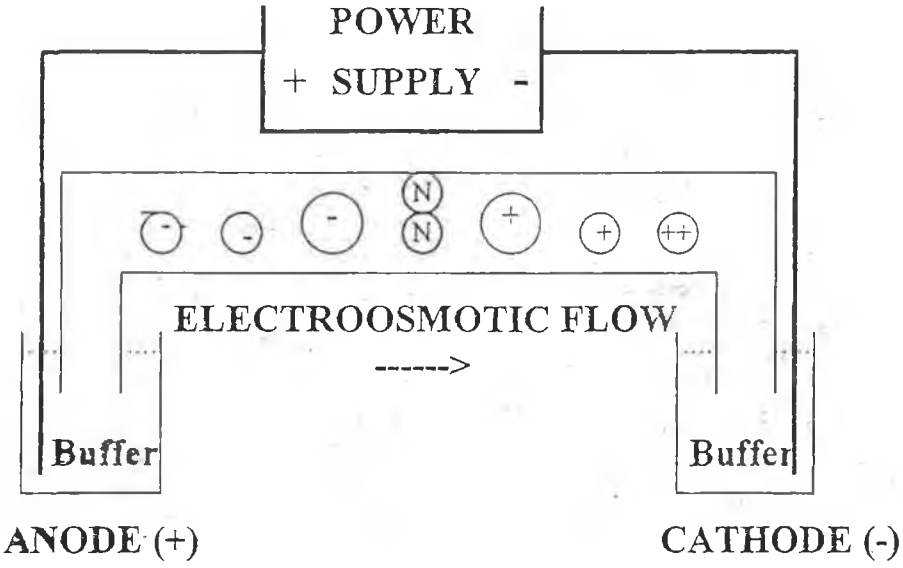


Diagram 2.4 Electroosmotic Flow

The second layer of positive charges are mobile. As soon as an electric field is applied to the outer mobile layer, this layer of cations is pulled towards the negative electrode. Thus, the movement of the bulk electrolyte causes electroosmotic flow as the positively charged cations are solvated.

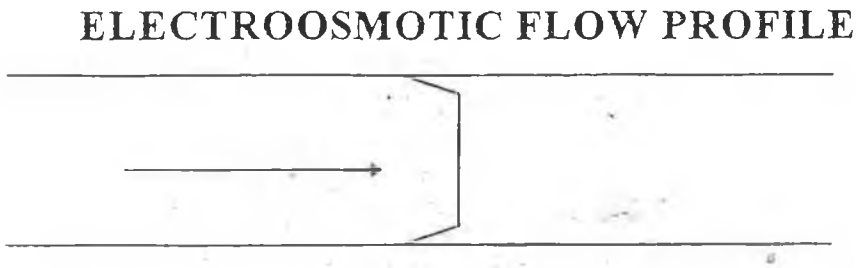


Diagram 2.5 Electroosmotic Flow Profile in CE

The velocity of the electroosmotic flow is given by

$$\nu_{EOF} = \varepsilon \zeta E / 4 \pi \eta \qquad \text{(Eqn. 2.4)}$$

where ν_{EOF} = electroosmotic velocity, ε = dielectric constant of the buffer, ζ = zeta potential, E = applied electric field in volts / cm and η = viscosity of the buffer.

2.1.6.2 Reversal of EOF

Normal polarity is to have the anode (+) at the inlet and the cathode (-) at the outlet. In this format, the EOF travels towards the cathode (detector). Order of elution follows positive ions, neutrals and negative ions. If the polarity is reversed, the direction of the EOF is away from the detector and the order of elution is reversed. Thus, depending upon the charge of the analyte, the electrode polarity is altered.

2.1.6.3 Measurement of EOF

The electroosmotic velocity (ν_{EOF}) and electroosmotic mobility (μ_{EOF}) can be determined by injecting an uncharged species (neutral marker) into the capillary.

$$\nu_{EOF} = l / t_{nm} = \text{distance / time (cm / } s^{-1}) \qquad \text{(Eqn. 2.5)}$$

l=length of the capillary from the inlet to the detector, t_{nm}=migration time.

$$\mu_{EOF} = \nu_{EOF} / E = cm^2 / V \cdot s^{-1} \quad \text{(Eqn. 2.6)}$$

Neutral Markers chosen in any system must be (a) uncharged at the pH of the buffer, (b) detectable by the detector used, (c) pure, (d) unreactive with the capillary walls and (e) soluble in the buffer.

2.1.6.4 Electroosmotic Mobility

The electroosmotic mobility of the buffer, μ_{EOF}, is given by

$$\mu_{EOF} = \varepsilon \zeta / 4 \pi \eta \quad \text{(Eqn. 2.7)}$$

where μ_{EOF} = electroosmotic mobility, ε = dielectric constant of the buffer, ζ = zeta potential, and η = viscosity of the buffe.r

Thus the electroosmotic mobility is dependent on buffer characteristics such as dielectric constant, viscosity, pH and concentration but is independent of the applied electric field.

2.1.6.5 Electrophoretic Mobility

Analytes are separated in CE on the basis of the rate at which they travel through the capillary, electrophoretic mobility (μ_{EP}). Electrophoretic Mobility (μ_{EP}) of a charged molecular species can be approximated from the Debye-Huckle theory[35],

$$\mu_{EP} = \frac{q}{6 \pi \eta r} \quad \text{(Eqn. 2.8)}$$

where q = charge on the particle, η = viscosity of the buffer and r = Stoke's radius of the particle.

The mass of the particle may be related to the Stoke's radius by

$$M = (^4/_3) \pi r^3 V \quad \text{(Eqn. 2.9)}$$

where M = mass of the particle, r = Stoke's radius of the particle and V = applied voltage.

Under the influence of an electric field an electrically charged solute will migrate through a buffer with an electrophoretic velocity, ν_{EP},

$$\nu_{EP} = \mu_{EP} E \ (cm / s) \quad \text{(Eqn. 2.10)}$$

$$\mu_{EP} = q / 6 \mu \eta r \ (cm^2 / V \cdot s) \quad \text{(Eqn. 2.11)}$$

μ_{EP} = electrophoretic mobility, E = applied electric field, q = charge of ionised solute, η = buffer viscosity and r = Stoke's radius.

The greater the mass:charge ratio the greater the electrophoretic mobility, and hence the electrophoretic velocity. Small molecules with a large charge move through the system fastest, large molecules with a small charge move slowly through the system. Neutral species have zero charge (q = 0) therefore they do not have electrophoretic mobility. Electrophoretic mobility is affected by solvent viscosity in the same way as electroosmotic velocity.

$$\nu_{OBS} = \nu_{EP} + \nu_{EOF} \qquad \text{(Eqn. 2.12)}$$

$$\mu_{OBS} = \mu_{EP} = \mu_{EOF} \qquad \text{(Eqn. 2.13)}$$

Electrophoretic velocity and mobility for any solute can be determined by measuring the electroosmotic velocity and the observed velocity,

$$\nu_{EP} = l / t_m - l / t_{nm} \qquad \text{(Eqn. 2.14)}$$

t_m = migration time of solute, t_{nm} = migration time of neutral marker, l = effective length of capillary.

$$\mu_{EP} = (l / t_m - l / t_{nm})\ (L / V) \qquad \text{(Eqn. 2.15)}$$

L = total capillary length, V = applied voltage.

Solvent viscosity, temperature, electrolyte pH and ionic strength will also affect the electroosmotic flow.

2.1.6.6 Migration Time

Migration time, t_m, is the time taken for the solute to travel from the capillary inlet to the detector and depends on both EOF and EPM.

$$t_m = l / \nu_{OBS} \qquad \text{(Eqn. 2.16)}$$

$$\nu_{OBS} = \mu_{OBS} E \qquad \text{(Eqn. 2.17)}$$

Therefore, $t_m = l / \mu_{OBS} E$ (Eqn. 2.18)

Also, since $E = V / L$ $t_m = l L / \mu_{OBS} V$ (Eqn. 2.19)

On substitution: $t_m = (\mu_{EP} + \mu_{EOF})\ V$ (Eqn. 2.20)

2.1.7 Background Electrolyte

The electrophoresis buffer is of key importance because its composition fundamentally determines the migration behaviour of the analytes. A suitable electrolyte must ensure[1] (a) the correct electrophoretic behaviour of all individual solutes, (b) the overall stability of the system and (c) satisfactory separation of the analytes. The majority of buffers used in CE are aqueous electrolytes[36].

2.1.7.1 Analyte Ionisation

It is important to consider the degree of ionisation of the analytes in the solution. In the case of weak acids or bases, their degree of ionisation depends on the pH of the solution. For a monovalent weak acid HA, which has a dissociation constant K_{HA} and the degree of dissociation is given by:

$$K_{HA} = \frac{C_{H^+} \cdot C_{A^-}}{C_{HA}} \qquad \text{(Eqn. 2.21)}$$

where, C_{A^-} =the concentration of the ion A^-, C_{HA} =concentration of the non-ionised weak acid, C_A =total concentration of the weak acid and C_{H^+}= is the concentration of the proton.

$$\alpha_A = \frac{C_{AH^+}}{C_A} = \frac{C_{H^+}}{K_{AH^+} + C_{H^+}} \qquad \text{(Eqn. 2.22)}$$

In weak electrolytes, both the non-ionised molecules and the corresponding ions may be present. Although each type of particle may have its own value of mobility, they behave as a uniform substance since both types of particles mutually interchange by rapid reversible acid-base equilibrium. The mobility of the substance is given by:

$$\mu_A = \mu_A \alpha_A \qquad \text{(Eqn. 2.23)}$$

where μ_A = mobility of the ion A^-.

The dependence of α_A on pH is similar to the dependence of μ_A on pH. For the protonation of a weak base, the dissociation constant of its protonated form, K_{BH^+}, the degree of protonation α_B and the effective mobility of the base B, μ_B are given by

$$K_{BH^+} = \frac{C_{H^+} \cdot C_B}{C_{BH^+}} \qquad \text{(Eqn. 2.24)}$$

or, $\mu_B = \mu_{BH}\alpha_{BH}$ (Eqn. 2.25)

where C_B= concentration of the non-ionised base, C_{BH}^+= concentration of the protonated form of the base, C_B= total concentration of the base, C_H^+= concentration of the base, C_H^+ =concentration of the proton and μ_{BH} is the mobility of the ion BH^+.

Thus mobilities of ions through the electrolyte is dependent upon their degree of ionisation within the system.

2.1.7.2 Electrolyte pH

Electroosmotic and electrophoretic mobilities are effected by changes in buffer pH.

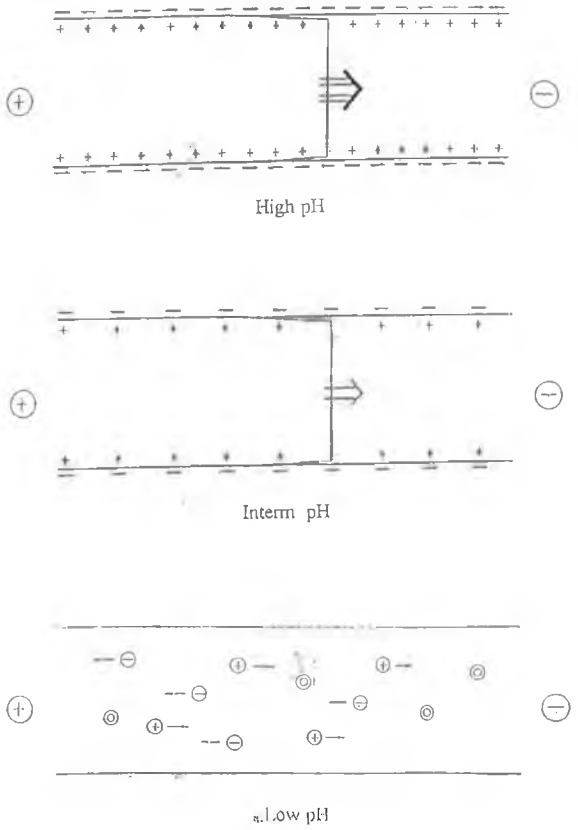


Diagram 2.6 Effect of Electrolyte pH

As the pH increases, the charge on the inner capillary wall increases (as more Si-OH groups dissociate to Si-O$^-$) therefore the electroosmotic flow increases. The zeta potential is proportional to the charge on the internal capillary wall and the buffer potential affects the electroosmotic flow. Electrophoretic mobility is also altered as a consequence of buffer pH as the degree of ionisation of solute depends on the pH of the solution. Differences in the degree of ionisation give rise to differences in electrophoretic and

electroosmotic mobilities. Consequently, both the separation efficiency and flow velocities may be affected by the buffer pH. By pH adjustment, both electroosmotic and electrophoretic mobilities can be altered.

At low pH values there is less ionisation of the silanol groups and therefore the zeta potential is low and the electroosmotic flow is low. At pH values below 2 there is no electroosmotic flow in fused silica capillaries as all the silanol groups are protonated. Ionisation of the silanol groups on the inside of the capillary start at values greater than pH 2. Between pH 2 and pH 5, the ionisation of the silanol groups is in a dynamic equilibrium. At higher pH values the EOF begins to slow down.

The pH of the buffer also effects the degree of ionisation of the buffer and analytes and hence the electrophoretic mobilities of the analytes. The electrophoretic mobility and charge of anions in the electrolyte is dependent on the pH of the buffer. Anionic mobility increases as the pH increases as the anionic analyte pass through the capillary unhindered and reduces when the pH is lowered. In the case of electrophoretic mobility for cationic species, their mobility decreases as the pH is increased.

2.1.8 Joule Heat

Joule heat arises when electric current is passed through the buffer inside capillaries. High voltages result in increased current, which in turn leads to an increase in heat production. If this heat produced is not quickly dissipated, the temperature inside the capillary will rise, causing the viscosity of the buffer to decrease, permitting even more current to flow.

2.1.8.1 Effects of Joule Heat

Joule heat generation effects the quality of the CE separation in the following way:

(a) Temperature gradients may be generated and if they are steep enough, density gradients in the buffer can be induced. This in turn can cause convection currents in the capillary. Any such convection would serve to remix separated sample zones and therefore reduce separation performance.

(b) If temperature gradients are not large enough to cause convection, separation performance can be compromised by introducing a spatial dependence (δ) on electrophoretic mobility. This spatial dependence of mobility can cause a deformation in the migrating zones.

(c) If temperature of the electrolyte becomes too high, the structural integrity of the analyte may be compromised.

(d) The buffer may begin to boil causing air bubbles and therefore causing the instrument to cut out.

Efficient removal of Joule heat allows CE to use high electric fields, to perform separations and achieve very low band dispersion. High operating voltages therefore favour the generation of Joule heat.

2.1.9 Ionic Strength

Typical buffer concentrations are chosen within the range 10-100 mM[1] and as a general rule the electrolyte concentration should be 100 times that of the sample. Changes in ionic strength of the buffer will effect electroosmotic and electrophoretic mobilities from equations (2.12) and (2.13) where μ_{EOF} and μ_{EP} are directly related to η, buffer viscosity. In general, when capillary temperatures are controlled, electroosmotic flow decreases in a logarithmic manner as the zeta potential is reduced with increasing ionic strength. Where capillary temperature is not controlled, increasing the ionic strength of the buffer may cause an increase in the electroosmotic flow. This in turn increases the current, and therefore, increases the temperature and reduces the viscosity of the buffer. Capillary cooling will allow the use of higher concentration buffers as at lower temperatures there will be less current produced. Effects of increasing ionic strength are (a) migration times increase, (b) number of theoretical plates increase, (c) better resolution and (d) improved peak shape.

2.1.10 Temperature

Increasing temperature decreases buffer viscosity, increases conductivity and hence increases electroosmotic flow[35]. However, increasing the temperature decreases the dielectric constant which decreases the electroosmotic flow. Separations should initially be attempted at close to ambient temperature in order to establish whether the need for temperature control arises. Variations in temperature can lead to a decrease in analysis time, thermal denaturation of the sample, alteration of buffer viscosity, electrophoretic mobility and injection volume.

.11 Organic Solvent Addition

Addition of organic solvents to the buffer permits the analysis of some analytes which are not normally aqueous soluble by improving their solubility in the buffer. Organic solvents, e.g. Methanol, Acetonitrile and Tetrahydrofuran[23] are also known to reduce the EOF, which may result in better resolution at the expense of longer analysis time. Organic solvent addition can affect the electroosmotic flow depending on the type and quantity of solvent used. System effects include viscosity, dielectric constant and zeta potential.

2.1.12 Organic Modifiers

Chemical additives may be covalently attached to the capillary wall or dissolved in the buffer to reduce the electroosmotic flow[1]. This is caused by the reduction of the zeta potential as the charges on the capillary wall are masked. Covalently bonded wall coatings can be used to eliminate electroosmotic flow and reduce sample adsorption onto the capillary wall while dynamic coatings such as surfactants and hydrophilic polymers[26-28] are added to the buffer and adsorb onto the walls of the capillaries, thus blocking the charges on the capillary wall.

2.1.13 Voltage

Optimum voltage is the maximum voltage at which insignificant Joule heating occurs. The voltage used should be chosen to give maximum efficiency without generating excessive heat that cannot be dissipated easily. This optimum voltage can be chosen from a graphical representation of voltage v current.

Optimum voltage is dependent on the capillary length and internal diameter. If the voltage is kept constant and the capillary length is reduced, the resistance will decrease and hence current will increase and more heat will be generated. The internal diameter of the capillary will also affect the maximum voltage. Smaller internal diameters will have higher resistances[6] and therefore lower currents than larger internal diameters. High operating voltages, short capillaries and high electroosmotic flow give shorter migration times. High electroosmotic flow can lead to poor resolution, therefore, to reduce migration times one should preferentially increase the applied voltage or shorten the capillary[6]. Both of these measures require heat

dissipation. Analyte mobility through the capillary can be enhanced by the application of high operating voltages. Increased electrical application facilitates electrophoretic and electroosmotic mobility as the current in the capillary is altered. Lower operating voltages induce diminished migration of the analyte. An optimum voltage chosen should consider the efficiency and resolution of analyte separations in a convenient analysis time.

2.1.14 Efficiency, Selectivity and Resolution

Efficiency can be determined by measuring migration time, t_m, and peak width at the base of the peak, w, or at half the peak height.

$$N = 16 (t_m / w)^2 \qquad \text{(Eqn. 2.26)}$$

$$N = 5.54 (t_m / w_{1/2})^2 \qquad \text{(Eqn. 2.27)}$$

where = N, is the number of theoretical plates, , t_m = migration time and , w = peak width at the base of the peak.

Narrow peaks with long migration times have highest efficiencies. The bulk electrolyte travels under the influence of EOF and thus solute plugs do not undergo significant spreading. Zone spreading can be represented by spatial variance,

$$\delta^2 = 2 D t \qquad \text{(Eqn. 2.28)}$$

where, δ^2 spatial variance, *t = time and D = solute's diffusion coefficient (cm^2 / s)*

if $t = t_m$ then,

$$t_m = l L / (\mu_{EP} + \mu_{EOF}) V \qquad \text{(Eqn. 2.29)}$$

and the number of theoretical plates can be expressed as

$$N = L^2 / \delta^2 \qquad \text{(Eqn. 2.30)}$$

Then,
$$N = (\mu_{EP} + \mu_{EOF}) V / 2 D \qquad \text{(Eqn. 2.31)}$$

High efficiencies are obtained by increasing electroosmotic flow (provided the electroosmotic and electrophoretic flow are in the same direction) or increasing applied voltage.

Selectivity is the distance between adjacent peaks as they pass through the detector and is represented by the equation

$$\alpha = (t_2 - t_{nm}) / (t_1 - t_{nm}) \quad \text{(Eqn. 2.32)}$$

where t_2 and t_1 are the migration times of two adjacent peaks and t_{nm} is the migration of the neutral marker.

$$\alpha = \nu 1 / \nu 2 \text{ . constant} \quad \text{(Eqn. 2.33)}$$

and $$\alpha = \mu 1 / \mu 2 \text{ . constant} \quad \text{(Eqn. 2.34)}$$

Thus, selectivity is dependent on differences in electrophoretic mobilites of solutes and can be altered by changing pH.

Resolution can be related to the operating voltage, electroosmotic flow, electrophoretic mobility and capillary length by the following equations:

It can initially be expressed in terms of peak efficiency and velocities of solutes as:

$$R = 1/4 (N)^{1/2} (\Delta \nu / \nu_{ave}) \quad \text{(Eqn. 2.35)}$$

where, R= resolution, N= plate number, $\Delta\nu / \nu_{ave}$ = relative velocity difference ($\Delta\nu$ is the velocity difference between two solutes and ν_{ave} is the average velocity of the two solutes)

The relative velocity difference between two solutes is related to the average electrophoretic mobilities of the solutes

$$\Delta \nu / \nu_{ave} = (\mu_2 - \mu_1) / (\mu_{AVE} + \mu_{EOF}) \quad \text{(Eqn. 2.36)}$$

where, μ_{EOF}= electroosmotic mobility, μ_{AVE} =average electrophoretic mobilities of the solutes.

Upon substitution of $N = (\mu_{EP} + \mu_{EOF}) V / 2 D$ into $R = 1/4 (N)^{1/2} (\Delta \nu / \nu_{ave})$ (Eqn. 2.33),(Eqn. 2.37)

$$R = 1/4 [(\mu_{EP} + \mu_{EOF}) V / 2 D]^{1/2} [(\mu_2 - \mu_1) / (\mu_{AVE} + \mu_{EOF})] \quad \text{(Eqn. 2.38)}$$

or $$R = 0.177 (\mu_2 - \mu_1) [V / (\mu_{AVE} + \mu_{EOF}) D]^{1/2} \quad \text{(Eqn. 2.39)}$$

Resolution is proportional to the square root of the voltage and high electroosmotic flow in the same direction as the electrophoretic mobility will decrease resolution[36]. Resolution will be good when there is a large difference in electrophoretic mobilities of solutes that can be enhanced by optimising the pH of the buffer. As resolution is proportional to $(l / L)^{1/2}$ the capillary length chosen should optimise analysis time and effect the best resolution.

Using high operating voltages may increase the sharpness of the detector response and improve resolution. If the sample matrix ionic strength is much greater than the running buffer ionic strength, resolution and efficiency will suffer from changes in band broadening. In general, high voltages will increase efficiency while low voltages will decrease resolution and the number of theoretical plates of the system[36].

2.2 Micellar Electrokinetic Capillary Chromatography (MECC).

2.2.1 Principles of Separation in MECC.

In MECC two phases exist: an aqueous phase and a micellar phase. Solutes partition between phases resulting in retention based on differential solubilisation in the micelles. MECC is used to separate anions, cations and electrically neutral compounds. Separation is achieved by adding detergents to the run buffer. Detergents or surfactants have hydrophobic and hydrophilic ends and are anionic, cationic, zwitterionic or non-ionic. Micelles are aggregations of individual surfactant molecules that form when a surfactant is present above its critical micelle concentration (CMC). These micelles are spherical in shape and form such that the hydrophilic groups are on the outside toward the aqueous buffer and the hydrophobic carbon molecules are in the center. The size of micelles are in the range 3 to 6 nm in diameter and exhibit properties of homogenous solutions. MECC is most commonly performed with anionic surfactants especially sodium dodecyl sulphate (SDS)[32-34], [CH -$(CH_2)_{11}$-O-SO]. SDS has a CMC of 8.27 mM and an aggregation number of 62[35]. The surface of SDS micelles have a large net positive charge and therefore exhibit large electrophoretic mobility (μ_{EP}) toward the anode, which is in opposite direction to the EOF towards the cathode.

The magnitude of EOF is slightly greater than that of μ_{EP}, resulting in a fast-moving aqueous phase and a slow-moving micellar phase. Consequently, the MECC technique provides a means of obtaining selective separations of neutral and organic compounds while retaining the advantages of the capillary electrophoresis format.

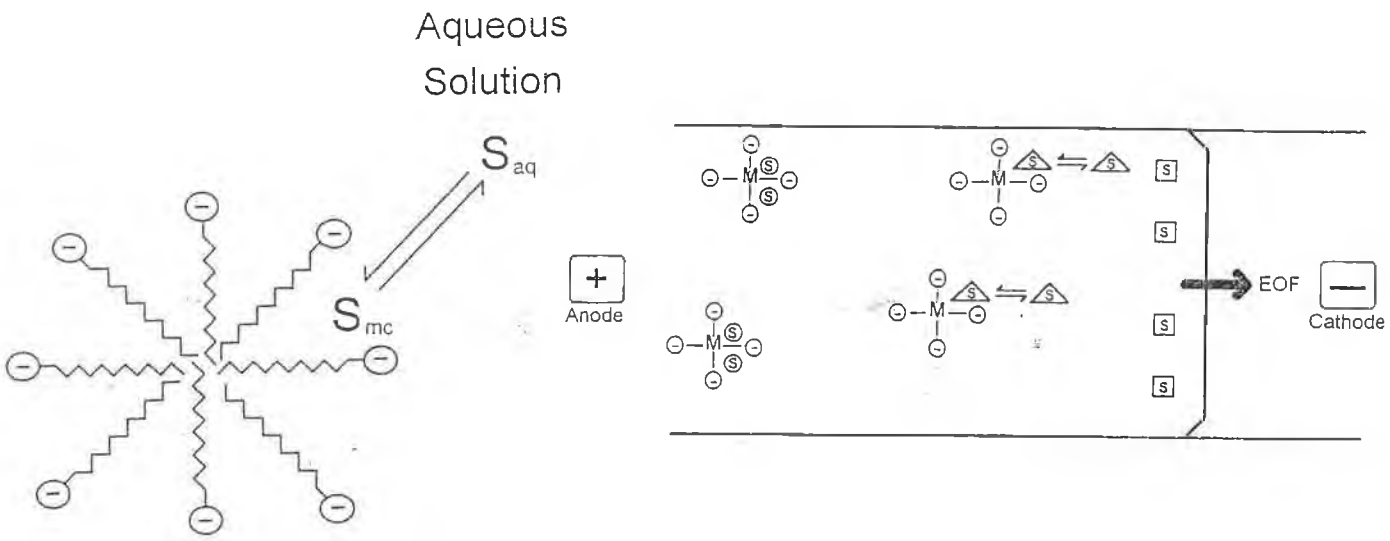


Diagram 2.7 (a) SDS micelle (b) SDS inside capillary

2.2.2 Migration of Analytes

Migration of analytes is generally governed by hydrophobicity. Micellar solutions may solubilise hydrophobic compounds that might otherwise be insoluble in water. More hydrophobic solutes interact more strongly with the micellar phase and thus migrate slower than hydrophilic compounds. The migration time (t_m) of a solute that interacts with the micelles will fall in a 'migration time window' between the migration time (t_o) of a solute that has little or no interaction with the micelles and the migration time of a solute that is 100% solubilised by the micelles (t_{mc}).

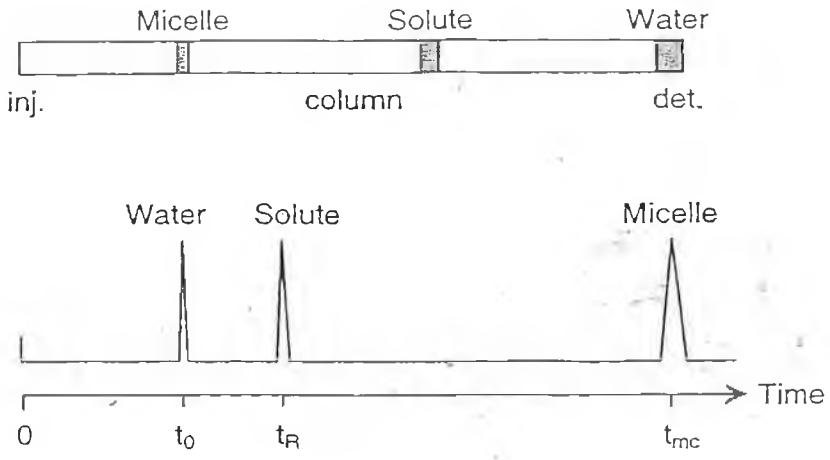


Diagram 2.8 Migration Time Window in MECC

The fact that neutral solutes must elute between t_o and t_{mc} is the most significant difference between MECC and conventional chromatography. The order of elution in MECC is illustrated by a migration time window where water travels at a faster rate than the solute and the solute in turn travels at a faster rate than the micelle along the capillary.

2.2.3 Electroosmotic Flow in MECC

EOF transports the bulk solution to the negative electrode due to the negative charge on the surface of fused silica. EOF is usually stronger than the electrophoretic migration of the micelle under neutral or alkaline conditions and therefore the anionic micelle also travels toward the negative electrode at a retarded velocity. When a neutral analyte is injected into the micellar solution, a fraction of it is incorporated into the micelle and it migrates at the velocity of the micelle. The remaining fraction of the analyte remains free from the micelle and migrates at the electroosmotic velocity. The migration velocity of the analyte thus depends on the distribution coefficient between the micellar and non-micellar or aqueous phase. The greater the percentage of analyte distributed in the micelle, the slower it migrates. The analyte must migrate at a velocity between the electroosmotic velocity and the velocity of the micelle, provided the analyte is electrically neutral. To illustrate this, a capacity factor k' is defined as:

$$k' = \frac{N_{mc}}{N_{aq}} \qquad \text{(Eqn. 2.40)}$$

where N_{mc} and N_{aq} are the amount of the analyte incorporated into the micelle and that in the aqueous phase, respectively.

A relationship can be derived between the capacity factor and the migration times i.e.

$$k' = \frac{t_r - t_o}{t_o (1 - t_r / t_{mc}} \qquad \text{(Eqn. 2.41)}$$

The migration time of the analyte is equal to t_o when $k' = 0$, or when the analyte does not interact with the micelle at all; the migration time becomes t_{mc} when k' is infinity or the analyte is totally incorporated into the micelle. Thus, the migration time window is limited to between t_o and t_{mc}. When t_o is infinity (when electroosmosis is completely surpressed) equation (2) becomes:

$$t_r = [1 + 1 / k'] t_{mc} \qquad \text{(Eqn. 2.42)}$$

In this case, the bulk solution remains stationary in the capillary and the micelle migrates only by electrophoresis. If the capacity factor, k', is defined as the reciprocal of equation (1), equation (3) becomes identical with the relationship between t_r, t_o and k' in conventional chromatography. Equation (2) can be related to electrophoretic processes. In CZE, the migration velocity of the analyte v_s is expressed as:

$$v_s = [\mu_{EOF} + \mu_{EP}(s)]\, E \qquad \text{(Eqn. 2.43)}$$

This equation can be applied in MECC by defining the electrophoretic mobilities, for the neutral analytes as:

$$\mu_{EP}(s)\ \frac{k'}{1+k'}\ \mu_{EP}(mc) \qquad \text{(Eqn. 2.44)}$$

where, μ_{EP} (mc)= electrophoretic mobility of the micelle and k' / 1 + k'= fraction of the analyte incorporated into the micelle,

as shown by the following equation:

$$\frac{k'}{1+k'} = \frac{nm}{naq + nmc} \qquad \text{(Eqn. 2.45)}$$

Thus the velocity of the analyte in MECC is given as

$$v_s = [\mu_{EOF} + \mu_{EP}(s)]\, E \qquad \text{(Eqn. 2.46)}$$

therefore the effective mobility indicates that a neutral analyte has mobility.

2.2.3.1 Reversal of EOF

The direction and rate of electroosmotic flow depends on the polarity and magnitude of the zeta potential. The direction of the EOF is normally towards the cathode in aqueous solutions. By introducing cationic surfactants in the MECC buffer[6], the direction of the EOF can be reversed due to adsorption of the cationic species on the capillary wall and subsequent reversal of the zeta potential.

2.2.4 Resolution in MECC

Resolution in MECC is given by the equation:

$$Rs = \sqrt{N}\,(\alpha - 1 / \alpha)\,(k_2' / 1 + k_2')\ \frac{1 - t_o / t_{mc}}{1 + (t_o / t_{mc})\, k_1'} \qquad \text{(Eqn. 2.47)}$$

where, N= theoretical plate number, α = separation factor and k_1' and k_2'= capacity factors of analytes 1 and 2, respectively.

2.2.5 Efficiency in MECC

The higher the applied voltage, the higher the plate number, unless the conditions are such that the applied voltage generates too much Joule heating. Average plate numbers for most analytes are usually in the range 100,000 to 200,000[6]. If the plate number is considerably lower, analytes are likely to be absorbed on the capillary wall. In such cases, experimental conditions must be optimised to produce more efficient separations. Cleaning of the capillary is a possible procedure as is changing the pH of the run buffer. Hydrophobic analytes, or those having longer migration times, typically yield high theoretical plate numbers because the micelle has a smaller diffusion coefficient. The plate number does not depend significantly on the capillary length. With short capillaries, however, the amount of sample volume injected must be minimised to avoid zone broadening.

2.2.6 Selectivity in MECC

Selectivity is the most important and effective term to maximise resolution. Selectivity reveals the relative difference of the distribution coefficient between two analytes and can be manipulated by chemical means. Since the distribution coefficient is a characteristic of a given separation system consisting of a micellar and aqueous phase, the selectivity can be manipulated by changing either the type of micellar phase or by modifying the aqueous phase.

2.2.6.1 Capacity Factor

From the resolution equation, the optimum value of the capacity factor is equal to $(t_{mc} / t_o)^{1/2}$. Under conditions of pH > 6, the optimum k' value is close to 2 for most long alkyl chain surfactants[31]. In most instances, the capacity must be adjusted to between 0.5 and 10. A large capacity factor means that the major fraction of the analyte is incorporated into the micellar and the aqueous phase in other words an equal distribution between two phases.

The capacity factor is related to the distribution coefficient, K, by

$$k' = K V_{mc} / V_{aq} \qquad \text{(Eqn. 2.48)}$$

where V_{mc} / V_{aq} = ratio of micelle and aqueous volumes and V_{mc} and V_{aq} =volumes of micelle and the remaining aqueous phase.

The capacity factor is approximately related to the surfactant concentration Csf, by

$$k' = K\, v\, (Csf - CMC) \qquad \text{(Eqn. 2.49)}$$

where, v = partial specific volume of the micelle.

This indicates that the capacity factor increases linearly with an increase of surfactant concentration. Adjusting the surfactant concentration provided the CMC is known can therefore vary the capacity factor.

2.2.7 Electroosmotic Velocity

The effect of EOF on resolution is related to the migration time ratio, t_o / t_{mc};

$$t_o / t_{mc} = [\, 1 + \mu_{EP}(mc) / \mu_{EOF}\,]\, E \qquad \text{(Eqn. 2.50)}$$

where, t_o / t_{mc} *= migration time window,* μ_{EP} **(mc) = electrophoretic mobility of analyte,** μ_{EOF} *= electrophoretic mobility of EOF and E = applied electric field.*

The mobility of μ_{EOF} and μ_{EP} (mc) usually have different signs and the ratio μ_{EP} (mc) / μ_{EOF} is smaller than 0 and larger than -1. Therefore, t_o / t_{mc} is less than one. The t_o / t_{mc} is also directly related to the width of the migration time window. The smaller the value of t_o / t_{mc}, the wider the migration time window, hence the higher resolution. In order to reduce the value of t_o / t_{mc}, it is necessary to reduce μ_{EOF} relative to μ_{EP} (mc) because in practice, increasing μ_{EP}(mc) is rarely possible[36]. It is also possible to reduce v_{EOF} by changing the pH of the buffer to acidic conditions.

2.2.8 Surfactants used in MECC

Mobility of micelles and binding constants of solutes are influenced by the nature of the hydrophobic moiety, charged head group, and counterion of surfactant-forming micelles. The applicability of a surfactant system in MECC depends on its solubility and CMC value.

Table 2.2 Surfactants Used in MECC[36]

Surfactant	CMC, mM	Aggregation Number
Anionic		
Cholic Acid, sodium salt	14	2-4
Deoxycholic acid, sodium salt	5	4-10
Glycocholic acid, sodium salt	13	2
Sodium Dodecyl Sulphate (SDS)	8.27	62

Taurocholic acid, sodium salt	10-15	4
Cationic		
Cetyltrimethylammonium chloride	1	-
Cetyltrimethylammonium bromide	1.3	78
Dodecyltrimethylammonium bromide	14	50
Hexadecyltrimethylammonium bromide	0.026	169
Zwitterionic		
CHAPS[a]	8	10
CHAPSO[b]	8	11
Nonionic		
n-Decyl-β-D-glucopyranoside	2.2	-
Triton-X-100	0.24	140

[a] three-[(three-cholamidopropyl)dimethylammonio]-1-propanesulfonate
[b] three-[(three-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate

Surfactants which have very large CMC values are unsuitable because of the high conductivity produced which may lead to undesirable thermal effects. Together with anionic, cationic, non-ionic and zwitterionic micelle systems, the use of mixed micelles and reversed micelles, and the addition of modifiers[36] further enlarges the scope and variety of the micellar phase available in MECC. With different systems, interactions that can occur between micelles and solutes vary and therefore selectivity changes are due to differences in solubilisation behaviour.

2.2.9 Organic Modifiers

Some of the most commonly used additives to enhance efficiency and selectivity in MECC are hydroxypropylcellulose (HPC), hydroxyproplymethylcellulose (HPMC), Brij-35 and Tween-20[26, 28-29]. The addition of methanol[10, 14, 16-18] is also known to decrease the electroosmotic velocity while that of urea[34] addition has been demonstrated to effect the formation of micelles with a resultant decrease in capacity factor for most solutes. The presence of organic solvents into a micellar solution causes a reduction in EOF and hence an extension of the elution time window and a decrease of the migration factor, k'.

2.3 Comparing CZE and MECC

MECC differs from CZE in that it uses an ionic micellar solution instead of a buffer salt. The micellar solution generally has a higher conductivity and hence causes a higher current than the buffer does in CZE. MECC can separate both ionic and neutral substances while CZE typically separates only ionic substances.

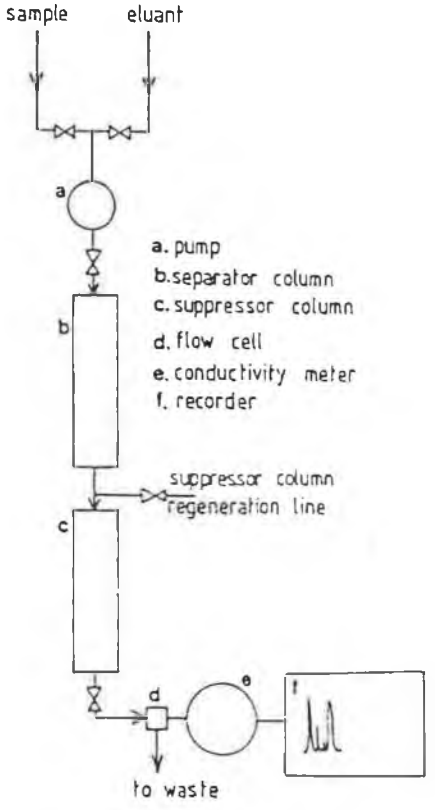
Thus MECC has a great advantage over CZE for the separation of mixtures containing both ionic and neutral compounds. In MECC, the size of sample molecules are limited to values of less than 5,000 a.m.u., whereas CZE virtually has no limitation in molecular size. The separation principle of MECC is based on the distribution of the solute between the micelle and water as CZE is based on the differential electrophoretic mobility.

2.4 Ion Chromatography

2.4.1. Principle Components of an IC System

The key components of an IC system comprise a high pressure pump, separator column, suppressor column and a recording conductivity instrument.

Diagram 2.10 Principle Components of an IC System



2.4.2 Ion Exchange Column

The ion exchange column contains a packing of pellicular resin which owing to their small particle size (10-25 μm) present a high surface area and short diffusion paths therefore promoting very fast rates of exchange and rapid resolution of mixed ions. The resins used are of low capacity (0.005-0.02 meq g^{-1}) which for cation analysis are surfaced sulfonated copolymer microspheres. An anion exchange resin is obtained by surface coating a pellicular cation exchanger with a quaternary ammonium latex (0.1-1.0 μm particles) co-ploymer which becomes irreversibly electrostatically bonded to the host particle. Because of the deliberate low column exchange capacity the elution times for total ion loadings of only a few mg are very short. The eluting agents are prescribed according to the known or anticipated ions present and are pumped at a controlled rate down the column.

2.4.3 Mechanism of Cation Analysis

The suppressor column contains a finely graded anion exchange resin which interacts with cations in the sample according to charge. Prior to elution, the suppressor column only sees the eluent (HY) and the following exchange takes place:

$$ROH + HY \Rightarrow RY + H_2O \qquad \text{(Eqn.4.1)}$$

The conductivity of the column effluent is that of water and therefore has been suppressed. During the elution of each cation the suppressor column only sees electrolytes BY and HY. The conductivity detector due to HY is nullified but BY exchanges to give highly conductive hydroxide ions:

$$ROH + BY \Rightarrow RY + B^+ + OH^- \qquad \text{(Eqn. 4.2)}$$

When elution of the first cation is complete, only the eluent is present and the conductivity falls again to that of water. The conductivity increases again as each of the other cations in the sample travel down the column. Each cation interacts with the ion exchange material in the column so that their retention time is proportional to their size and charge. Ion detection is determined through a series of peaks detected by the conductivity detector whose retention time and size is characteristic of the ion present and its concentration, respectively.

2.4.4 Order Of Elution.

The order of elution follows that the greater the size and charge of the cation, the slower it will travel through the column. In a mixture of four cations, M^{4+}, M^{3+}, M^{2+} and M^{+}, the cation with the smallest charge will elute more quickly than all others as it is retarded the least. The partition coefficient is related to the ions interaction between the stationary phase and the mobile phase. The more strongly a solute favours the stationary phase, the lower the retardation.

Order of elution follows:

$$M^{4+} > M^{3+} > M^{2+} > M^{+}$$

CHAPTER THREE

BENZOIC ACID

3.1 Introduction

In order to become familiar with the CES 1 instrument, a previously reported analytical procedure[47] for the determination of Benzoic Acid was carried out.

Structure of Benzoic Acid

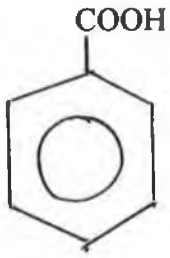


Diagram 3.1 Structure of Benzoic Acid

Benzoic acid contains a carboxylic acid group attached to an aromatic ring and absorbs UV light at 215 nm. Direct UV detection was used to analyse a 10-50 mg / L sample using a borate buffer at pH 7.

3.2 Literature Review

Benzoic acid has been analysed by GC[37-39], IC[40], HPLC[41-45] and CE.[46-49] Benzoic acid is routinely added to food where it functions as a preservative, anti-oxidant and sweetner.

Table 3.1 Benzoic Acid Literature Review

Matrix	Separation Technique	Analysis Time	Linear Range	R^2	LOD	Precision and Accuracy	Recovery	Ref
Food	GC	7 min	0.4-25 mg/L	0.999	0.15 mg/L	RSD=3.0%	103%	37
Oils and Cheese	GC	5 min	1.5-500 µg/ml	-	0.8 µg/ml	RSD=3.5%	92.8-102.5%	38
Urine	GC	5 min	-	-	-	-	-	39
Sweetners	IC	6 min	1-40 µg /ml	0.9997	10 ng/ml	RSD=1.0%	85-104%	40
Wine	RP-HPLC	<10 min	0.005-100 ng.ml	0.999	10 µg/ml	RSD=0.6%	-	41
Fruit Juice	HPLC	27 min	-	-	-	-	-	42
Syrup	HPLC	15 min	0.1-0.3 mg/ml	0.9995	-	RSD=1.38%	100%	43
Drugs	RP-HPLC	7 min	0.25-50 µg/ml	0.9996	0.5 µg/ml	RSD=3.0%	85-95%	44
Cosmetics	HPLC	7 min	1-20 µg/ml	>0.999	4 ng/ml	RSD=2.6%	99%	45
Drugs	CZE	15 min	0.006-0.113 mg/ml	0.9998	2.6 µg/ml	RSD=0.53-1.46%	95.1%	46
Soft Drinks	CZE	2 min	1-400 µg/ml	0.9958	4 µg/ml	RSD=0.37-2.4%	100.6%	47
Sweetners	MECC	7 min	1-50 µg/ml	0.9995	1.5 µg/ml	RSD<3.3%	98.6%	48
Drugs	MECC	8 min	-	0.995	3 µg/ml	RSD<2.0%	100.89%	49

3.3 Method Application

3.3.1 Materials and Operating Conditions

Electrolyte:	10 mM Disodiumtetraborate, 50 mM Boric Acid, pH 7	
Sample:	10-50 mg / L Benzoic Acid	
Operating Conditions:	Capillary Dimensions	75 µm (i.d.) x 67 cm (l) x 375 µm (o.d.)
	Detection System	Direct UV, at 215nm
	Polarity	(+), Detector side Cathodic
	Control Mode	Constant Voltage, 20 kV
	Injection	Gravity, 50 mm for 10 sec
	Temperature	Ambient

(i) Electrolyte Preparation

Buffer was prepared by accurately weighing 5.283g of solid Disodiumtetraborate and dissolving in ultra pure water. The solution was transferred to a 1 L volumetric flask and made up to the mark with ultra pure water. Electrolyte pH was adjusted using 50 mM Boric acid, placed in an Ultrasonic bath for 10 min to degas and filtered through a Gelman Acrodisc (0.45µM) filter paper. Electrolyte was prepared fresh daily. (All future electrolyte preparations carried out in this work were prepared fresh daily, degassed and filtered as above, unless otherwise stated. All stock standard and working standard solutions were also prepared fresh daily, unless otherwise stated.)

(ii) Standard Preparation

A 100 mg / L stock solution of benzoic acid was prepared by accurately weighing 100 mg of the compound, dissolving in ultrapure water and diluting to 1 L in a volumetric flask.

(iii) Working Standard Solutions:

10, 20, 30, 40 and 50 ml of the stock solution were pipetted into 100 ml volumetric flasks and made up to the mark with ultrapure water. Standard solutions were prepared fresh daily.

(iv) Capillary Preparation

The capillary was prepared by measuring accurately 50 cm in length and cutting using a ceramic scoring tool supplied by Dionex. A flat even capillary end was ensured. Creation of a capillary cell window at a distance of 4.5 cm from one end was achieved by burning the polyimide coating using a lighter. Remnants of the polyimide coating were removed by gentle cleaning of the cell window with lens tissue soaked in methanol. The capillary was installed into the instrument, conditioned by washing with 1 M NaOH for 30 min, followed by ultrapure water for 30 min and equilibrated with the run buffer for 30 min. The capillary was rinsed with electrolyte for 120 sec between injections to remove any residue from previous analysis and to maintain reproducibility from injection to injection. Performing injections of water and electrolyte ensured baseline stability. Each standard was injected three times.

3.3.2 Results:

A response appeared at approximately 5 min.

Diagram 3.2 (a) Electropherogram of 50 mg/L Benzoic Acid in ultra pure water.

Conditions: 10 mM Disodium Tetraborate – 50 mM Boric Acid Buffer (pH 7); UV detection at 215 nm; Capillary: 50 cm x 50 μm i.d.;Voltage, 20 kV; Gravity Injection, 50 mm for 10 s.

Peak height values were more appropriate than peak area values for constructing a calibration graph as the variation in readings was less obvious. Concentrations of benzoic acid were plotted against peak height to examine the correlation coefficient obtained.

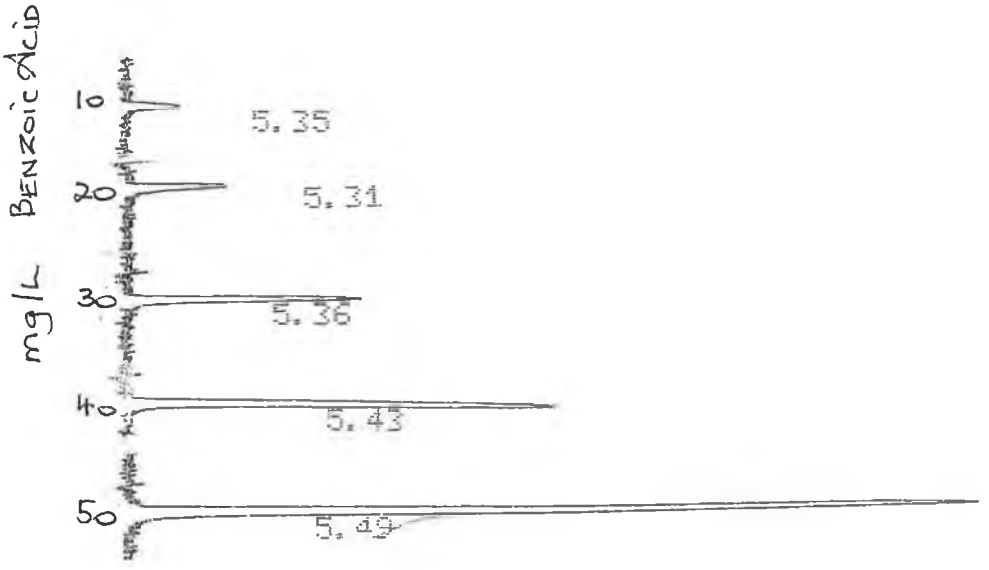


Diagram 3.2 (b) Electropherogram of 10, 20, 30, 40 and 50 mg/L Benzoic Acid in ultra pure water.

Conditions: 10 mM Disodium Tetraborate – 50 mM Boric Acid Buffer (pH 7); UV detection at 215 nm; Capillary: 50 cm x 50 µm i.d.;Voltage, 20 kV; Gravity Injection, 50 mm for 10 s.

CHAPTER FOUR

INORGANIC CATION ANALYSIS

4.1 Introduction

The first compound chosen for determination by CE was tromethamine. Since this compound has no intrinsic UV absorbing properties, it was decided to use previously reported methods[50,51,52] using indirect photometric detection for analysing inorganic cations Li^+, Na^+, K^+, Mg^{2+}, Ca^{2+} and NH_4^+. Tromethamine contains an amino group that forms an ammonium ion at pH 4.8. This method was chosen to examine suitability of operating conditions for the migration of tromethamine as a cation.

4.2 Literature Review

Inorganic cations are smaller and thus have higher charge densities than most organic ions therefore their electrophoretic mobilities are higher. The problems connected with CE analysis of inorganic cations are due to small differences in their migration rates and their low absorption of UV radiation. Complexation of the cation is used to change the selectivity and / or to facilitate the detection. Many of these references contain indirect UV detection, those which do not involve off-line preparation of complexes prior to CE analysis.

Table 4.1 Literature Review for Inorganic Cations

Matrix	Separation Technique	Detector	Analysis Time (min)	Analytical Performance	Ref
Drinking Water	CZE	Indirect UV Detection	< 6 min	(Method Only)	52
Drinking Water	CZE	Indirect UV Detection	< 4.5 min	(Method Only)	53
Metal Salts	CZE	Indirect UV Detection	< 6 min	L.R.=50-150 µg/ml R=0.9996 RSD=1.26% LOD=33µg/ml	54
Metal Salts	CZE	Indirect Fluorometric Detection	< 5min	(Method Only)	55

Table 4.1 Literature Review for Inorganic Cations

Pharmaceutical Matrix	CZE	Indirect UV Detection	< 4.5 min	L.R.=5-50 µg/ml R=0.9999 RSD<0.35% LOD=0.5µg/ml	56
Cation Standards	IC	UV Detection	< 6 min	L.R.=0.5-5 mg / L LOD=0.5mg /L	57
Brine	IC	UV Detection	< 8 min	(Method Only)	58
Aerosols	CZE	UV Detection	< 6 min	RSD=0.5%	59
Metal Salts	CZE	Indirect UV Detection	< 7 min	(Method Only)	60
Beverages	CZE	Indirect UV Detection	< 8 min	R=0.9987 L.R.=10-100µg/ml RSD=0.6%	61
Silicone Products	CZE	Indirect UV Detection	<3.5 min	R=0.9999 15-100µg/ml RSD<5%	62
Vegetables	CZE	UV Detection	< 5 min	LR=1-5 mg / L	63
Juice	CZE	Indirect UV Detection	< 6 min	LR= $4x10^{-5}$-$2x10^{-2}$ M	64
Soft Drink	CZE	Indirect Fluorometric Detection	< 5 min	LR= $1x10^{-7}$-$3x10^{-6}$M	65

Table 4.1 Literature Review for Inorganic Cations

Milk	CZE	Indirect UV Detection	< 4 min	Method Only	66
Tears	CZE	Indirect UV Detection	< 6 min	Method Only	67
Seawater	CZE	Indirect UV Detection	< 5 min	LOD $4x10^{-4}$ M	68
Trace Explosives	CZE	Indirect UV Detection	< 9 min	LOD=$2x10^{-4}$M	69
Vitreous Humour	CZE	Indirect UV Detction	< 7 min	Method Only	70

4.3 CE Method Application

4.3.1 Materials and Operating Conditions

Electrolyte:(a) 4 mM Copper (II) Sulphate, 4 mM Formic Acid, 4 mM 18-Crown-6-Ether, pH 5

(b) 50 mM Phosphate, 5 mM 18-Crown-6-Ether, pH 7 with 1 M NaOH

(c) 5 mM DDP, 5mM 18-Crown-6-Ether, 6 mM α-hydroxyisobutyric acid, pH 4.6

Sample: 1-20 mg / L Li^+, Na^+, K^+, Mg^{2+}, Ca^{2+}, and NH_4^+

Operating Conditions:

Capillary Dimensions	50 µm (i.d.) x 50 cm (l) x 375 µm (o.d.)
Detection System	Indirect UV, at 215 nm
Polarity	(+), Detector side cathodic
Control Mode	Constant Voltage, 20 kV
Injection	Gravity, 100 mm for 30 sec
Temperature	Ambient

(i) Electrolyte Preparation

(a) 0.4993g of Copper (II) Sulphate Pentahydrate and 0.52864g of 18-Crown-6-Ether were accurately weighed and dissolved in ultrapure water. The solution was transferred to a 500 mL volumetric flask and made up to the mark with ultrapure water. Electrolyte pH was adjusted to 5 using 4 mM Formic Acid.

(b) 7.056g Disodium Hydrogen Phosphate and 1.2216g 18-Crown-6-Ether were accurately weighed and dissolved in ultrapure water. The solution was transferred to a 1 L volumetric flask and made up to the mark with ultrapure water. Electrolyte pH was adjusted to 7 using 1 M NaOH.

(c) DDP Buffer was purchased in concentrate form from Dionex U.K. Ltd. and required a 1 in 4 dilution with ultrapure water.

(ii) Standard Preparation

A 100 mg / L Stock Standard solution of each of the cations (Lithium, Sodium, Potassium, Magnesium, Calcium and Ammonium) were prepared by weighing 100 mg of the compound in its chloride form, dissolving with ultrapure water and diluting to a final 1 L volume.

(iii) Working Standards

1, 5, 10, 15 and 20 ml of the stock solution were pipetted into 100 ml volumetric flasks and made up to the mark with ultrapure water.

(iv) Capillary Preparation: As per 3.3.1 (iv)

Each standard was injected three times using the operating conditions above for each electrolyte.

4.3.2 Results: Linear responses were observed for the DDP electrolyte. Interferences were obtained using the Copper Sulphate electrolyte. Non-linear responses attained using the phosphate electrolyte.

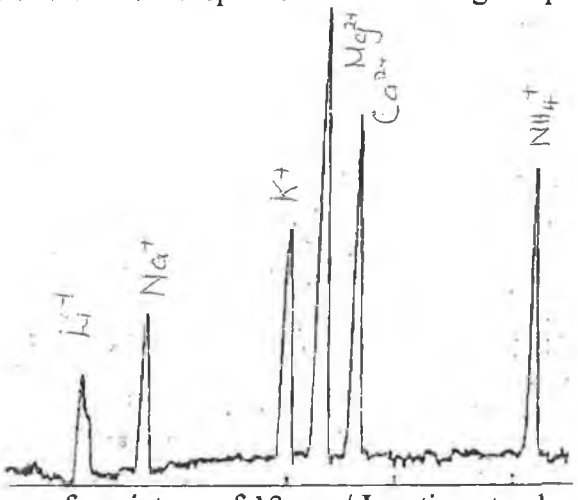


Diagram 4.1 Electropherogram of a mixture of 10 mg / L cation standards (Lithium, Sodium, Potassium, Magnesium, Calcium and Ammonium).

Conditions: 5 mM DDP Buffer, pH 4.5; UV Detection at 215 nm; Capillary: 50 cm x 50 µm i.d.; Voltage, 20 kV; Gravity Injection 50 mm for 10 s.

4.4 IC Method Application

4.4.1 Materials and Operating Conditions

A previously reported method for analyzing cations by IC[71] was used to examine interference and non-linear response observed during CE analysis with the Copper Sulphate and Phosphate buffers.

Eluent 0.11 mM H_2SO_4

Sample	1-20 mg / L Li^+, Na^+, K^+, Mg^{2+}, Ca^{2+}, NH_4^+
Operating Conditions	Cation-Exchange Column, Guard Column, Cationic Supressor Column
Flow Rate	1 ml / min

(i) Eluent Preparation

0.1051ml of AnalaR Grade Sulphuric Acid was accurately weighed and added to a large volume of ultrapure water. This was transferred to a 1 L volumetric flask and made up to the mark with ultrapure water. The eluent was filtered through a Gelman Acrodisc filter paper (0.45μm) and placed in the eluent container. Eluent was prepared fresh daily.

(ii) IC Operation

The system was purged with the eluent for 2 minutes. The flow rate was set at 1 ml / min. The cation-exchange column was allowed to equilibrate for approximately half an hour, until a stable background conductivity was obtained. Injections of ultrapure water were performed to ensure baseline stability. Once a steady baseline was obtained injections of the cation standards were carried out.

(iii) Stock Solution

A 100 mg/L stock solution of each cation was prepared by weighing 100 mg of their chloride salt and dissolving in ultrapure water and diluting to 1 L in a volumetric flask.

(iv) Working Standards

1, 5, 10, 15 and 20 ml of the stock solution were pipetted into individually labelled 100 ml volumetric flasks and made up to the mark with ultrapure water.

Each cation standard was injected three times.

4.4.2 Results: The elution order of inorganic cations followed results obtained in the previously reported method. It was decided to continue use of the DDP buffer for the initial determination of the first analyte, tromethamine.

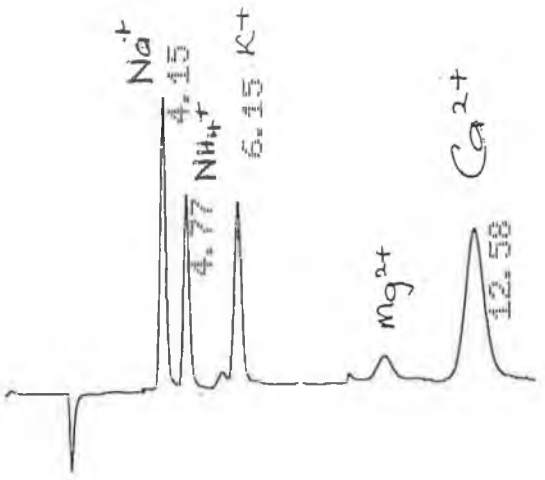


Diagram 4.2 Chromatogram of a mixture of 10 mg / L cation standards (Sodium, Ammonium, Potassium, Magnesium and Calcium).

Conditions: Eluent 0.11 M H_2SO_4 ; Flow Rate 1 ml / min.

CHAPTER FIVE

TROMETHAMINE ANALYSIS

5.1 Introduction

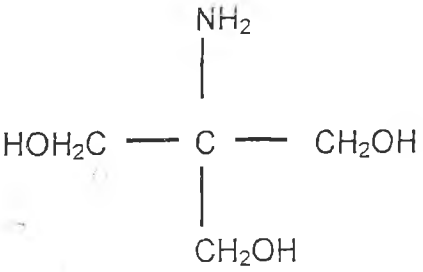


Diagram 5.1 Tris (hydroxymethyl)aminomethane, (Tromethamine)

Tromethamine is a small inorganic molecule that is easily ionised and readily soluble in water[72]. It is commonly used as an alkaliser and emulsifying agent in pharmaceutical and cosmetic preparations[73]. It is a symmetrical molecule that consists of a central carbon atom attached to three -CH_2OH groups and an -NH_2 group. The presence of these groups will determine the electrophoretic mobility of the analyte in the capillary. As tromethamine is a weak amine base, ionisation will take place within a narrow pH range above or below it's pK value. Tromethamine has a pKb value of 7.8[72] at body temperature.

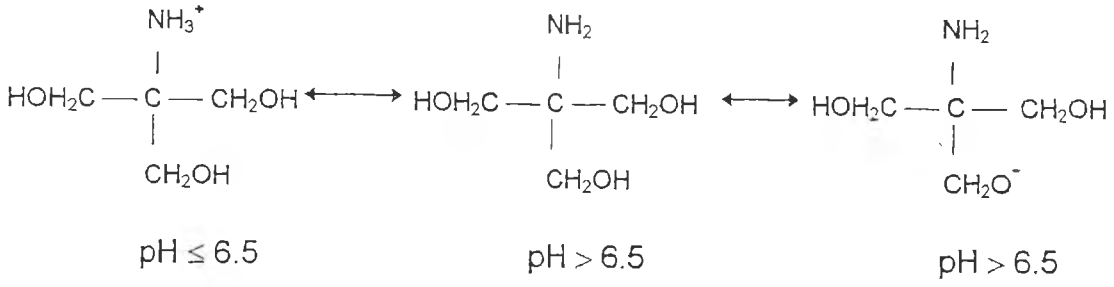


Diagram 5.2 Ionisation Mechanism of Tromethamine

At low pH the amino group acquires a net positive charge where the amino group is protonated and is reduced to -NH_3^+, while at higher pH values the -CH_2OH groups becomes oxidised to CH_2O^-. Tromethamine is relatively non-complex and possesses no UV absorbing properties. Having considered its non-complex structure and known chemical properties, it was anticipated that the analyte would undergo electrophoretic mobility on the basis of charge to mass ratio in a charge environment.

Table 5.1 Tromethamine Analysis

Matrix	Separation Technique	Analysis Time (min)	Linear Range	R^2	LOD	Precision and Accuracy	Recovery	Ref
Opthalmic Solution	IC	5-7 min	0.195-0.325 mg / ml	0.9998	-	RSD=0.3-0.5%	100.5%	73
Tablet	UV-Vis (Derivatisation)	-	20-100 mcg / ml	0.99977	20 mcg / ml	-	99.34%	74
Tablet	Titration	-	250 mg-500mg	-	-	-	-	75
Tablet	TLC	-	-	-	-	-	-	75
Tablet	UV-Vis	-	4-16µg / ml	-	-	-	98.49%	75
Tablet	HPLC	5 min	20-100 µg / ml	-	-	-	98.76%	75
Tablet, Injection	HPLC	3.5 min	0.125-2.5 µg / ml	-	0.2%	99.3-99.8%	99.7%	76
I.V. Fluid	UV-Vis (Derivatisation)	>30 min	10-100 µg / ml	-	10 µg / ml	99.87%		77

Table 5.1 Tromethamine Analysis

Matrix	Separation Technique	Analysis Time	Linear Range	R^2	LOD	Precision and Accuracy	Recovery	Ref
Plasma	HPLC (SPE)	7 min	25-2500 ng / ml	0.9986	5 ng / ml	RSD= 0.08%-2.2%	72.9-87.7%	78
Drug Matrix	UV-Vis	>30 min	10-80 µg / ml	0.9997	-	-	98.52-100.26%	79
Opthalmic Solution	HPLC	11 min	1-25 µg / ml	0.99886	1µg / ml	RSD=0.05	101.103%	80
Plasma	HPLC (Derivatisation)	15 min	1-25 µg / ml	0.997	0.282 µg / ml	<16.4% 90.4-108%	90.4-101.2%	81
Plasma, Urine	HPLC (Derivatisation)	12 min	1-500 µg / ml	0.9986-00.9998	1µg / ml	RSD=0.38-0.99%	97.6-99.6%	82
Serum	RP-HPLC	10 min	10-150 ng / ml	0.9944	10 ng / ml	4.0%, 3.7%	92%	83
Injection	HPLC (Derivatisation)	8 min	0.5-1.25 mg / ml	0.9991	0.5 mg / ml	3.0%	101.2%	84
Ophthalmic Solution	RP-HPLC	6 min	5-15 mg / ml	0.9998	-	2.0%	95.105%	85

Hall and co-workers[73] have described the development of an Ion Chromatography method with conductivity detection for the determination of tromethamine in an ophthalmic solution. Using direct detection to avoid diminished performance, the elution of tromethamine was found at less than 5 min however the method suffered form interference from sodium ions that could only be resolved from the analyte peak at the expense of elution time. It is evident from the procedure that the method induced a significant loss in column efficiency due to the build up of the cation on column.

A complexation reaction between KT and p-dimethylamino benzaldehyde in concentrated HCl was necessary for the spectrophotometric determination of the drug in a pharmaceutical dosage form[74], however the complex generated was chemically unstable as it degraded within 24 h, even though the method achieved recoveries of approximately 99.34%. Sane et al.[75] developed a non-aqueous titration method, thin-layer chromatographic (TLC) method, UV and RP-HPLC for the detection of KT in a bulk pharmaceutical form were developed and compared. They found that the non-aqueous titrimetric method provided linearity only in the range 250 mg to 500 mg of the drug while the UV method required derivatisation, the RP-HPLC system involved the use of an internal standard while the separation of KT from its impurities was only partially successful for TLC. Various other methods[76-77] have been developed for the investigation of tromethamine in tablets and urine however strict control of experimental conditions was essential to ensure adequate precision and accuracy. The spectrophotometric evaluation of tromethamine was achieved by reaction of the analyte with ortho-phthalaldehyde in alkaline medium in the presence of a reducing agent that was specific only for detection in intravenous fluids.

Tromethamine has been analysed previously in its ketorolac form that has been demonstrated to be effective in the management of acute pain as well as in the improvement of ocular inflammation[78]. This procedure reported by Sola et. al. involves the development and use of an automated solid phase extraction method for the determination of ketorolac tromethamine (KT) in human plasma using HPLC. The method describes a lengthy extraction procedure and the use of an internal standard for quantitation. The method suffers from poor precision and accuracy at low KT concentration low recoveries from plasma samples

approximately 73%. Ketorolac has also been analysed by colorimetry and HPLC[80] in an ophthalmic solution and by charge transfer and ion pair complexation reactions in which reaction with various reagents was required. The charge transfer procedure suffered from poor sensitivity as the ion pair preparation method was inconvenient and time consuming. Gumbhir and Mason[81] also attempted the derivatisation of tromethamine by reaction with benzoyl chloride and subsequent analysis by GC. Derivatisation was achieved via reaction of the molecule with the UV absorbing chromophore by first reacting with acid chloride in the presence of a base and subsequent generation of the benzoylated derivative. The GC method reported uses UV/vis detection. These authors describe an extraction procedure prior to analysis of the derivative that proved suitable only for tromethamine determination in specific bodily fluids. Morris and Hsieh[82] detected tromethamine in human plasma and urine using fluorescence detection however an additional derivatisation step was required. Analysis of tromethamine has been accomplished by several analytical methods. Early reports describe the use of colorimetry[86] in the evaluation of tromethamine. This involves a diazotisation reaction coupled with a coupling procedure, and the use of boiling sulphuric acid. Gas Chromatography (GC)[87] methods were developed subsequently in which derivatisation reactions were required. Vincent et al.[88] derivatised tromethamine for GC analysis where the three hydroxy groups were converted to their corresponding silyl derivatives however the derivatised products were unstable and therefore the procedure was deemed inconvenient for incorporation as a routine analytical method. Omission of extraction and derivatisation steps would speed up analysis. Other spectrophotometric methods also require derivatisation procedures.

5.3 Method Development

5.3.1 Choosing an Electrolyte

The first electrolyte system chosen for determining a response for tromethamine was a Copper Sulphate electrolyte. This buffer determined the NH_4^+ cation as described in Section 4.3.2. The CE method used however suffered from interference, but not at the migration time of the ammonium ion, thus it was thought that a response would also be generated for tromethamine at a similar migration time.

5.3.1.1 Copper Sulphate

Electrolyte: 4 mM Copper (II) Sulphate, 4 mM Formic Acid, 4 mM 18-Crown-6-Ether, pH 5

Sample: 20 mg / L tromethamine

Operating Conditions: As per Section 4.3.1

Electrolyte Preparation: As per Section 4.3.1

(i) Stock Standard Solution

0.0989g of tromethamine solid (99.8% pure) was accurately weighed and dissolved in ultra pure water. The solution was transferred to a 1 L volumetric flask and diluted to the mark with ultrapure water.

(ii) Working Standard

20 ml of the stock solution was pipetted into 100 ml volumetric flasks and diluted to the mark with ultrapure water.

Result: A response appeared at approximately 5 min, however other peaks were present in each electropherogram.

Subsequent standards of tromethamine were prepared and injections performed.

Working Standard

1, 5, 10, 15 and 20 ml of the stock solution was pipetted into 100 ml volumetric flasks and diluted to the mark with ultrapure water.

Result: Responses occurred at approximately 5 min. Peaks recorded increased in height and area upon increasing concentration injected.

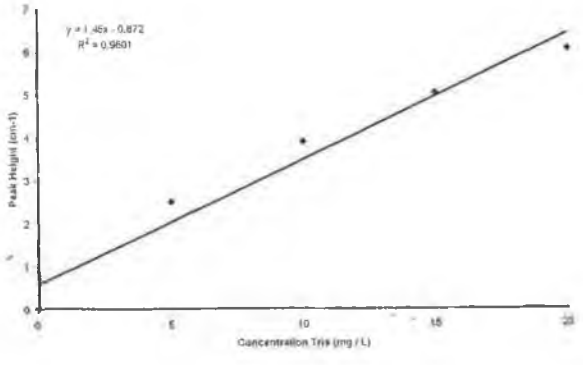


Diagram 5.3 Calibration Plot of Tromethamine using Copper (II) Sulphate Electrolyte

An unacceptable linear response was obtained in the calibration plot of concentration v peak area however, a correlation coefficient of R^2=0.9601 was obtained from a plot of concentration v peak height, (n=3).

Using the above electrolyte system the occurrence of peaks at approximately 2.5, 3, 3.5 and 10 min also appeared with each injection. Large peak areas were observed when 20 mg/L tromethamine standards were injected, thus it was thought that concentrations above 15 mg/L deviated form linearity. Linearity was further investigated in the 5-10 mg/L concentration range to improve the correlation coefficient values and results indicate significant variations from those obtained previously. Upon repeating injections (5-10 mg/L), non-linear responses were demonstrated. Together with the persistent presence of unknown peaks and unacceptable linearity, it was decided to choose a more suitable electrolyte system.

5.3.1.2 Imidazole

Imiadzole-Acetic Acid electrolyte has been previously reported for the analysis of inorganic cations.[68,89-92] Imidazole possesses strong UV absorbing properties and is structurally similar to the analyte of interest. Imidazole was chosen for this investigation as it is thought it would have similar electrophoretic mobility to tromethamine.

Electrolyte: 5 mM Imidazole, 1 M Acetic Acid, pH 5

Sample: 10-50 mg / L tromethamine

Operating Conditions As per Section 4.3.1

(i) Electrolyte Preparation

Imidazole (4.0098g) was accurately weighed, dissolved in ultrapure water and transferred to a 1 L volumetric flask and made up to the mark. Electrolyte pH was adjusted using 1 M Acetic Acid.

(ii) Stock Solution As per Section 5.3.1.1

(iii) Working Standards: As per Section 5.3.1.1

Working standards were diluted in electrolyte in an attempt to remove unknown peaks observed in Section 5.3.1.1.

Result: Responses occurred at approximately 5 min. Peaks still remained at approximately 2.5, 3, 3.5 and 10 min with each injection. Below 10 mg / L sample no detector response was observed. Peak splitting was observed at higher concentrations.

Due to poor results obtained with imidazole-acetic acid electrolyte, it was decided to examine the effect of adding18-Crown-6-Ether. Crown ethers have been added in previous studies CE analysis[65,68-70,91] and it was hoped that this compound would complex with unknown peaks in the system.

Electrolyte: 5 mM Imidazole, 5 mM 18-Crown-6-Ether, 1 M Acetic Acid, pH 5

Sample: 10-50 mg / L Tromethamine

Operating Conditions: As per Section 4.3.1

(i) Electrolyte Preparation

Imidazole (4.0098g) and 0.52721g of 18-Crown-6-Ether were accurately weighed, dissolved in ultrapure water and transferred to a 1 L volumetric flask and made up to the mark. Electrolyte pH was adjusted to pH 5 with 1 M Acetic Acid.

(ii) Stock Solution: As per Section 5.3.1.1

(iii) Working Standards: As per Section 5.3.1.1

Result: Inclusion of the complexating agent did not remove unknown peaks. Responses at 5 min were still the same.

Electroosmotic Flow:

The flow of neutrals in the system was determined by injecting 2-naphthol to investigate whether the EPM and EOF had similar mobility using these conditions. Approximately 2g (5 mM) was weighed accurately and dissolved in methanol. EOF was apparent at approximately 10 min.

Cation standards prepared as per Section 4.3.1 were injected onto this system. Peaks were identified as K^+, (2.4 min), Ca^{2+} (2.96 min) and Na^+ (3.48 min).

As a result of satisfactory outcomes using DDP as electrolyte for the determination of cations by indirect-UV detection, this was the next electrolyte of choice.

5.3.1.3 DDP

Dimethyldiphenylphosphonium Iodide Buffer (DDPI)

DDPI has been used in the analysis of a wide variety of cations from simple alkali metals to transition metals[52,94]. It is reported that the mobility of the electrolyte is similar to metal ions Li^+, Na^+, Mg^{2+}, Ca^{2+} and K^+, resulting in high peak efficiencies and good peak shapes. Indirect photometric detection was used as the DDPI compound acted as the UV absorber. In order to determine the most appropriate wavelength to use in the CE, UV spectrophotometric scans of DDP were obtained.

(i) Stock Electrolyte Solution

A Stock Solution of DDPI (0.2 mM) was prepared in ultrapure water by weighing 0.0612g of the solid, dissolving in ultrapure water, transferring to a 100 ml volumetric flask and making up to the mark with ultrapure water. An absorbance reading against a water blank was taken. Serial dilutions of the stock solution were made by pipetting 1-50 ml, transferring to 100 ml volumetric flasks and making up to the mark with ultrapure water. Corresponding absorbance values were read.

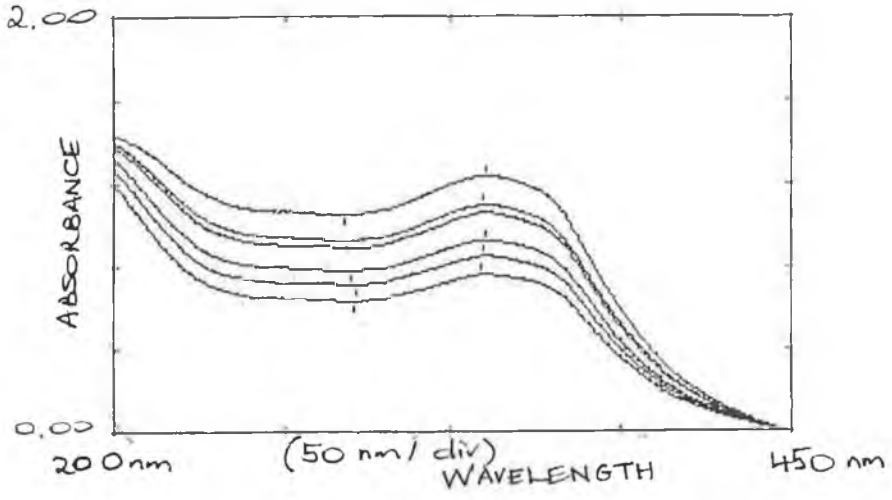
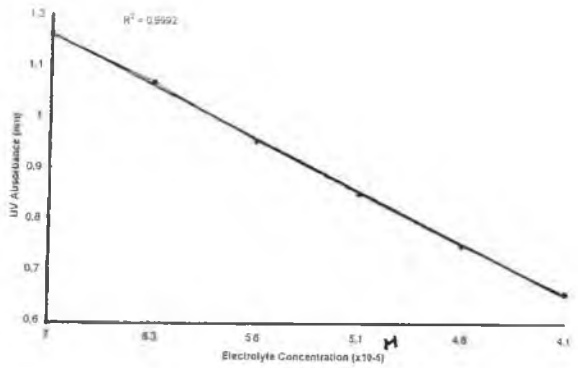


Diagram 5.4 (a) UV Spectrophotometric Scans of DDP



(b) Calibration Plot of DDP (Electrolyte Concentration v UV Absorbance)

Result: DDPI was found to have a molar absorptivity of 1.65 x 10^5 mol^{-1} dm^2 at a wavelength of 215 nm.

DDPI-18-Crown-6-Ether-α-hydroxyisobutyric Acid

In previous studies[52,94] DDPI has been used with 18-Crown-6-Ether and α-hydroxyisobutyric acid thus the electrolyte was prepared using these methods.

Electrolyte	5 mM DDP, 6 mM α-hydroxyisobutyric acid, 8 mM 18-Crown-6-Ether pH 4.6
Sample:	1-5, 10-50 mg / L tromethamine

Operating Conditions: As per Section 4.3.1

(i) Electrolyte Preparation

A stock solution of DDPI (25 mM) was prepared by weighing 0.8854g of the compound, dissolving in ultrapure water and making up to a final 100 mL volume. A stock solution of 18-Crown-6-Ether (40 mM) was prepared by weighing 1.057g of the compound, dissolving with ultrapure water and making up to a final 100 mL volume. A stock solution of α-hydroxyisobutyric acid (100 mM) was prepared by dissolving 1.041g of the compound in ultrapure water, transferring to a 100 mL volumetric flask and making up to the mark.

DDP electrolyte was purchased from suppliers in its iodide form. By solid phase extraction, this solution was converted to its corresponding hydroxide form by the following method.

(ii) Solid Phase Extraction (SPE)

Conditioning of the SPE cartridge was carried out as follows: 10 ml of NaOH (1 M) was passed through an On Guard A Cartridge, followed by 10 ml ultrapure water at a rate of 2 ml / min. 10 ml of the DDPI stock

solution was then passed through the cartridge, the first 2 ml was discarded. To a 100 ml volumetric flask was added the remaining 8ml of the converted DDPI solution, 5 ml of the 18-Crown-6-Ether solution and 6 ml of the α-hydroxyisobutyric acid solution. The mixture was made up to the mark with ultrapure water.

DDPI DDP

Diagram 5.5 DDPI converted to DDPOH

(iii) Stock Standard Solution As per Section 5.3.1.1

(iv) Working Standards As per Section 5.3.1.1

Result: Responses occurred at less than 5 min. Detector responses generated symmetrical peaks without interference.

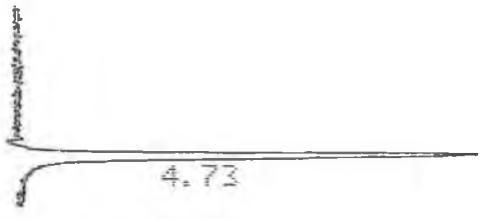
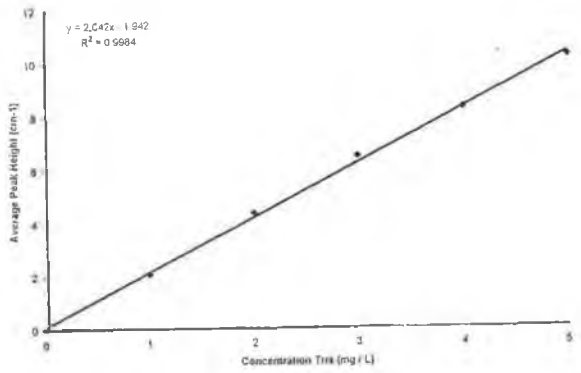
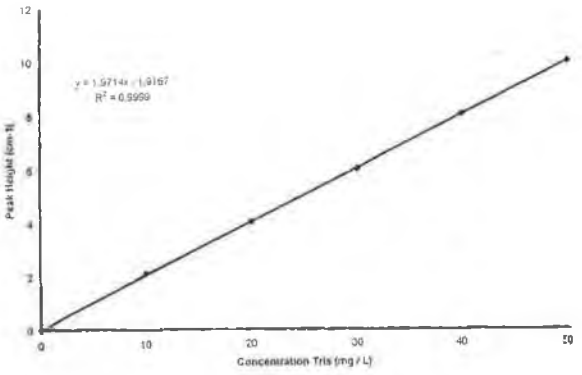


Diagram 5.6 Initial Tromethamine Response

Calibration graphs constructed for injections 1 - 5 mg / L tromethamine, at n = 3, indicated good linearity with correlation coefficient 0.9984 for average peak height. Injections of tromethamine (10-50 mg / L) were then performed to examine linearity at higher concentrations. Calibration plots constructed (10-50 mg / L) displayed linearity with a correlation coefficient = 0.9999.

(a)





(b)

Diagram 5.7 Calibration Plot of (a) 1-5 mg / L and (b) 10-50 mg / L Tromethamine

DDPI electrolyte was found to be the most suitable for the analysis of the analyte as peak shape appeared to be much improved compared to previously used electrolytes. The function of the crown ether in the buffer was next examined as injections of ultrapure water and electrolyte alone did not generate peaks (identified in Section 4.4.1) as before in Section 5.3.1.2.

Result: No difference was observed from results collected with or without 18-crown-6-ether, thus it was decided to omit the inclusion of the complexating agent from all further analysis.

5.3.2 Electrolyte pH Variation

The pH of the electrolyte was varied to examine its effect on the electrophoretic mobility of the analyte.

Electrolyte 5 mM DDP, 6 mM α-hydroxyisobutyric acid, pH 3-11

Sample: 20 mg / L tromethamine

Operating Conditions: As per Section 4.3.1

Electrolyte Preparation:

A Stock Solution of DDPI (25 mM) was prepared by weighing 0.8862g of the compound and dissolving in ultrapure water and making up to a final 100 mL volume. A Stock Solution of α-hydroxyisobutyric acid (100 mM) was prepared by dissolving 1.039g of the compound in ultrapure water, transferring to a 100 mL volumetric flask and making up to the mark.

(ii) Solid Phase Extraction (SPE): As per Section 5.3.1.3

By adding various amounts of α-hydroxyisobutyric acid dropwise to the electrolyte, a range of pH values were obtained. Electrophoretic and electroosmotic mobility were monitored in the range pH 3-11. EOF was measured by injecting 2-naphthol (prepared as before).

Result: Optimum mobility can be observed at pH 5.2, Table (i).

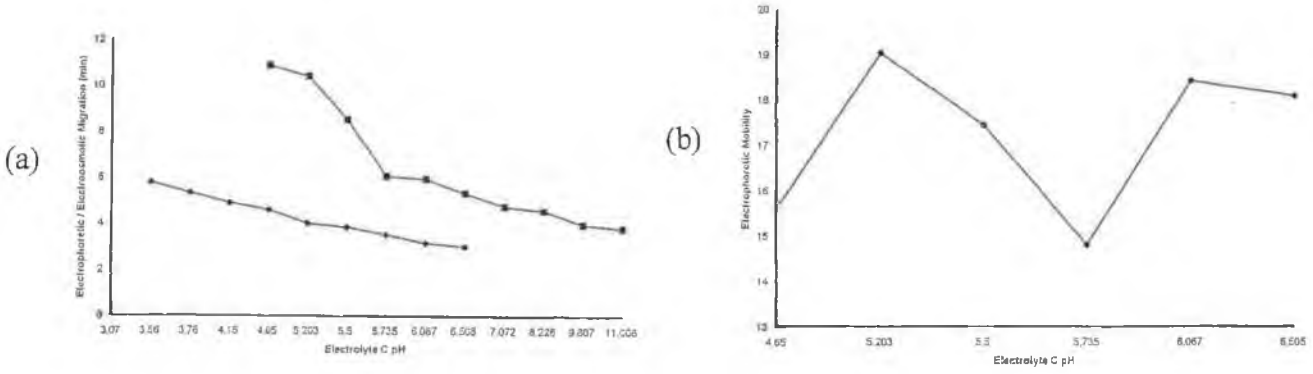


Diagram 5.8 DDP pH v (a) Electrophoretic and Electroosmotic Migration and (b) Electrophoretic Mobility

Analyte mobility was apparent only in the narrow pH range 3.5-6.5, Table (ii), Appendices. The electroosmotic flow was retarded at low pH values whereas at higher pH values the flow of the bulk electrolyte was rapid throughout the capillary. Tromethamine is therefore uncharged in this alkaline environment, pH > 6.5 and is detected as a cationic species in acidic conditions, pH > 3.5. Electrolyte pH 5.2 was used for all further analysis.

5.3.3 Voltage Variation

The operating voltage was varied from 15 kV to 25 kV, Table (iii), Appendices, to examine the effect of electrophoretic and electroosmotic mobility. Voltages above 20 kV required the application of a cooling jacket. The resulting current and observed power generated were monitored from the display screen on the CES instrument, placing a thermometer inside the hood of the instrument monitored temperature variation. A 20 mg / L standard was used to examine electrophoretic mobility and electroosmotic mobility monitored upon injections of 2-naphthol.

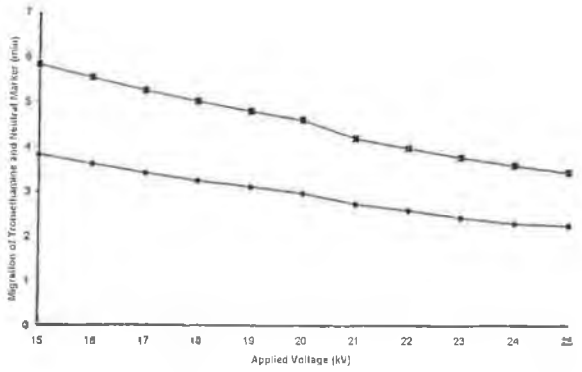


Diagram 5.9 Applied Voltage v Migration of Tromethamine and 2-Naphthol

Result: Increased electrical application is seen to enhance electrophoretic and electroosmotic mobility whereby analysis time is reduced by approximately 2 min. An optimum voltage of 20 kV was chosen.

5.3.4 Temperature Variation

A thermometer was placed on the inside of the hood. Injections of 20 mg / L tromethamine standard were performed at extreme temperatures.

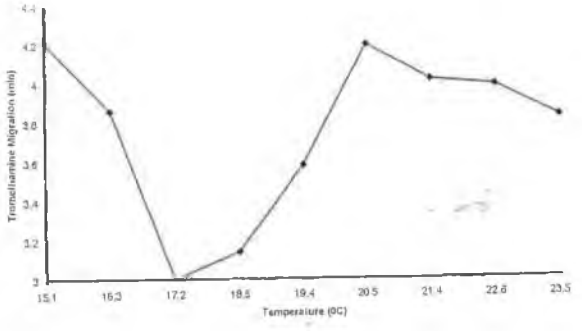


Diagram 5.10 Electrophoretic Mobility v Temperature

As the instrument did not have an automated temperature control system, thermometer readings, were taken early in the morning when average temperatures were 15^0C, early in the afternoon when laboratory humidity was highest and temperatures averaged 23.5^0C and finally, late at night when laboratory temperature was reduced, Table (iv). Injections were performed at various temperatures, readings collected over three days and results averaged.

Result: Electrophoretic mobility is most significant at 20^0C and less significant at 17^0C.

5.3.5 Ionic Strength Variation

A series of electrolyte concentrations were prepared to examine the effect of variation of ionic strength on migration time of 20 mg / L tromethamine.

(i) Ionic Strength Concentrations

To a series of labelled 100 ml volumetric flasks were added 8, 4, 2, 1 and 0.5 ml of the converted DDPI solution and 6 ml of α-hydroxyisobutyric acid (As per Section 5.3.1.3) to a final pH 4. The mixture was made up to the mark with ultrapure water. Final buffer concentrations were 5 mM, 2.5 mM, 1.25 mM, 0.625 mM and 0.312 mM.

Result: Analyte migration increases from 3 min to approximately 5 min upon reducing ionic strength in the range examined, Table (v). Concentrations above 5 mM DDPI were not examined as too much heat was generated inside the capillary and the system automatically shut down. An ionic strength of 5mM electrolyte was used for all further analysis.

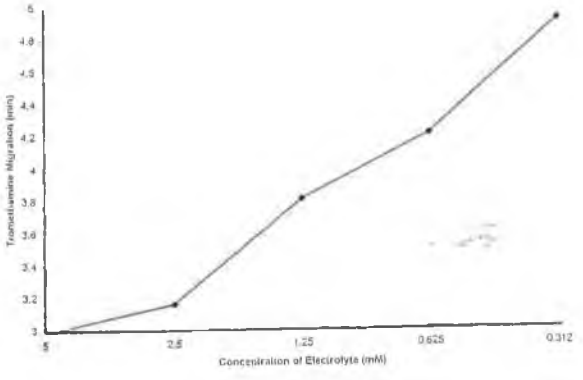


Diagram 5.11 Concentration DDP v Tromethamine Migration

5.4 Method Validation

5.4.1 Linearity Linear Range and Limit of Detection

Linearity of the method was determined initially between 1-5 mg / L followed by 10-50 mg / L tromethamine.

(i) Working Standards

1, 2, 3, 4 and 5 ml and 10, 20, 30, 40, 50 and 60 ml of the Stock Solution were pipetted into 100 ml volumetric flasks and made up to the mark with ultrapure water. Each standard was injected three times.

Result: The method provided linearity in the range 2.5 mg / L to 25 mg / L. The lowest operating limit of detection was found to be 2.5 mg / L, as responses below this concentration remained relatively constant and independent of changing concentrations. At higher concentrations, deviations from linearity were observed in excess of 25 mg / L where line plateau was evident, Table (vi). Calibration curves were compiled from data generated upon injection of 2.5 mg / L - 25 mg / L standard solutions where n = 5. y = 0.098x - 0.029, R^2=0.9992. The sensitivity of the system was found to be 0.98 absorbance units / $mg.^{-1}$

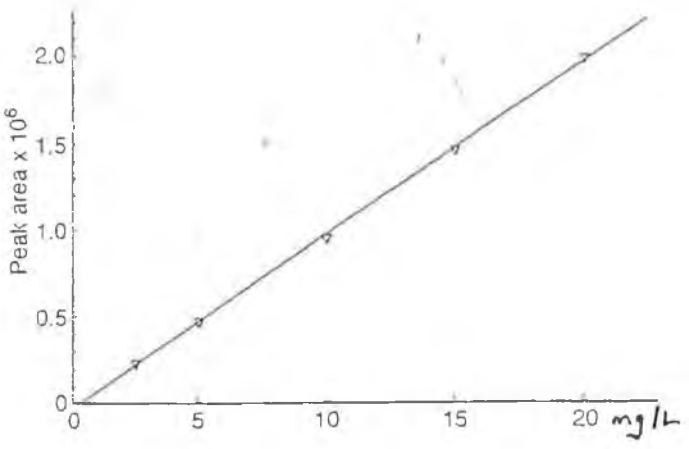


Diagram 5.12 Calibration Plot Tromethamine

5.4.2 Repeatability

The repeatability of the method was examined by injecting ten samples of 10 mg / L tromethamine solutions from similar and different carousel vial positions to ascertain variability between injections.

Result: Standard Deviation and Relative Standard Deviation were calculated from peak height and found to be 0.14% and 2.01% respectively for injections from the same vial position and 0.36% and 3.43% respectively for injections from different vial positions.

5.4.3 Precision and Accuracy

The precision and accuracy of the method was determined by conducting intra- and inter-assay variability tests. The intra-assay variability test consisted of analyzing five concentrations of each standard, 5 to 25 mg / L and injecting each standard six times in one batch, Table (vii). The amount of tromethamine in

each standard was plotted against the average peak height to determine the correlation coefficient. Results from four different intra-assay variability tests, collected over four days were used to find the % coefficient of variation and % deviation.

Result: Over the concentration range studied the average RSD was found to be 0.42% and the average deviation was found to be 1.62%. The average deviation is inflated owing to the relatively poor deviation obtained for the concentration of 5 mg / L. The remaining four concentrations gave an average of 0.94%. As the concentration of the sample is 12 mg ml^{-1}, the result is acceptable.

5.4.4 Specificity

The specificity of the method was examined by comparing the response from injections using standard tromethamine and a placebo solution to examine matrix effects. The placebo solution contains other ingredients such as Polyhexamethylene Biguanide, Tromethamine, Disodium EDTA and Tyloxapol. Injection of the placebo were made (n=3).

Result: A steady baseline was obtained when placebo solutions were injected. This suggests that the method developed is specific for the determination of tromethamine. Recovery studies were therefore conducted.

5.4.5 Recovery Studies

Recovery of the method was applied for the determination of tromethamine in a pharmaceutical product 'Complete', containing 12 mg ml^{-1} tromethamine.

(i) Spiked Placebo Solutions

Stock solutions of spiked placebo were prepared as follows: 5, 10, 15, 20 and 25 mg of tromethamine were accurately weighed and dissolved in the placebo. A 1 in 1000 dilution of each of the spiked solutions was made using ultrapure water to generate a series of placebo solutions containing 5, 10, 15, 20 and 25 mg / L. Six injections of standard tromethamine solutions and six injections of spiked placebo solutions were performed.

CHAPTER SIX

OXYTETRACYCLINE ANALYSIS

6.1 Introduction

As the method for determining tromethamine using indirect photometric detection was developed and validated, it was decided that another method using direct photometric detection could also be developed and validated. The aim of this investigation was to develop a CE method using direct photometric detection for the analysis of oxytetracycline (OTC).

The pKa values of OTC are 3.27, 7.32, 9.11 and 10.7. The presence of a hydroxyl group at C-6 enables acid degradation to 4-epioxytetracycline (EOTC) through an equilibrium reaction, and to anhydrooxytetracycline (ADOTC) by irreversible dehydration.

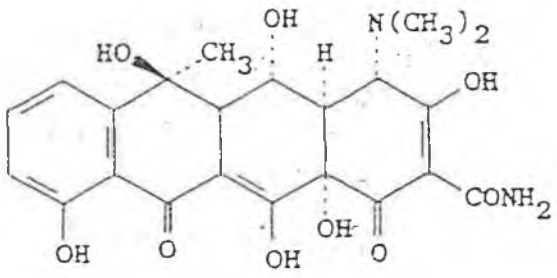


Diagram 6.1 Structure of Oxytetracycline

OTC is a broad spectrum antibiotic of the family of tetracyclines which are used to control bacterial infections in both humans and animals. OTC is found in a variety of pharmaceutical products for human and veterinary use. OTC is commonly found in topical ointments used in dermatology therapy. OTC has also been added to animal feed in order to control lice infestations on the animal's skin. The use of oxytetracyline in animal feed to control animal disease leads to problems with residues in medicated fish and in the environment. The use of oxytetracyline may result in residues being present in animal-derived food products and these residues pose a health threat to consumers. Oxytetracyline has also found applications in the preservation of harvested fruits and vegetables.

6.2 Literature Review

HPLC methods[95,96] have been developed for the analysis of OTC from fish samples of skin, muscle and tissue. Sample preparation involves a number of steps including spiking with internal standard, extracting, shaking, sonication, centrifugation, filtration, dilution followed by extraction with C18 and C8 Bond Elut SPE columns, prior to analysis by HPLC. Even though analysis times are less than 5 min, these methods suffer from low recoveries, 89-95%. A CE method for determining OTC from its impurities[97] in a pharmaceutical product has been described. Triton-X-100 and methyl-β-cyclodextrin were required to separate OTC from another tetracycline, demeclocycline. Analysis time for OTC was measured at 17 min. Electrolyte pH was restricted to the alkaline region to avoid sample adsorption onto the capillary wall and OTC could not be resolved completely from its impurity ADOTC. CZE techniques[98,99,100] report the detection of OTC from pharmaceutical and biological samples. OTC is reported to easily complex with metals and thus this was the basis for the non-aqueous CZE technique[98] using laser-induced fluorescence detection. Metal complexation was reported to obtain separation selectivity. The non-aqueous CE method[99] demonstrates the separation of OTC from its degradation products as metal chelates with magnesium ions. The electrolyte was magnesium acetate tetrahydrate with NMF. This organic solvent was found to harm the material of the lever arm of the instrument and parts of the injection system had to be cleaned and trimmed due to leaks resulting in variations of injection pressure.

The separation and characterisation of OTC and other tetracycline products has been reported by Tavares and McGuffin.[100] A recovery of 95% was obtained from a tablet matrix in approximately 5 min. Acid-base equilibrium constants were determined for each of the tetracyclines analysed. Low recoveries were also reported[101] for the analysis of OTC from milk samples with HPLC. The matrix also contained tetracycline and chlortetracycline and the authors report difficulty optimising separation of OTC from these. An isocratic RP-HPLC method[102] was also unsuccessful in isolating OTC from its degradation

impurities. An MECC technique[103] using 50 mM borate, 50 mM phosphate and 10 mM SDS at pH 8.5 successfully resolved OTC from its impurities however analysis time was 13.3 min. CZE[104] has detected OTC at 4.5 min using 20 mM sodium carbonate electrolyte with 1 mM EDTA. The method however is less selective than other reported HPLC methods. CE has also been used to detect OTC in a pharmaceutical[105] and biological matrices.[106] The CEC[105] method involved etching the inner wall of the capillary surface and modifying with a silation/hydrosilation reaction in order to attach a C18 moiety. The CZE method[106] determined OTC in 11 min however sample preparation was complex. Three HPLC methods[107-109] have determined OTC from milk and environmental samples with recoveries approximately 80%. Analysis times range from 4.5 min to 12 min and methods are not selective for OTC in each of the respective matrices. The IC determination[110] of OTC has also been reported for its determination in milk and milk powder. OTC has been resolved from other tetracyclines in the matrix using a cation exchange column. It was necessary to use an organic solvent, ACN, in this technique to facilitate sample suolubility and column cleanup.

Table 6.1 Literature Review for Oxytetracycline

Matrix	Separation Technique	Analysis Time (min)	Linear Range	R^2	LOD	Precision and Accuracy	Recovery	Ref
Fish Tissue	HPLC (SPE)	3.1-3.4 min	0.2-10 µg/ml	0.9991	0.05 µg/ml	RSD=0.8-1.0%	91.3%	95
Fish Tissue	HPLC (SPE)	5 min	10-20 µg/ml	0.978	5 ng/g	RSD=4.2%	89.3-94.8%	96
Antibiotics	CZE	17 min	0.25-1.75 µg/ml	0.9994	0.05 µg/ml			97
Milk, Plasma	CZE	13 min	50-1000 ng/ml	0.999	25 ng/ml	RSD=3.6%	97.2%	98
Ointment	CZE	14 min	0.2-3 mg/ml	0.999	0.2 mg /ml		100%	99
Tablet	CZE	5.41 min		0.9989	1×10^{-5} M		95%	100
Milk	HPLC	4 min	100-3200 ng/ml	0.982-0.996	100 ng/ml	RSD=1-9.3%	63.5-93.3%	101
	RP-HPLC	8 min		0.9998	0.05%	RSD=0.3%	92.7%	102

Milk, Serum, Urine	MECC	13.3 min	0.992	0.992	4.2 µg/ml	RSD=0.07%	75.7-82.1%	103
-	CZE	4.5 min	0.9982	0.5-1.8 mg/ml	10^{-5} %w/v	RSD=4.1%	-	104
Antibiotics	CEC	10 min			5-10 µg/ml			105
Fish Tissue	CZE	11 min	0.999	0.1-25 µg/ml	0.05 µg/ml	RSD=2.6-3.5%	92.9%	106
-	HPLC	10.5 min	0.9995	16-24 µg/ml	16 µg/ml	RSD=0.9-1.2%	80-120%	107
Milk	HPLC	12 min	0.998	0.1-5 µg/ml	113 µg/Kg	RSD=1-5%	81-83%	108
Environmental Samples	HPLC	4.5 min	0.9974	3.6-3.8 µg/ml	0.21 µg/L	RSD=1%	83%	109
Milk	IC	12 min	0.9996	0.05-2mg/L	10 µg/L	RSD=1.4-2.1%	88.15%	110

6.3 Method Development

6.3.1 Solubility of OTC

Oxytetracycline is an amphoteric compound with high polarity and an isoelectric point between 4 and 6.[110] Therefore, as it was insoluble in ultrapure water it was necessary to establish solubility data. Sample preparation: 100 mg of Oxytetracycline Dihydrate was accurately weighed and placed in a 50 ml b eaker. 20 ml of each solvent was pipetted into the beaker. The solution was mixed with a glass rod and resultant appearance recorded.

Solvent Preparation: (a) Dilute Acid Solution: 36.54g of concentrated Hydrochloric acid was weighed and added to a large volume of ultrapure water in a 1 L beaker. The solution was transferred to a 1 L volumetric flask and made up to the mark with ultrapure water. (b) Dilute Alkali Solution: 40.010g of Sodium Hydroxide pellets were accurately weighed and added to a large volume of ultrapure water contained in a 1 L beaker. The solution was mixed with a glass rod and transferred to a 1 L volumetric flask and made up to the mark with ultrapure water.

Result: Methanol and dilute acid were selected as sample solvent for further investigations.

Table 6.2 Oxytetracycline Solubility Data

Solvent	Solubility	Appearance
Methanol	Readily Soluble	Clear
Ethanol	Insoluble	Opaque yellow suspension
Propanol	Insoluble	Opaque yellow suspension
Butanol	Insoluble	Opaque yellow suspension
Pentanol	Insoluble	Opaque yellow suspension
Acetonitrile	Sparingly soluble	Opaque yellow suspension
Tetrahydrofuran	Sparingly soluble	Opaque yellow suspension
1 M HCl	Readily soluble	Clear
1 M NaOH	Readily soluble	Clear

6.3.2 UV Measurements

UV absorbance measurements of OTC were recorded in order to ascertain its maximum wavelength of absorbance.

(i) Stock Solution Preparation

A 1000 mg / L stock solution was prepared by weighing 0.010g of OTC Dihydrate, dissolving in Methanol and transferring to a 100 ml volumetric flask. The solution was made up to the mark with the solvent.

(ii) Working Standard Solutions

1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 ml of the stock standard solution were pipetted into 100 ml volumetric flasks labelled 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 mg / L OTC. The solutions were made up to the mark with methanol.

Methanol was placed in the UV Spectrometer and a background reading taken. Absorbance readings for each of the standard OTC solutions were read.

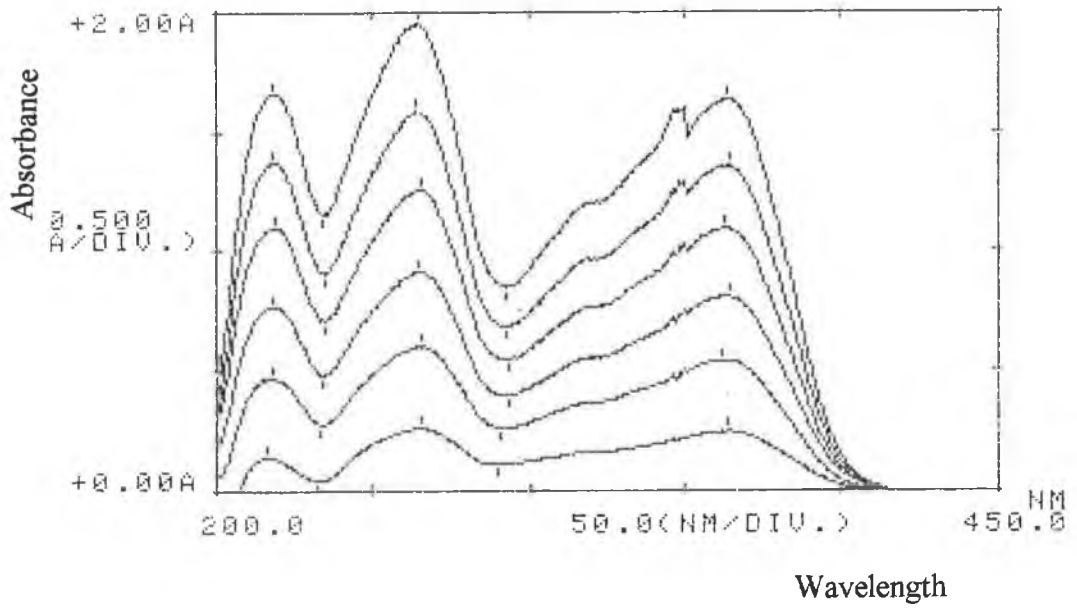


Diagram 6.2 UV Scans of OTC

Result: The maximum wavelength of absorbance is 270 nm.

6.3.3 Choosing a Suitable Electrolyte:

Tris-HCl buffers have been previously reported for the analysis of OTC[104,105] therefore this electrolyte was used to determine an initial response. The pH of the electrolyte was maintained neutral to avoid degradation. Injections of 100 mg / L OTC and 2-naphthol (5mM) were made to examine electrophoretic and electroosmotic behaviour.

Electrolyte	50 mM Tris-HCl, pH 7	
Sample	100 mg / L OTC in Methanol, 2-Naphthol	
Operating Conditions	Capillary Dimensions	50 µm (i.d.) x 50 cm (l) x 375 µm (o.d.)
	Detection System	UV, at 270 nm
	Polarity	(+), Detector side Cathodic
	Control Mode	Constant Voltage, 20 kV
	Injection	Gravity, 100 mm for 30 sec
	Temperature	Ambient

(i) Electrolyte preparation

6.059g of tromethamine was weighed and dissolved in ultrapure water in a 50 ml beaker. This solution was transferred to a 1 L volumetric flask and adjusted from pH 10.4 to pH 7 using the previously prepared 1 M dilute HCl solution. The solution was made up to the mark with ultrapure water.

The stock standard, working standard, naphthol solution and capillary were prepared as in Section 6.3.2.

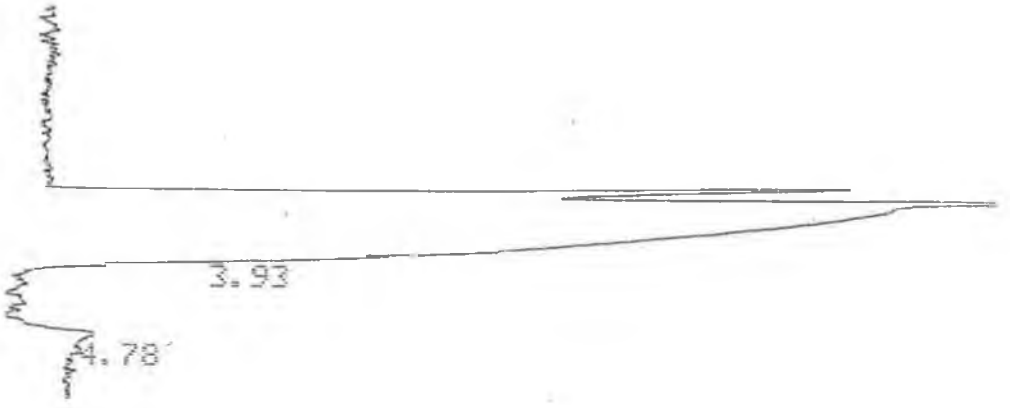


Diagram 6.3 Initial OTC Response

Result: A response appeared at approximately 3.5 min showing two reponses eluting together. Electroosmotic mobility at approximately 3 min however peak shape was poor.

Initial detector responses indicate peak efficiency was poor as peak shapes were asymmetric thus the sensitivity of the detector was low. Attempts were made to improve peak efficiency by optimising UV wavelength used, changing the sample solvent and increasing sample injection volume.

6.3.4 Optimising UV Absorbance

The output signal generated from the detector was examined to see which wavelength would give the greatest response. Injections of 100 mg / L OTC were performed at wavelengths 270 to 210 nm.

Result: The greatest detector response was at 210 nm. Peak Height readings were not recorded due to poor peak shape as the sample solvent used was methanol. Satisfactory agreement has been reached upon detector output in terms of peak area as these remain relatively constant regardless of peak shape. From results obtained it can be seen that as the operating wavelength decreases there is a steady decrease in electrophoretic mobility in the system. This may be due increased absorption of water near 210 nm. A UV wavelength of 210 nm was chosen for all further analysis.

6.3.5 Changing Sample Solvent

100 mg / L standard OTC solution was dissolved in previously prepared 1 M HCl, 0.1 M HCl, electrolyte. Injections of 100 mg / L were made using the conditions above to investigate if peak shape improved.

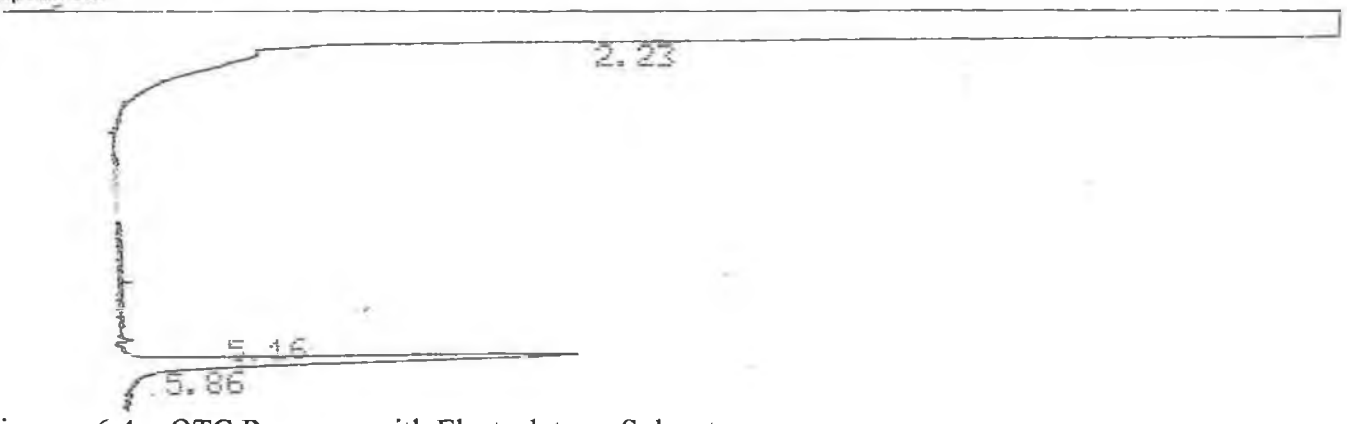


Diagram 6.4 OTC Response with Electrolyte as Solvent

Result: The best sample solvent is electrolyte as the peak shape has significantly improved.

6.3.6 Varying Injection Volume

Detector sensitivity was low because greater peak height and area readings were obtained for much smaller concentrations during tromethamine analysis. By increasing the volume of sample injected onto the capillary, the output signal from the detector can be increased. Programming the instrument to raise the head height and inject for longer periods, increased the volume of sample loaded onto the capillary. Injections of 0.142 nl, 0.852 nl, 1.704 nl, 2.13 nl and 2.556 nl of 200 mg / L OTC were performed. Sample volumes were calculated using the equation:

$$V = \frac{2.84 \times 10^{-8} H T D}{L} \qquad \text{(Eqn 6.1)}$$

where, V = Volume injected (nL), H = Head height (mm), T = Injection time (sec), D = Internal capillary Diameter (m) and L = Total capillary length (cm).

Result: An injection volume of 1.704 nl was chosen for further analysis as this volume produced the greatest detector signal.

The optimum operating UV wavelength was found to be 210 nm, sample solvent was electrolyte and optimum injection volume used a head height of 100 mm and injection time of 40 sec. Once these parameters were chosen, it was possible to examine the electrolyte pH and composition.

6.3.7 Electrolyte pH Variation

The pH of the electrolyte was varied to examine the effect on the electrophoretic and electroosmotic mobility of the analyte.

(i) Electrolyte Preparation as per Section 6.3.3

For each pH value, 200 ml of the electrolyte was pipetted into a beaker and 2 M HCl was added drop wise with a Pasteur pipette to generate a series of pH values.

(ii) Capillary Conditioning

The capillary was washed sequentially with 1 M NaOH for 10 min, ultrapure water for 15 min followed by capillary conditioning with run buffer for 30 min upon application of each pH buffer systems.

Electrophoretic mobility (μ_{EP}) was calculated by injecting 200 mg / L OTC, n = 3.

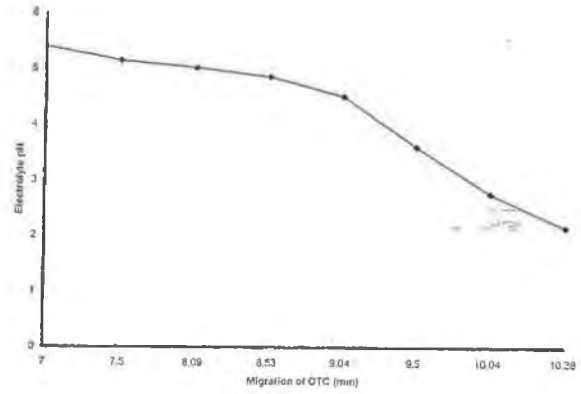


Diagram 6.5 Migration of OTC v Electrolyte pH

Result: At high pH>10, electrophoretic and electroosmotic mobility flow through the capillary has increased however peak shape has deteriorated, Table (viii). At pH 3-7, peak splitting was obvious and the response due to OTC was no longer apparent. It is thought that acid degradation of the sample may have occurred inside the capillary. From initial investigations, electrolyte at pH 7 indicated acceptable peak shape in reasonable migration time, thus this pH was chosen for all further analysis.

6.3.8 Ionic Strength Variation

(i) Electrolyte Preparation: A series of electrolytes of varying Tris concentration (10, 20, 30, 40, 50, 60, and 70 mM) were prepared by dissolving 1.210g, 2.422, 3.632g, 4.843g, 6.059g, 7.264g and 8.475g of the compound in ultrapure water in a 50 ml beaker. Each solution was adjusted to pH 7 with 1 M HCl and transferred to labelled 250 ml volumetric flasks. The resulting solutions were made up to the mark with ultrapure water.

(ii) Capillary Conditioning: As per Section 6.3.3

6.3.9 Electrophoretic and Electroosmotic Mobility

The electrophoretic mobility was examined by injecting 200 mg / L OTC and recording the migration time obtained with each buffer concentration. The electroosmotic mobility was calculated by injecting

of 5 mM 2-naphthol solution prepared as before. Current and temperature readings were taken throughout. The resulting current was read from the CES screen and temperature fluctuation was monitored by placing a thermometer inside the hood of the instrument.

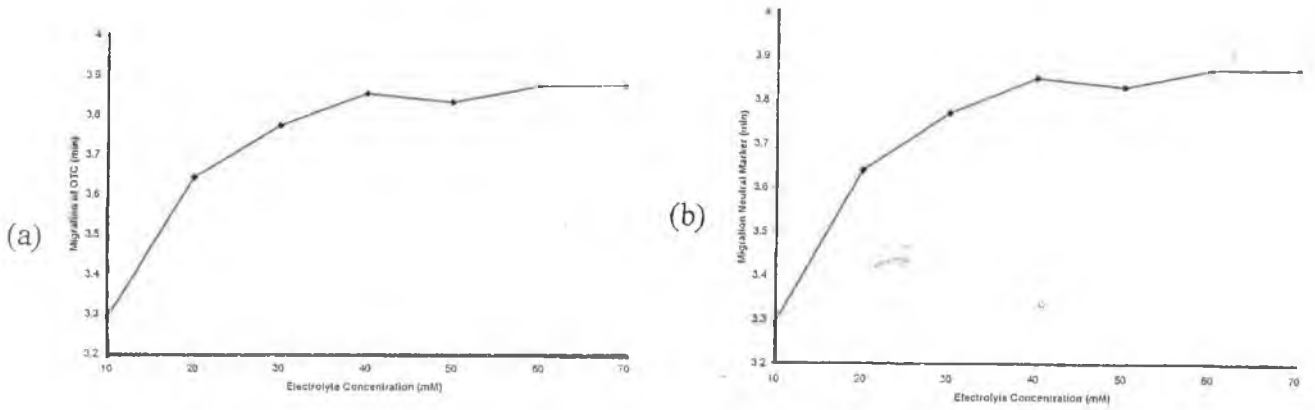


Diagram 6.6 Electrolyte Concentration v Migration Time

Result: From graphs constructed of electrolyte concentration v electrophoretic and electroosmotic migration, analysis time for OTC is reduced by 2 min upon decreasing ionic strength from 70 mM to 10 mM, Table(ix). The reduction in migration for the EOF is not as significant. Therefore electrolyte ionic strength using 10 mM Tris was employed for all future procedures.

6.3.10 Electrolyte Composition

The composition of electrolyte was altered as previously reported methods[103-106] have explored the effect of adding organic solvents and surfactants to buffer systems in tetracycline analysis to resolve similar chemical structures. It was decided to add methanol and sodium dodecyl sulphate to establish the overall effect on electroosmotic and electrophoretic mobility within the CE system.

6.3.10.1 Addition of Methanol

(i) Electrolyte Preparation

1.211g of Tris was weighed and dissolved in ultrapure water in a 50 ml beaker. This solution was transferred to a 1 L volumetric flask and adjusted from pH 10.4 to pH 7 using 1 M dilute HCl solution. The solution was made up to the mark with ultrapure water. 60, 70, 80, and 90 ml of the electrolyte

solution were pipetted into 4 x 100 ml beakers. 10, 20, 30 and 40 ml of methanol were pipetted into each of 100 ml beakers containing the electrolyte solution ensuring 10- 40% (v/v) methanol was contained in each.

(ii) Capillary Conditioning: As per Section 6.3.3

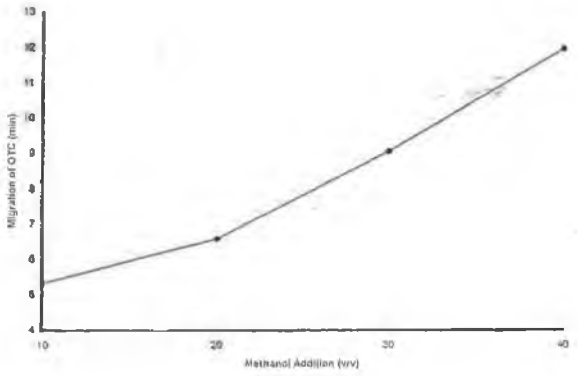


Diagram 6.7 Effect of Adding Methanol to Electrolyte

Result: As analysis time has significantly increased it was decided not to add methanol to the electrolyte.

6.3.10.2 Addition of Sodium Dodecyl Sulphate (SDS)

(i) Electrolyte Preparation: As before

4 x 100 ml of the electrolyte solution were pipetted into 4 x 200 mL beakers. 10, 20, 30, 40 mM SDS were weighed and added to each of the electrolyte solutions and stirred using a glass rod.

(ii) Capillary Conditioning:

The capillary was washed sequentially with isopropanol for 30 min, ultrapure water for 15 min, 1 M NaOH for 10 min and rinsed again with ultrapure water for 15 min. The capillary was equilibrated with run buffer for 30 min upon application of each new electrolyte system.

200 mg / L standard OTC solution prepared as before was injected three times.

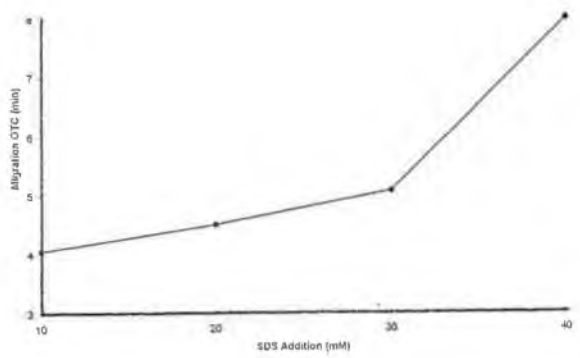


Diagram 6.8 Effect of Adding SDS to Electrolyte

Result: Analysis time has increased by 5 min. Concentrations of SDS above 40 mM caused excessive build up of current in the capillary that could not be efficiently dissipated and initiated the automatic shut down system. No real advantage was offered for the addition of SDS.

6.3.11 Voltage Variation

The operating voltage was varied from 15 kV to 25 kV to examine its effect on the speed of analysis, Table (x). Voltages above 20 kV required the application of a cooling jacket. The resulting current was monitored from the display screen on the CES instrument.

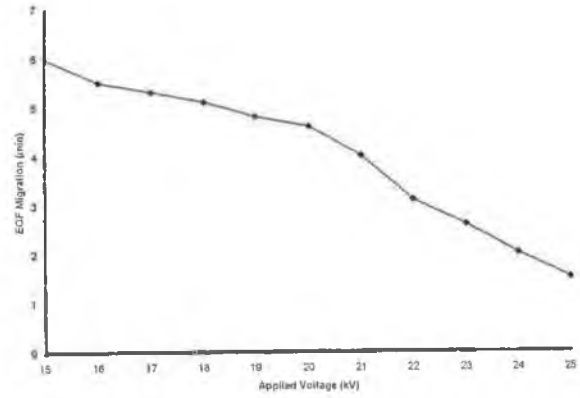


Diagram 6.9 Applied Voltage v Migration Time

Result: High voltages reduce electrophoretic and electroosmotic mobility in the capillary. Electrophoretic mobility is affected to a greater extent then the electroosmotic mobility as the analysis time is reduced by over 5 min as the applied voltage is increased from 15-25 kV. Change in the

migration of the neutral species was substantially less and amounted to less than 3.5 min over the applied voltage range. Optimum voltage chosen from an Ohm's Law Plot of 25 kV was used for all further analysis.

6.4 Method Validation

6.4.1 Linearity, Linear Range and Limit of Detection

(i) Stock Standard Solution: As per Section 6.3.3

(ii) Working Standard Solutions: A series of working standards were prepared to investigate the linear range and limit of detection of the method developed. 40, 30, 20 and 10 ml of the stock OTC solution were pipetted into 100 mL volumetric flasks labeled 400, 300, 200 and 100 mg / L and made up to the mark with electrolyte. The 100 mg / L standard was diluted 1 in 2 sequentially to generate 50, 25, 12.5 and 6.25 mg / L standards. Each working standard was injected three times.

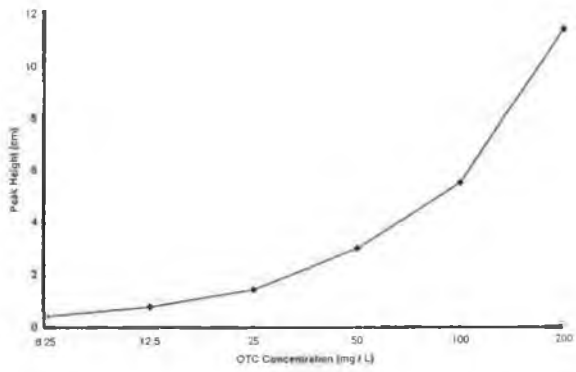


Diagram 6.10 (a) Linear Range, (b) Calibration Curve

Result: Injections of concentrations above 200 mg / L OTC demonstrate no variance in peak height or area, Table (xi). This was taken to be the upper detection limit. Linearity was displayed between concentrations 12.5 mg / L and 200 mg / L. Average correlation coefficient was found to be 0.9994 with slope 0.055. No change in peak height / area was recorded below 6.25 mg / L, thus the limit of detection was taken to be 6.25 mg / L.

6.4.2 Repeatability

The repeatability of the method was examined by injecting ten samples of 200 mg / L OTC solutions from similar and different carousel vial positions to ascertain variability between injections.

Result: Standard Deviation and Relative Standard Deviation were calculated from peak height and found to be 0.28% and 2.62% respectively for injections from the same vial position and 0.32% and 2.99% respectively for injections from different vial positions.

6.4.3 Precision and Accuracy

The precision and accuracy of the method was determined by conducting intra- and inter-assay variability tests. The intra-assay variability test consisted of analyzing five concentrations of each standard, 12.5 mg / L – 200 mg / L and injecting each standard six times in one batch. The amount of OTC in each standard was plotted against the average peak height to determine the correlation coefficient. Results from four different intra-assay variability tests, collected over four days were used to find the % coefficient of variation and % deviation.

Result: Over the concentration range studied the average RSD was found to be 2.82% and the average deviation was found to be 0.41%.

6.4.4 Recovery

The CE method developed was applied for the determination of oxytetracycline in a pharmaceutical product. Recoveries were calculated from an antibiotic tablet containing the following ingredients; Oxytetracylcine Dihydrate (250 mg), Lactose (17.66 mg), Starch Maize (21 mg), Povidone (10 mg), Sodium Lauryl Sulphate (3.33 mg), Stearic Acid (15.66 mg) and Sodium Starch Glycollate (15.66 mg). Recoveries were examined at 75%, 87.5%, 100%, 112.5% and 125% OTC Label Claim.

Stock Standard and Working Standard Solutions

A 2,512 mg / L stock OTC solution was prepared by weighing 0.2512g OTC dihydrate, dissolving in buffer, transferring to a 1 L volumetric flask and making up to the mark with electrolyte. To generate a series of working standard solutions containing 75% to 125% OTC, a series of concentrations were

prepared. 7.5, 8.75, 10, 11.25 and 12.5 ml of the stock OTC solution were pipetted into 100 ml volumetric flasks labelled 75%, 87.5%, 100%, 112.5% and 125%. These standards solutions were made up to the mark with electrolyte.

(ii) Placebo Solutions

A placebo solution was prepared by weighing 0.01764g Lactose, 0.02123g Starch Maize, 0.01011g Povidone, 0.0033g Sodium Lauryl Sulphate, 0.0157g Stearic Acid and 0.0156g Sodium Starch Glycollate dissolving in electrolyte, transferring to a 1 L volumetric flask and making up to the mark with electrolyte. The placebo solution was placed in an Ultrasonic bath for 30 min to degas and filtered through a Gelman Acrodisc (0.45μm) filter.

(iii) Spiked Placebo Samples

Five placebo solutions were prepared by weighing an appropriate amount of each ingredient and placing in separate 100 ml beakers. To each of these was added 0.0187g, 0.0218g, 0.02512g, 0.0282g and 0.03125g of OTC dihydrate. These solutions were dissolved in electrolyte and transferred to 1 L volumetric flask. The final volume was made up to the mark with electrolyte, placed in an Ultrasonic bath for 30 min to degas and filtered through a Gelman Acrodisc (0.45μm) filter.

The placebo was injected to see if there was any response at the migration time of the OTC analyte.

Result: As no detector response was apparent, six injections of each OTC standard and spiked placebo samples were made. % Recovery was calculated by comparing expected and found peak areas and peak heights.

Result: Peak area and height values are higher for spiked placebo sample than those obtained from injections of standard OTC solutions indicating that the method developed may not be appropriate for this pharmaceutical product. % Recovery = 107.94%. This result was thought to be very high and thus this procedure was not repeated therefore statistical analysis could not be carried out.

6.4.5 Specificity

Each product ingredient was prepared ten times their concentration label claim to investigate the selectivity of the method developed.

Ingredient Preparations

0.01760g Lactose, 0.02133g Starch Maize, 0.0099g Povidone, 0.0031g Sodium Lauryl Sulphate, 0.0162g Stearic Acid and 0.0158g Sodium Starch Glycollate were weighed, dissolved in electrolyte, transferred to separate 6 x 100 mL volumetric flask and made up to the mark with electrolyte. Each solution was degassed for 30 min and filtered as before. Each solution was injected using the same operating conditions as before.

Result: A large response appeared when 1000 mg / L povidone was injected at the same retention time as OTC.

6.4.5.1 Separation of OTC and PVP

(i) Electrolyte Preparation

10 mM SDS was weighed and added to 250 ml of the electrolyte solution. The solution was then transferred to a 1 L volumetric flask and made up to the mark.

(ii) Capillary Conditioning As in Section 6.3.3

(iii) Standard Solutions

Three standard solutions containing 1000 mg / L PVP, 250 mg / L OTC and a mixture of both were prepared. 0.0010g PVP was weighed, dissolved in electrolyte, transferred to a 100 mL volumetric flask and made up to the mark. 0.0025g OTC Dihydrate was weighed, dissolved in electrolyte, transferred to a 100 mL volumetric flask and made up to the mark. 0.0010g of PVP was weighed and added to 0.0026g OTC Dihydrate, dissolved in electrolyte, transferred to a 100 mL volumetric flask and made up to the mark. Injections of each solution were performed (n=3).

Result: The migration of PVP was altered upon addition of the surfactant from approximately 5 min toapproximately 6 min whereas the OTC response remained unaffected. Recovery studies were then repeated with SDS in the electrolyte and final recovery was found to be 100.01%.

CHAPTER SEVEN

POVIDONE ANALYSIS

7.1 Introduction

Povidone was chosen as the next analyte as the CE method developed for OTC also generated responses for PVP. The aim of this study was to develop and validate a method for the determination of PVP so that the method could be employed for the simultaneous analysis of both OTC and PVP contained in the same product.

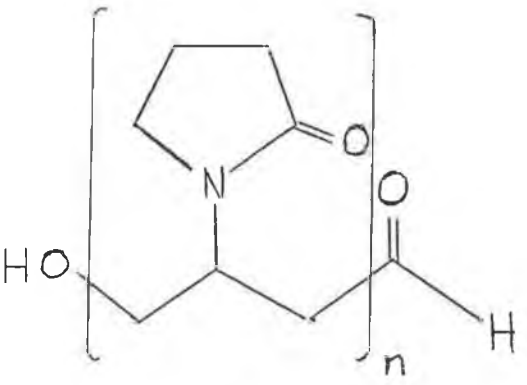


Diagram 7.1 Povidone $n = 4 - 50$

Povidone, (poly(vinyl)pyrrolidone), (PVP), is a low molecular weight polymer (average molecular weight 100,000 to 400,000 atomic mass units (a.m.u.)). It is used in pharmaceutical products as a binding agent. Povidone contains both ionic and hydrophobic characteristics and may be sparingly soluble in aqueous solutions. This compound has been extensively used for a variety of functions both in CE and HPLC applications. Reports indicate that it is used as a sieving matrix, binding agent, viscosity builder, electroosmotic flow suppressor and complexating agent.

7.2 Literature Review

A literature search indicates that PVP has not been determined previously by capillary electrophoresis. It has been used in CE applications but no CE method has been validated for the determination of PVP itself. PVP is commonly added directly to the electrolyte in CE, or can be used to coat the inner capillary wall depending on the matrix and chemical structure of the compounds to be analysed. A CE technique[111] for a single run determination of iodine and iodide in commercially available antiseptic such as povidone-iodine is reported. Tris-HCl electrolyte at pH 8.5 with direct UV detection at 214 nm was used. Reponses due to povidone occurred at 4 min. The method was validated only for I_2 and I^-.

Povidone is used as a viscosity builder[112] for an electrically conducting interface for a biomedical sensor. This paper discusses bioadhesive performance and demonstrates povidone's suitability for its purpose. Oligonucleotides[113] have been analysed by CE coupled with electrosrpay spectrometry in a PVP matrix. The coated fused silica capillary was filled with an aqueous solution of PVP that behaved as a pseudo-phase so that oligomers with hydrophobic modifications were retained longer than their unmodified analogs. PVP has also been used to influence the mobilities of diastereomeric derivatives of enantiomers separated by CZE[114]. PVP was added to a 30 mM phosphate electrolyte prior to pH adjustment and capillary equilibrated to ensure baseline stability. The spreading of mobility values by interaction with PVP was examined as a function of the electrolyte's viscosity. PVP has also been used as a sieving matrix[115] for the determination of DNA fragments by CE. The effects of PVP concentration and electric field strength on separation efficiency were examined. A selection of fragments were dissolved both in PVP and electrolyte and it was demonstrated that PVP improved separation efficiency better than electrolyte.

A qualitative PVP screening technique was devised by Trimpin et al.[116] This technique involved examining PVP's biodegradability by Matrix-Assisted Laser Desorption-Ionisation Time-Of- Flight Mass Spectrometry (MALDI-TOF-MS). Results indicate the degradation course of PVP. PVP has been used as an electroosmotic flow suppressor in the CGE[117] determination of cyanine-labelled amino acid enantionmers modified with cyclodextrin. The separation of six amino acid enantiomers could be achieved by using a 1% PVP solution containing 70 mM γ-cyclodextrin. It was also shown that PVP does not effect the selectivity of optical isomers.

PVP has been used as a complexating agent in the CZE[118] separation of ten nitrophenols. PVP was present at a concentration of 2.5% ($^{w}/_{v}$) in the electrolyte. Nitrophenols were detected in the 20-80 ppb range. Another CEC[119] method for determining chiral proteinogenic α-amino acids included PVP in the electrolyte to optimise separation between chemically similar compounds. PVP 0.5% ($^{w}/_{v}$) was added to

a 20 mM sodium citrate buffer containing 9% 2-propanol and 1% tert.-butylmethyl ether. PVP concentration was optimised to enhance stereoselectivity of the structures analysed. PVP has been used to shield sepharose samples in order to prevent the matrices from binding foreign proteins in their analysis by HPLC.[120]

7.3 Method Development

7.3.1 Confirming PVP Response

Electrolyte: 10 mM Tris-HCl, 10 mM SDS, pH 7

Sample: 1000 mg / L PVP

Operating Conditions: As per Section 6.3.3

(i) Electrolyte Preparation

1.210g Tris and 10 mM SDS were weighed and dissolved in ultrapure water, transferred to a 1 L beaker containing approximately 900 ml ultrapure water and stirred with a glass rod. The electrolyte pH was adjusted to 7 upon addition of 1 M HCl drop wise with a Pasteur pipette.

(ii) Stock Standard Solution

A standard solution of PVP was made by weighing 0.0099g of PVP solid, dissolving in electrolyte, transferring to a 100 ml volumetric flask and making up to the mark. Injections of 1000 mg/L were made, (n=3).

Result: An assymmetric response was observed at approximately 5 min.

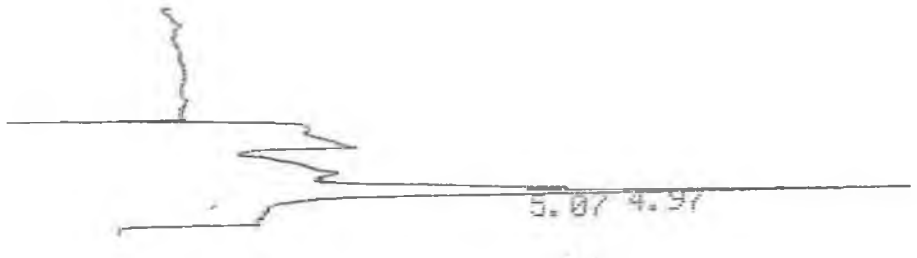


Diagram 7.2 Initial PVP Response

Due to the shape of peaks obtained from initial responses, it was decided to examine the effect of adding an organic solvent to see if peak shape could be improved. Organic solvents chosen were methanol and acetonitrile. These solvents are routinely used to improve peak shape in CE, as discussed earlier Section 5.2, Section 6.2.

7.3.2 Addition of Organic Solvent

Electrolyte-Organic Solvent Preparation

25, 50 and 75 ml methanol and 25, 50 and 75 ml acetonitrile were added to 6 x 250 ml of electrolyte. These solutions corresponded to 10%, 20% and 30% organic solvent ($^{v}/_{v}$). Solutions were placed in an Ultrasonic bath and filtered as before. Three injections of 1000 mg / L PVP solution were made using each of the six electrolyte systems.

Result: Additions of methanol resulted in slower migration times and poorer peak shape, however upon additions of acetonitrile, peak shapes were symmetric and migration times unchanged. Acetonitrile, 10% (v/v) was added to the electrolyte for all future investigations.

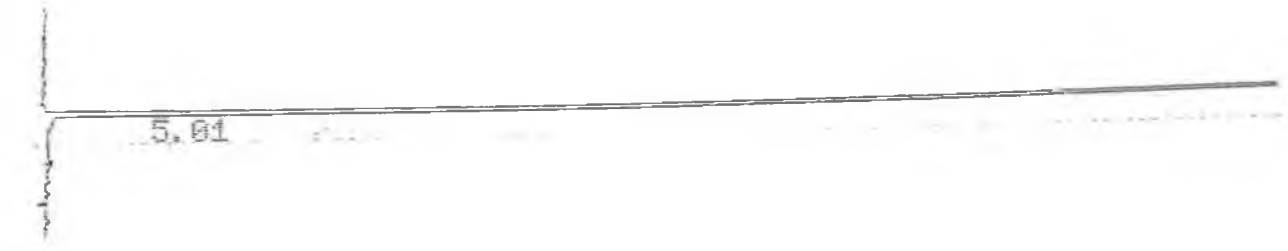


Diagram 7.3 Symmetric PVP Peaks with ACN

7.3.3 Electrolyte pH Variation

(i) Electrolyte Preparation:

1.210g Tris and 10 mM SDS were weighed and dissolved in ultrapure water. This solution was transferred to a 1 L beaker containing approximately 900 ml ultrapure water and stirred with a glass rod.

Acetonitrile (25 ml) was added to 250 ml of electrolyte. Electrolyte pH was adjusted to 3, pH 5 and pH 10 upon addition of 2 M HCl drop wise with a Pasteur pipette.

Result: No response was observed using these conditions.

Subsequent injections of electrolyte and sample using the same conditions were made however no response was obtained. This result would suggest that conditions inside the capillary have changed. Due to the analytes polymeric nature, it is possible that sample may have adsorbed onto the capillary wall. In order to try to remove sample from inside the capillary, the capillary was washed sequentially with isopropanol for 30 min, ultrapure water for 30 min, 1 M NaOH for 20 min and ultrapure water for 30 min followed with electrolyte for 30 min.

Three injections of electrolyte alone were performed to ensure all sample had been removed.

Result: No response was observed at the migration time of the sample thus this was thought to confirm adhesion of the analyte onto the capillary wall.

A new capillary was created using the previous method however it was necessary to coat the inside of the capillary wall with a suitable wall modifier to ensure that sample would travel unhindered through the capillary.

7.3.4 Capillary Wall Modification

Cetyl Trimethyl Ammonium Bromide (CTAB) is a cationic surfactant previously reported in capillary wall modification[26-29].

Electrolyte Preparation: As in Section 7.3.1

0.109g CTAB was added to previously prepared electrolyte solution and made up to the mark. To 250 ml of electrolyte was added 25 ml acetonitrile. The pH was adjusted to 7 upon addition of 1 M HCl drop wise with a Pasteur pipette. Electrolyte was placed in an Ultrasonic bath for 30 min and filtered as before.

Injections of 1000 mg / L PVP in electrolyte were made.

Result: No response was observed upon injection of PVP standard and it was thought that the EOF was reversed.

Another capillary was created using the previous method and electrolyte in order to regenerate PVP response. Injections of 1000 mg / L PVP in electrolyte were made, n = 3.

Result: Symmetric responses were observed at approximately 5 min.

A range of PVP concentrations were prepared in order to (a) establish whether sample adsorption is independent of amount injected.

7.3.5 Investigating Linearity

Working Standards: A 300 mg / L standard concentration was prepared by weighing 0.0030g of PVP solid, dissolving in electrolyte, transferring to a 100 mL volumetric flask and making up to the mark.

This standard solution was diluted sequentially with 1 in 2 and 1 in 10 dilutions to generate a series of standards containing total PVP concentrations of 300 mg / L, 150 mg / L, 75 mg / L, 30 mg / L, 15 mg / L and 3 mg / L. Each standard was injected three times.

Result: A linear response was obtained over the concentration range studied.

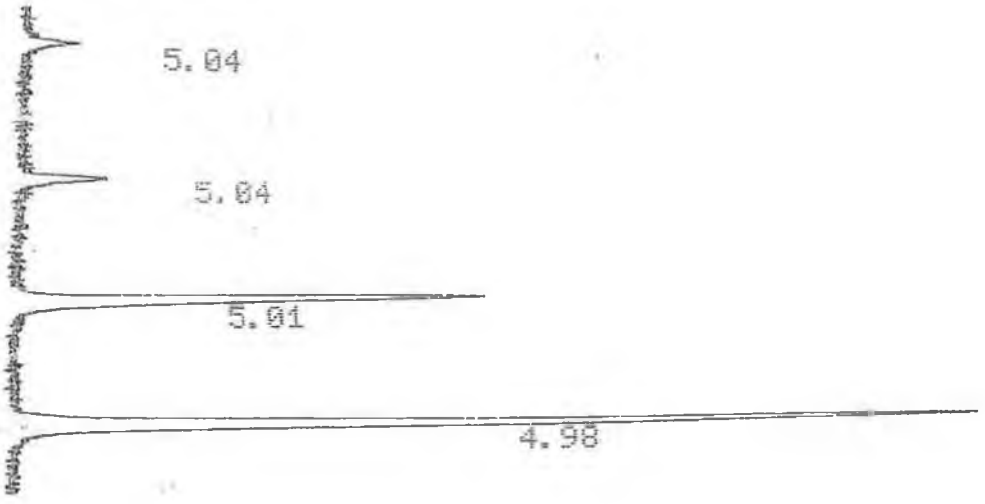


Diagram 7.4 Peaks Increasing Linearly with Concentration PVP

7.3.5.1 Limit of Detection

(i) Working Standards

A series of standards were prepared by pipetting 1, 2, 3, 4, 5, 10, 20 and 30 ml of the stock solution and transferring to separate 100 ml volumetric flasks. Each working standard was made up to the mark with electrolyte and injected onto the CE system. Each standard was injected three times.

Result: Linear responses were not observed as peak shape was dramatically altered and peak area or peak height values obtained were not consistent.

When injections were repeated, any response due to sample was not detected.

Another capillary was created as the previous capillary was thought to be irreversibly effected by sample adhesion. When the initial response due to PVP was made during validation of OTC method, product ingredients such as lactose and starch maize were injected prior to PVP. A similar capillary environment was created in an attempt to establish whether these ingredients had coated the capillary.

7.3.6 Effect of Products Ingredients

The creation of a new capillary and subsequent injections of product ingredients at similar concentration as before were performed. Each ingredient was injected six times followed by injections of PVP (n=3). It was thought that the presence of sample ingredients Lactose, OTC and Starch Maize had in some way modified the capillary wall that provided good PVP peak symmetry. When these injections were performed as before however, peak height and area values together with peak shape and migration time did not improve.

7.3.7 Electroosmotic Mobility:

The μ_{EOF} was determined upon injection of 2 mM 2-Naphthol (0.288g in Methanol) and was found to have a migration time at 6 min which is close to the migration time of the compound.

CHAPTER EIGHT

DISCUSSION

8.1 Benzoic Acid Analysis

Direct UV detection was used for the analysis of this compound as it intrinsically absorbs UV light at 215 nm. Analysis time at 5 min compares favourably with methods reported in the literature, Section 3.2. The calibration graph obtained demonstrated that the detector responded according to variations in concentration of analyte. Familiarisation with the CE instrument was established.

8.2 Inorganic Cation Analysis

Indirect photometric detection at 215 nm was used for the analysis of inorganic cations employing three different electrolyte systems. As tromethamine acquires a positive charge at pH 4.8, it was thought that it would be detected as a cation with similar electrophretic behaviour as the ammonium cation reported in the literature[73-85]. Electropherograms using the copper sulphate and phosphate electrolytes displayed responses at the migration time of Na^+ ion, 2.5 min. Following injections of ultra-pure water only, these responses remained. As all standard solutions were prepared with ultra-pure water, it was thought that there was a higher concentration of sodium in the water than expected. IC analysis however demonstrated that the concentration of sodium was acceptable. Non-linear behaviour was also noted using the phosphate electrolyte. This was possibly due to the build up of sodium residues on the capillary wall. Upon creation of a new capillary and application of the third electrolyte system, DDP, linear responses were obtained, free from interference. This electrolyte was deemed best for the analysis of tromethamine. The ammonium ion was detected at less than 5 min.

8.3 Tromethamine Analysis

8.3.1 Selection of Detection System

As tromethamine has no intrinsic UV response, indirect UV detection was necessary for the determination of the analyte within the CZE system. Indirect UV detection has been used previously for the detection of small inorganic cations. There are two methods of approach using this detection system. The first is to derivatise the compound into a form that allows direct UV detection. As these procedures

are laborious and often non-specific the most convenient method is to add a UV absorber to the electrolyte and thus facilitate the indirect detection of the compound in the CE system. A UV absorber in the buffer will generate a high background absorbance and as the analyte passes the detector, a proportional amount of the UV absorber will be displaced and cause a dip in the background absorbance. The main consideration in the choice of UV absorber was to match its mobility with tromethamine.

8.3.2 Choosing a Suitable Electrolyte

(i) Copper Sulphate Electrolyte: The first buffer system chosen for initial determination of tromethamine was a Copper (II) Sulphate-18-Crown-6-Ether-Formic Acid buffer because it has been used previously for the analysis of cations, Section 4.2. Results from inorganic cation analysis exhibited a response for the ammonium cation at 5 min. The pH of the buffer was adjusted to 5 with formic acid as this is one unit above the pKa of the analyte. The addition of 18-Crown-6-Ether was chosen as the UV absorbing species and using indirect detection with reversed polarity injections of the non-UV absorbing analyte generated a response at approximately 5 min. Gravity injection was chosen as this is reported to be the simplest injection form and the amount injected does not depend on the electrophoretic mobility which was considered appropriate for the initial determination of the analyte. All electropherograms obtained showed responses at 2.5, 3, 3.5, and 10 min as well as the response at 5 min due to sample. No attempt was made at this stage to determine the identity of these peaks as it was more convenient to choose a more suitable electrolyte.

(ii) Imidazole Electrolyte: The second buffer system was chosen on the basis that imidazole would act as the UV absorber and has been previously reported for inorganic cation analysis. Both imidazole and tromethamine are structurally similar as they both form ammonium ions at low pH. Results show responses at 5 min however, peak splitting is observed. Differences in degree of peak split are observed upon injecting different concentrations of analyte. The general trend follows that on increasing

concentration, the calculated peak height ratio decreases, within the response observed. From electropherograms obtained at higher concentrations, the second peak appears as a shoulder instead of an individual peak as seen with lower concentrations. It was thought that the ionisation of the component was different to that observed using copper sulphate. The pH of the electrolyte could have been altered to determine the optimum pH in order improve peak shape.

As peak symmetry was acceptable using copper sulphate electrolyte, it was thought that the presence of 18-Crown-6-Ether was in some way responsible for the modification of the capillary wall. Addition of the inclusion complexating agent at the same concentration as the copper sulphate electrolyte however did not improve peak shape. All electropherograms obtained showed responses at 2.5, 3, 3.5, and 10 min as well as the response at 5 min due to sample.

8.3.3 Identification of Unknown Peaks

When injections of tromethamine, ultra pure water and electrolyte were performed using both copper sulphate and imidazole electrolytes, peaks consistently appeared with average migration times at 2.40, 2.96, 3.48 and 9.13 min, (n=10). It was necessary to establish the nature of these responses before another buffer system was chosen. As both electrolytes have been successfully used for the analysis of cations previously, and as standard tromethamine solutions were prepared with ultra pure water (copper sulphate) and with buffer as solvent (imidazole) it was considered the presence of these responses were possibly due to cations in water. Cation standards were injected into the CE system using imidazole as electrolyte and identified as K^+, (2.4 min), Ca^{2+} (2.96 min) and Na^+ (3.48 min). A neutral marker, 2-naphthol, was introduced into the system and migrated at approximately 10 min therefore the unidentified peak at 9.13 min was thought to be due to the movement of the bulk electrolyte.

8.3.4 DDP Electrolyte

This electrolyte was the most successful for analyzing inorganic cations as interferences previously found were now absent from all electropherograms and detector showed sharp symmetrical responses. Responses due to tromethamine at approximately 5 min were also acceptable for these reasons.

8.3.5 Electrolyte Composition

Because DDP functions as a UV absorber, it was decided to investigate the role of the organic additive 18-Crown-6-Ether in the buffer system. Unlike the copper sulphate and imidazole electrolytes, on injection of sample, electrolyte or standard, no other peaks appeared other than that due to tromethamine. The DDP buffer was prepared without crown ether and injections of sample made. It was found that no difference in retention time was seen and that the capillary wall was not altered by the presence / absence of the additive. It was decided to omit the addition of crown ether to the electrolyte in subsequent investigations.

8.3.6 Effect of Electrolyte pH

The degree of ionisation of a species present in the electrolyte system depends on the pH of the solution. By altering the pH of the DDP buffer with α-hydroxyisobutyric acid, the flow rate (EOF) inside the capillary was controlled. Low buffer pH values were thought to enhance the protonation of the monoacidic amine giving it an overall positive charge however as tromethamine contains a weakly ionisable group it was expected to be charged within a narrow range above or below its pKa value. The pKa value is 4.8 and results demonstrate that it is charged within pH 3.5 – 6.5. The EOF was apparent in the range pH 4.5 – 11. Optimum mobility was observed at pH 5.2.

8.3.7 Effect of Voltage Variation

Voltage variation affects the electrophoretic and electroosmotic mobilities in the CE separation. This results in altering the conducting medium in the capillary in terms of changing current, temperature inside the capillary. In order to determine the optimum operating voltage for the electrophoretic separation, variation of this control mode was achieved in the mid-voltage range from 15 kV to 25 kV

in increments of one. Changes in voltage can affect Joule Heat generation and thus a capillary cooling jacket was applied during all voltage variations. Due to the use of ambient temperature conditions, the generation of any Joule Heat was thought to be adequately dissipated as there was no significant change in peak symmetry and thus the analyte was thought to be chemically stable under conditions of increasing voltage. Analysis time was reduced from 5 min to 3 min using an optimum voltage of 20 kV chosen from an Ohm's Law Plot.

8.3.8 Effect of Temperature Variation

Direct automated control of capillary temperature was not an option using the Dionex CES 1 Model. Electrophoretic mobility is best at close to mid-day laboratory temperatures, approximately 17^0C Fig 31. There is no evidence to suggest that the structural nature of the sample or the injection volume was in any way altered with changing temperatures as peak response was maintained. As temperature changes usually affect buffer viscosity, these measurements could have also been taken.

8.3.9 Effect of Ionic Strength Variation

Ionic strength of the electrolyte has significant effects on solute mobilities and separation efficiency. To determine the effect of altering ionic strength of the DDP electrolyte, concentrations in the range 0.312 – 5 mM were examined. As the temperature was not controlled, the EOF increased with ionic strength, as expected. It was also expected that the migration time of the analyte would increase with increasing ionic strength, by increasing the ionic strength, the migration time decreases by approximately 2 min in the range examined. Concentrations above 5 mM could not be examined as the instrument automatically shut down due to excessive heat inside the capillary. As this instrument did not have a temperature control system, it was impossible to further examine effects of increasing ionic strength.

The method developed was validated in accordance with ICH3[111]. The parameters for method validation include linear range, linearity, limit of detection, repeatability, precision, accuracy, specificity and recovery.

8.3.10 Linearity, Linear Range and Limit of Detection

The linear range of the method was investigated from 1-50 mg / L which accommodates the required 80-120% of the test concentration (12 mg / ml). A wide concentration range was investigated to determine at which concentrations the method deviated from linearity. From table (vi), it can be observed that concentration below 2.5 mg / L and above 25 mg / L deviated from linearity. The linearity of the method was examined in the range 5 mg / L to 25 mg / L, n = 6. Calibration curves compiled gave a correlation coefficient 0.9992 ± 0.029 with an intercept of $-2.9 \times 10^{-2} \pm 0.017$ and slope 0.098. The sensitivity of the system was found to be 0.98 absorbance units / mg^{-1}. The lowest operating limit of detection was found to be 2.5 mg / L, as responses below this concentration remained relatively constant and thus independent of changing concentrations.

8.3.11 Repeatability

System repeatability was assessed in terms of run to run variation between responses obtained from similar and different vial positions by the same operator. In order to examine the repeatability of the method ten successive injections of 10 mg / L standard from the same vial position were carried out. Standard deviation and relative standard deviation values were calculated for peak height and found to be 0.14 and 2.01%, respectively. Variations between injections collected from samples located in different vial positions displayed standard deviation values calculated by peak height data was found to be 0.360 with an relative standard deviation of 3.43 %.

8.3.12 Precision and Accuracy

The precision and accuracy of the method was determined by conducting intra- and inter-assay variability tests. Concentrations in the range 5 to 25 mg / L were prepared over a series of four days and

analysed. Variations within and between days were calculated at n = 6. Standard deviation and relative standard deviation results were calculated form peak height data in order to examine the co-efficient of variation of the method developed. Average RSD was found to be 0.42% and the average deviation was found to be 1.62%. The average deviation is inflated owing to the relatively poor deviation obtained for the concentration of 5 mg / L. The remaining four concentrations gave an average of 0.94%.

8.3.13 Specificity

The method developed was applied to a commercial eye-care product containing tromethamine at 12 mg / ml in order to assess the specificity of the method. Injections of a placebo solution containing other product ingredients did not generate detector response at the retention time of tromethamine. The selectivity of this method could further be tested if the method was applied to a matrix containing tromethamine and its synthetic precursors, degradation products or excipients. A small peak was observed at 1.91 min in all electropherograms and was thought to be due to sodium residues remaining from capillary conditioning. This response did not interfere with the response due to analyte.

8.3.14 Recovery

Recovery was calculated from injecting spiked placebo and standard solutions and comparing the peak heights obtained. Initial recovery studies were low at approximately 98% and it was thought that the viscosity of the sample solution had in some way affected the inner capillary wall. Standard solutions, prepared with ultrapure water and in sample matrix, were tested with a viscometer however no significant variations in measurements were observed. Mean recovery was found to be 102.98 ± 2.59%

8.4 Oxytetracycline Analysis

8.4.1 Choice of CE Technique

Capillary Zone Electrophoresis was the method of choice for initial determination of the analyte in the pharmaceutical matrix. It was thought that on considering the structure of OTC which consists of a

tricarbonyl system attached to polar constituents, electrophoretic mobility would readily be achieved on the basis of charge:mass ratio.

8.4.2 Selection of Detection System

Direct UV detection was an obvious method of choice for this compound. A UV scan of various concentrations of OTC in methanol yielded a maximum wavelength at 270 nm. Previous CE methods have reported using a wavelength of 254 nm. For initial investigations, this was the wavelength chosen. A Beer-Lambert Plot was constructed which displayed linearity up to concentrations of 60 mM.

8.4.3 Choice of Suitable Electrolyte

Due to availability of tromethamine (Tris), and its prevalent use as an aqueous electrolyte in both CZE and MECC, it was therefore decided to examine its suitability as the buffer for initial determination of OTC response. Tris electrolytes are alkaline electrolytes at pH 10.4. However, it was decided to lower buffer pH to ensure OTC was charged in the capillary. Due to OTC instability in strongly alkaline media and degradation in highly acidic solutions it was decided to reduce the pH of the electrolyte to neutral. Tris has no UV absorbing properties due to its chemical structure, as has already been discussed, and thus it was considered that there would be no interference from the background electrolyte during analysis. Oxytetracycline was found to be soluble in this electrolyte system that further enhanced the choice of electrolyte.

Upon application of the Tris-HCl electrolyte at pH 7, initial response due to OTC was noted at approximately 5 min. Peak efficiency and peak shape were poor.

8.4.4 Electroosmotic Flow

Injection of 2-naphthol (5mM) onto the system gave an indication of the migration of the bulk electrolyte. The migration of the EOF at 3 min was close to the analyte migration at 5 min. Suppression of the electroosmotic flow has been reported using various methods however, it was decided to examine the effect of varying EOF dependent factors before a decision was made to supress the EOF.

8.4.5 Peak Efficiency

CE is reputed for lack of sensitivity primarily because of the length of the detector cell window, as A = ε c t. Thus, from the Beer-Lambert Law, the absorbance is directly related to the cell path length. The fused silica capillaries used in this report consist of a cell path length of 0.5 cm. The larger the cell path length value, the lower the concentration of analyte detected. Poor peak efficiency and low detector response were observed in initial investigations. When the operating wavelength was changed from 254 nm to 210 nm in an attempt to enhance detector response, a decrease in electrophoretic migration inside the capillary was observed. When wavelengths above 254 nm were used, no detector response was observed. The operating wavelength was maintained at 254 nm.

Detector response is proportional to the amount of sample loaded onto the capillary and therefore this response dependent parameter was investigated subsequently. Injection volume was doubled by changing program conditions and increasing the sample load from 0.814 nl to 1.704 nl by injecting for a further 10 sec. Detector response generated peak heights double the value of original heights for the 200 mg / L standard. Changing the sample solvent from methanol to electrolyte resulted in producing sharp, symmetric peaks.

The selection of 1 M HCl and 0.1 M HCl as sample solvent caused poor peak shapes probably due to acid degradation of OTC. This could have been confirmed by injecting EOTC standards.

8.4.6 Electrolyte Composition

The pharmaceutical matrix selected to validate this method was known to contain non-aqueous ingredients. Organic solvents were added to the electrolyte in the hope that they would improve the solubility of other product ingredients in the buffer. Organic solvents are routinely used to alter the mobility of one analyte to improve resolution or enhance selectivity. Methanol was added to electrolyte systems in other OTC methods as discussed earlier thus, the impact of methanol addition upon electrophoretic and electroosmotic behaviour was investigated in this method. Organic solvent addition

can also alter electroosmotic flow depending on the type and quantity of solvent used. Other system effects include viscosity, dielectric constant and zeta potential.

8.4.6.1 Effect of Methanol Addition

The effect of adding methanol to the aqueous electrolyte was established in terms of monitoring both electroosmotic and electrophoretic mobilities. OTC migration was reduced by approximately 7 min and the migration of the EOF reduced by approximately 5 min with 40% ($^{v}/_{v}$) methanol, Fig 42. Electrophoretic and electroosmotic speed was hindered upon addition of the methanol as expected.

8.4.6.2 Effect of SDS Addition

Addition of surfactants to electrolyte systems for OTC and tetracycline analysis in other MECC applications have been reported and thus the effect of adding SDS to the tris buffer was examined. SDS has a CMC value of 8.27 mM. No advantage was offered for analysis of OTC in this electrophoretic analysis at this stage of development. SDS addition caused electrophoretic and electroosmotic mobility reduction.

8.4.7 Effect of Electrolyte pH

As the pH increases electroosmotic flow increases as the charge on the inner capillary wall increased. The degree of ionisation of OTC is also altered by increasing pH of the buffer, as its speed through the capilliary is increased by approximately 3 min over the pH range examined. Initial Buffer pH variations involved selecting a 200 mg / L standard OTC solution and injecting at various pH values at n = 3. Electrolyte pH values from pH 7 to pH 3 were also carried out however, peak shape deteriorated possibly due to analyte degradation. At extreme acidic conditions, peak splitting was obvious and the response due to OTC was no longer apparent. These results demonstrate that the electrophoretic mobility of this anion increased as it passed through the capillary unhindered, as the pH of the buffer increased, as was expected.

8.4.8 Effect of Voltage Variation

Mid-range voltages (15 - 25 kV) were examined by programming the instrument accordingly. Electrophoretic and electroosmotic mobilities were monitored as a function of varied applied voltage. Application of a cooling jacket above 20 kV was essential during operation as high applied voltages caused increased currents which resulted in Joule heating in the capillary. The current produced did not exceed 60 μA. It is evident that altering the applied voltage affected both electrophoretic and electroosmotic mobility. Results obtained follow those expected as higher voltages reduced analysis times. Electrophoretic mobility is affected to a greater extent than the electroosmotic mobility as the analysis time is reduced by over 5 min as the applied voltage is increased from 15-25 kV. Change in the migration of the neutral species was substantially less and amounted to less than 3.5 min over the applied voltage range.

8.4.9 Effect of Temperature Variation

The effect of varying temperature was not carried out during this procedure as inconclusive evidence was obtained from results displayed compared to the analysis of tromethamine. Temperature fluctuations inside the hood of the instrument could not be accurately followed upon application of a thermometer only. Results obtained could not be directly correlated with laboratory humidity. It may therefore be necessary to monitor smaller fluctuations both outside and inside the instrument and over longer periods of time to truly appreciate and understand the effect of laboratory temperature on analyte mobility through the capillary, if any.

8.4.10 Effect of Ionic Strength Variation

Ionic strength of the electrolyte is related to the viscosity of the electrophoretic and electroosmotic mobility. The concentration of electrolyte was varied from 10 mM to 100 mM. Ionic strengths greater than 70 mM caused a build up of current. The highest current recorded was 71 μA. The CE system usually shuts down when currents between 60 to 70 μA are produced. Above 50 mM tris, the electroosmotic flow remains almost unaffected by the change in electrolyte concentration. The

electrophoretic mobility of OTC reduces as ionic strength increases by approximately 2 min on increasing from 10 to 70 mM. The fastest rate of analyte mobility was seen at tris concentration of 70 mM however electrolyte concentration of 50 mM was deemed best for future procedures.

8.4.11 Linearity, Linear Range and Limit of Detection

The linear range of the method was investigated from 6.25 - 400 mg / L which accommodates the required 80-120% of the test concentration (250 mg / ml). A wide concentration range was investigated to determine at which concentrations the method deviated from linearity. Concentrations below 6 mg / L and above 200 mg / L deviated from linearity. The linearity of the method was examined in the range 12.5 mg / L to 200 mg / L, n = 6. Calibration curves compiled gave a correlation coefficient 0.9994 with an intercept of 1.7×10^{-2} and slope 0.055. The sensitivity of the system was found to be 0.55 absorbance units / mg^{-1}. The lowest operating limit of detection was found to be 6 mg / L, as responses below this concentration remained relatively constant and thus independent of changing concentrations.

8.4.12 Repeatability

System repeatability was assessed in terms of run to run variation between responses obtained from similar and different vial positions by the same operator. In order to examine the repeatability of the method ten successive injections of 200 mg / L standard from the same vial position were carried out. Standard deviation and relative standard deviation values were calculated for peak height and found to be 0.28 and 2.62%, respectively. Variations between injections collected from samples located in different vial positions displayed standard deviation values calculated by peak height data was found to be 0.32 with an relative standard deviation of 2.99 %.

8.4.13 Precision and Accuracy

The precision and accuracy of the method was determined by conducting intra- and inter-assay variability tests. Concentrations in the range 12.5 mg / L to 200 mg / L OTC were prepared over a series of four days and analysed. Variations within and between days were calculated at n = 6. Standard

deviation and relative standard deviation results were calculated form peak height data in order to examine the co-efficient of variation of the method developed. Average RSD was found to be 0.415% and the average deviation was found to be 2.82%.

8.4.14 Recovery

The method developed was applied to an antibiotic tablet containing oxytetracycline at a label claim of 250 mg ($^{w}/_{w}$). Injections of standard solutions (75-125% label claim), placebo solutions containing each product ingredient (concentration label claim) and spiked placebo solutions containing 75-125% label claim of OTC were made. No response was observed at the migration time of OTC. Recovery was calculated to be 107.94%.

8.4.15 Specificity

In order to assess the specificity of the method injections of a placebo solution containing other product ingredients at ten times their label claim were performed. A large response appeared at the same migration time of OTC. This response was due to povidone (1000 mg ($^{w}/_{w}$)). Upon addition of 10 mM SDS the PVP response slowed to 5 min thus allowing the recovery studies to be repeated. Final % recovery was calculated to be 100.01%. As ICH3 guidelines stipulate the method should be selective for the analyte to be examined in ten times the concentration of each other ingredient found in the product, the method was deemed specific for the analysis of OTC in this product.

8.5 Povidone Analysis

8.5.1 Choice of CE Technique

Highly ionisable groups in PVP suggest that the analyte will undergo electrophoretic mobility within the capillary. Capillary Gel Electrophoresis would seem to be the most appropriate CE technique as solutes are separated due to size as analytes migrate through the pores of a gel filled capillary. Polymers with similar monomer units but differing chain lengths can thus separate on the basis of molecular sieving as they migrate with constant but different velocities. During the CZE analysis of oxytetracycline using an

aqueous buffer and fused silica capillary however, a response was observed for PVP. The technique had to be adequately modified to facilitate unhindered mobility of the analyte through the bare capillary by choice of a suitable capillary wall additive.

8.5.2 Selection of Detection System

PVP is strongly UV absorbing confirmed by the presence of conjugated double bonds. UV absorbance at 210 nm generated weak asymmetric signals at 8 min. A UV scan of PVP showed multiple UV absorbance thus it was decided to remain using 210 nm. By programming the instrument to detect over a series of wavelengths, reponses due to analyte could have been improved with regard to peak shape and height / area obtained. Using this wavelength however, responses were obtained for concentrations as low as 3 mg / L PVP, which is comparable to limit of detections obtained for both tromethamine (2.5 mg / L) and oxytetracycline (6.25 mg / L).

8.5.3 Choice of Suitable Electrolyte

Tris-HCl buffer was adequate for the determination of the analyte in the matrix concerned. The addition of SDS has already been demonstrated upon separation of OTC and PVP during selectivity studies to alter the electrophoretic mobility of the compound significantly but at the expense of peak efficiency. No real advantage has been observed with addition of SDS. Injections could have been performed without SDS to examine whether the analysis time could have been reduced from 5 min. Other commonly used electrolytes e.g. phosphate, borate, acetate at pH 7 could also have been employed to estimate the best electrolyte for this determination. Other electrolyte systems have not been attempted for the analysis of the analyte due to solute interaction with the capillary wall. Aqueous or non-aqueous electrolyte systems must be modified with the selection of a suitable additive to facilitate reproducibility from run to run and also to ensure that the analyte does not undergo chemical modification inside the capillary. Initial determination of PVP response that displayed linearity could not be repeated upon

further injections even though separation conditions were not altered. This was thought to be due to solute adhesion onto the capillary wall.

8.5.4 Electroosmotic Flow

The flow of neutral solutes in the capillary was achieved upon injections of 2-naphthol to examine the electroosmotic mobility in the capillary but in addition to assess the alteration of the environment on the capillary wall. Injections were performed on damaged and newly created capillaries before and after repeated injections of PVP. Results indicate however that no significant effect had occurred as the migration of the neutral species had not been altered. It is evident that the analyte has experienced chemical degradation due to declining peak symmetry and detector response even though electrophoretic migration did not appreciably change.

8.5.5 Organic Solvent Addition

The effect of organic solvent addition to the electrolyte system was necessary to improve peak symmetry. Methanol was added from 10-40% ($^{v}/_{v}$) but displayed no meaningful benefit. The addition of acetonitrile, 10-40% ($^{v}/_{v}$), on the other hand greatly improved peak shape. Acetonitrile at 10% ($^{v}/_{v}$) was therefore added to the buffer for subsequent analysis.

8.5.6 Electrolyte pH Variation

Using a low buffer pH the internal capillary wall is further protected with a layer of H^{+} ions that prevent analyte-wall interaction. As the pH of the electrolyte was lowered to 3, no response was observed for PVP injections. It may be assumed that the analyte is uncharged at low pH. Higher pH values were examined thereafter however similar results were seen, thus electrolyte pH is thus limited to near neutral conditions using this system.

8.5.7 Capillary Wall Modification

Many additives have been incorporated into buffer systems in CE to enhance analyte mobility through narrow bore capillaries. These additives provide wall coatings that prevent interaction between the

solute and capillary wall. Wall coatings affect electroosmotic flow and electrophoretic mobility to different degrees depending on the additive chosen. Alteration of the zeta potential and shielding of ionised silanol groups is necessary in this application. When a cationic surfactant is added to an electrolyte system at a concentration below its CMC, a monolayer of positive charges forms on the capillary wall. CTAB was added at a concentration of 1.5 mM that is below its CMC but injections of the analyte demonstrated that the electroosmotic flow had been altered as EOF reversal was evident as the direction and rate of electroosmotic flow depend on the polarity and magnitude of the zeta potential.

8.5.8 Effect of Products Ingredients

In order to establish the same conditions when PVP responses were repeatable, it was necessary to create the same electrophoretic environment. The creation of a new capillary and subsequent injections of product ingredients at similar concentration as before were performed. Each ingredient was injected six times followed by injections of PVP (n=3). It was thought that the presence of sample ingredients Lactose, OTC and Starch Maize had in some way modified the capillary wall that provided multiple, successive responses for PVP. When these injections were performed as before however, peak height and area values together with peak shape and migration time did not improve. Upon further injections of product ingredients, responses due to analyte were not obtained.

CHAPTER NINE

CONCLUSION

9.1 Summary of Results

The results of each CE technique developed for the three compounds investigated can be summarised as follows:

Table 9.1 Summary of Results

Analyte	CE Mode	Detection	Matrix	Electrolyte	Linear Range, Correlation Coefficient, LOD	Repeatability	Precision and Accuracy	Recovery	Analysis Time
(1)Tromethamine	CZE	Indirect UV at 215 nm	Eye-Care Pharmaceutical	5 mM DDP, 5 mM α-hydroxyisobutyric acid, pH 5.2	2.5 mg/L–25 mg/L, R^2=0.9999 LOD=2.5 mg/L	SD=0.14-0.36% RSD=2.01-3.43%	SD=1.62% RSD=0.42%	102.98%	3 min
(2)Oxytetracycline	MECC	UV Detection	Antibiotic Tablet	10 mM Tris-HCl,10 mM SDS, pH 7	12.5-200 mg/L, R2=0.9994, LOD=6.25mg/L	SD=0.28-0.32% RSD=2.62-2.99%	SD=0.41% RSD=2.82%	100.01%	3 min
(3)Povidone	MECC	UV Detection	Antibiotic Tablet	10 mM Tris-HCl,10 mM SDS, pH 7	3-300 mg/L	-	-	-	5 min

Operating Conditions (1) 50 µm (i.d.) x 50 cm (l), x 375 µm (o.d.), Detector Cathodic, (20kV), 100 mm for 30 sec, Temp. Ambient.
Operating Conditions (2) 50 µm (i.d.) x 50 cm (l), x 375 µm (o.d.), Detector Cathodic, (25kV), 100 mm for 40 sec, Temp. Ambient.
Operating Conditions (3) 50 µm (i.d.) x 50 cm (l), x 375 µm (o.d.), Detector Cathodic, (25kV), 100 mm for 40 sec, Temp. Ambient.

9.2 Benefits of this Study

The methods developed and validated in this work are beneficial to the pharmaceutical industry as they present rapid, precise, cost effective alternatives to conventional analytical techniques currently used to analyse these compounds.

The tromethamine method is free from interference, specific, has low limits of detection and good recovery, it does not use organic solvents and has an analysis time of 3 min. Previously reported methods suffer from interference and require derivatisation and solid phase extraction steps prior to injection. This method could be employed as a routine analytical technique for the determination of tromethamine in a variety of pharmaceutical products.

The method for determining OTC is faster and has a better recovery than any of the methods reported in the literature. It has low limits of detection and is accurate and precise. The method does not require extensive pre-injection steps and does not use organic solvents. This method could be employed as a routine analytical technique for the determination of oxytetracycline in a variety of pharmaceutical products.

The method for determining PVP provides a basis for evaluation by CE as there are currently no methods reported in the literature for this determination.

9.3 Future Investigations

The method developed for tromethamine could be applied to a variety of pharmaceutical products and thus contribute to the existing body of knowledge for determining this compound. As many commercially available OTC containing products also contain impurities, further work in this area could involve its determination by this method from a mixture of it's degradation products.

The method for determining PVP would benefit from the application of a suitable wall additive so that the analyte would travel freely through the capillary. The method could then further be developed and validated in accordance with ICH3 guidelines.

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APPENDICES

Table (i) Changing Electrolyte pH for Tromathamine Analysis

Volume of acid added (ml)	pH	Concentration tromethamine (mg / L)	Tromethamine migration (min)	EOF migration (min)
25.8	3.070	20	-	-
7.86	3.560	20	5.79	-
5.36	3.780	20	5.33	-
3.86	4.150	20	4.88	-
3.56	4.650	20	4.58	10.85
3.11	5.203	20	4.00	10.39
3.86	5.500	20	3.84	8.50
2.65	5.735	20	3.51	6.05
2.43	6.067	20	3.15	5.93
2.41	6.505	20	2.99	5.31
2.40	7.072	20	-	4.73
1.65	8.226	20	-	4.55
1.60	9.807	20	-	3.96
1.25	11.006	20	-	3.80

Table (ii) Electrosmotic/Electrophoretic Mobility as a Function of Electrolyte pH

PH	t_m (min)	t_{nm} (min)	ν_{EP}	μ_{EP} ($cm^2V^{-1}s^{-1} \times 10^4$)
4.650	4.58	10.85	6.245	15.612
5.203	4.00	10.39	7.611	19.027
5.500	3.84	8.50	6.977	17.442
5.735	3.51	6.05	5.921	14.802
6.067	3.15	5.93	7.367	18.417
6.505	2.99	5.31	7.233	18.082

Table (iii) Altering Voltage for Tromethamine Analysis

Voltage (kV)	Migration Analyte (min)	Migration EOF (min)	Current (μA)	Temperature (°C)	Power (mW)
15	3.83	5.84	2	18.7	20
16	3.62	5.54	2	18.9	22
17	3.42	5.25	3	19.0	24
18	3.25	5.01	3	19.3	28
19	3.10	4.78	3	19.5	38
20	2.96	4.59	3	20.0	40
21	2.72	4.17	3	20.3	41
22	2.58	3.96	3	20.5	42
23	2.41	3.75	3	20.7	46
24	2.28	3.57	3	21.0	51
25	2.23	3.41	4	21.3	54

Table (iv) Altering Temperature for Tromethamine Analysis

Temperature (°C)	Migration Analyte (min)	Peak Area (x 10^6)	Peak Height (cm^{-1})
15.1	4.21	1.534	10.05
16.3	3.86	1.610	10.10
17.2	3.00	1.527	10.00
18.5	3.14	1.401	10.20
19.4	3.58	1.498	10.15
20.5	4.19	1.550	10.05
21.4	4.01	1.670	10.10
22.6	3.98	1.590	10.10
23.5	3.82	1.611	10.05

Table (v) Altering Ionic Strength for Tromethamine Analysis

Concentration of DDPI Electrolyte (mM)	Analyte Migration (min)	EOF Migration (min)	Current (μA)
5	2.98	5.30	3
2.5	3.15	6.72	2
1.25	3.80	7.08	2
0.625	4.20	9.23	1
0.312	4.90	10.09	1

Table (vi) Linearity Studies for Tromethamine Analysis

Concentration of tromethamine (mg / L)	Migration time (min)	Average Peak Area (x 10^6)	Average Peak Height (cm^{-1})
0.315	3.23	0.086	0.51
0.625	3.30	0.106	0.58
1.25	3.23	0.170	0.50
2.5	3.27	0.427	2.10
5	3.29	0.492	3.98
10	3.30	0.918	5.95
15	3.33	1.588	8.10
20	3.16	2.200	9.95
25	3.13	2.848	12.00
30	3.11	3.387	11.90
35	3.09	3.648	12.00

Concentration (mg / L)	N	Correlation Coefficient	Intercept	Slope
5 – 25	6	0.9993	-2.63×10^{-2}	0.0959
5 – 25	6	0.9992	-4.04×10^{-2}	0.1013
5 – 25	6	0.9998	-2.07×10^{-2}	0.0989
5 – 25	6	0.9995	-3.25×10^{-2}	0.0987
Average ± S.D.	6	$0.9992 \pm 2.9 \times 10^{-2}$	$-2.9 \times 10^{-2} \pm 0.017$	0.098 ± 0.00

Table (vii) Precision and Accuracy for Tromethamine Analysis: Day One

Conc.Analyte (mg / L)	Average Peak Height (cm^{-1})	S.D.	X + S.D.	X - S.D.	% R.S.D.
5	2.65	1.144	3.769	1.481	4.35
10	6.825	0.6993	7.524	6.125	10.24
15	10.40	0.3829	10.782	10.017	3.68
20	12.525	0.206	12.731	12.319	1.64
25	15.00	0.6633	15.663	14.336	4.42

Day Two

Conc.Analyte (mg / L)	Average Peak Height (cm^{-1})	S.D.	X + S.D.	X - S.D.	% R.S.D.
5	2.20	0.3741	1.825	2.575	17.00
10	5.125	0.05	5.175	5.075	0.9756
15	6.70	0.959	5.741	7.659	13.31
20	9.175	0.7228	8.452	9.897	7.877
25	10.90	1.9949	8.905	12.895	18.25

Day Three

Conc.Analyte (mg / L)	Average Peak Height (cm^{-1})	S.D.	X + S.D.	X - S.D.	% R.S.D.
5	0.36	0.0478	3.58	3.68	1.314
10	7.12	0.1699	6.96	7.30	2.381
15	10.53	0.0478	10.48	10.58	0.453
20	13.61	0.3009	13.31	13.91	2.210
25	16.00	0.0125	16.06	16.08	0.070

Day Four

Conc.Analyte (mg / L)	Average Peak Height (cm^{-1})	S.D.	X + S.D.	X - S.D.	% R.S.D.
5	3.225	0.0645	3.289	3.160	2.00
10	6.163	0.1844	6.347	5.978	2.992
15	9.025	0.2060	9.231	8.819	2.282
20	11.52	0.8224	12.344	10.695	7.156
25	13.62	4.2224	17.842	9.397	30.99

Table (viii) Changing Electrolyte pH for OTC Analysis

pH	Concentration OTC (mg / L)	OTC migration (min)	EOF migration (min)
7.04	200	5.50	4.32
7.50	200	5.14	4.1
8.09	200	5.01	3.98
8.53	200	4.85	3.77
9.04	200	4.50	3.35
9.50	200	3.60	2.89
10.04	200	2.76	1.65
10.38	200	2.16	1.12

Table (ix) Altering Voltage for OTC Analysis

Voltage (kV)	Migration Analyte (min)	Migration EOF (min)	Current (μA)
15	7.20	5.98	31
16	6.70	5.60	33
17	6.30	5.32	36
18	5.90	5.14	38
19	5.40	4.80	41
20	5.02	4.63	42
21	4.80	4.00	44
22	4.01	3.17	46
23	3.60	2.60	50
24	2.89	2.01	54
25	2.03	1.50	58

Table (x) Altering Ionic Strength for OTC Analysis

Concentration of Tris (mM)	Analyte Migration (min)	EOF Migration (min)	Current (μA)	Temperature (°C)
10	3.54	3.29	14	20.6
20	3.98	3.64	20	20.8
30	4.22	3.77	25	21.0
40	4.39	3.85	30	21.1
50	5.00	3.83	48	21.2
60	5.24	3.87	58	21.2
70	5.54	3.90	71	21.2

Table (xi) Linearity Studies for OTC Analysis

Concentration of OTC (mg / L)	Migration time (min)	Average Peak Area (x 10^6)	Average Peak Height (cm^{-1})
12.5	5.01	0.0615	0.725
25	5.13	0.0326	1.375
50	4.98	0.3210	2.940
100	5.00	0.5016	5.425
200	5.01	1.1631	11.260

Table (xii) Precision and Accuracy for OTC Analysis: Day One

Conc.Analyte (mg / L)	Average Peak Height (cm^{-1})	S.D.	X + S.D.	X - S.D.	% R.S.D.
12.5	0.725	0.0134	0.7384	0.7116	1.848
25	1.375	0.0532	1.4282	1.3218	3.869
50	2.940	0.0567	2.9967	2.8833	1.928
100	5.425	0.0654	5.4909	5.3596	1.205
200	11.623	1.1640	12.787	10.459	10.01

Day Two

Conc.Analyte (mg / L)	Average Peak Height (cm^{-1})	S.D.	X + S.D.	X - S.D.	% R.S.D.
12.5	0.780	0.02114	0.80114	0.7588	2.710
25	1.512	0.04221	1.5542	1.4697	2.790
50	3.060	0.03012	3.0901	3.0290	0.980
100	6.400	0.0324	6.4132	6.3861	0.206
200	10.480	0.9124	11.3924	9.567	8.706

Day Three

Conc.Analyte (mg / L)	Average Peak Height (cm^{-1})	S.D.	X + S.D.	X - S.D.	% R.S.D.
12.5	0.700	0.0106	0.7106	0.6894	1.514
25	1.375	0.0523	1.4273	1.3227	3.803
50	3.375	0.0489	3.4239	3.3261	1.448
100	7.310	0.0700	7.3800	7.240	0.957
200	14.150	0.0980	14.248	14.052	0.6925

Day Four

Conc.Analyte (mg / L)	Average Peak Height (cm^{-1})	S.D.	X + S.D.	X - S.D.	% R.S.D.
12.5	0.722	0.0653	0.7873	0.6567	9.044
25	1.454	0.0412	1.4952	1.4128	2.833
50	2.998	0.0191	3.0171	2.9789	0.637
100	6.056	0.0298	6.0858	6.0262	0.492
200	11.997	0.0954	12.092	11.901	0.795

Determination of tromethamine in an eye-care pharmaceutical by capillary electrophoresis

Fiona A. McArdle* and Catherine J. Meehan

Department of Applied Science, Institute of Technology, Sligo, Ireland

Tromethamine [tris(hydroxymethyl)aminomethane] is used as an emulsifying agent, alkaliser and buffering agent in pharmaceutical preparations such as eye-care solutions. A new method for the determination of this compound in an eye-care preparation was developed using capillary zone electrophoresis with indirect UV detection. The method displayed linearity between 2.5 and 25 mg l^{-1} with a correlation coefficient of 0.9992. The limit of detection was 2.5 mg l^{-1}. Precision and accuracy were determined as relative standard deviation and % deviation, and were found to be 0.42% and 1.62%, respectively. Recovery studies of tromethamine in the pharmaceutical preparation gave a 102.98% recovery.

Keywords: *Capillary electrophoresis; eye-care pharmaceutical; indirect UV detection; tromethamine*

Capillary electrophoresis (CE) is gaining widespread acceptance as a standard analytical technique in pharmaceutical analysis. It has been employed in the determination of a variety of compounds including pharmaceuticals,[1–4] proteins,[5–7] polymers,[8] amines[9] and food constituents.[10–12] The eye-care industry has used CE to determine the constituents of contact lens cleaning fluids and ionic components in tears.[13] The main limitation of CE in pharmaceutical analysis is its lack of sensitivity and this is particularly true when determining trace chemicals in complex matrices.[14] However for the analysis of bulk pharmaceuticals or where low limits of detection are not required, this is not nesscessarily a problem.

In this study, CE was used to determine tromethamine [tris(hydroxymethyl)aminomethane]. Tromethamine is a weak amine base which is readily soluble in water. It is easily ionised and has a pK_b of 7.8 at body temperature.[15] It is commonly used as a buffering agent, alkaliser and emulsifying agent in pharmaceutical and cosmetic products. This work was focused on determining tromethamine in a contact lens cleaning solution.

Tromethamine has been determined by high-performance liquid chromatography (HPLC) with fluorescence[16] and UV[17] detection with retention times of 12 and 15 min, respectively. It has also been determined by ion chromatography (IC) with conductivity detection[18] and by gas chromatography (GC) with flame ionisation detection[19] with retention times of 5.4 and 3.6 min, respectively. For fluorescence, UV and flame ionisation detection, derivatisation of tromethamine is required, which leads to lengthy sample preparation times.

Tromethamine in its ionised state possesses electrophoretic mobility and, although it cannot be detected by direct UV detection, indirect UV detection was used in this study. Indirect UV detection has been used previously for the detection of inorganic ions by CE.[20]

Dimethyldiphenylphosphonium hydroxide (DDP) was chosen as the UV-absorbing species in this study as it possesses the same charge as the analyte under the conditions used. It is a highly mobile ion and can act as both electrolyte, facilitating electroosmotic flow, and absorbing species, allowing indirect UV detection. It is obtained by passing dimethyldiphenylphosphonium iodide (DDPI) through an anion-exchange cartridge. The UV detector displays a decrease in absorbance as the non-absorbing analyte passes through the detection cell. The polarity of the output signal is reversed to allow positive peaks to be observed.

This paper presents a validated method for the determination of tromethamine in a contact lens cleaning solution by capillary zone electrophoresis (CZE). The method uses indirect UV detection and requires minimal sample preparation.

Experimental

Reagents

DDPI and α-hydroxyisobutyric acid were purchased from Sigma–Aldrich (Dublin, Ireland), anion-exchange solid-phase extraction cartridges from Dionex UK, Camberely, Surrey, UK), tromethamine (ultra-pure grade, 99.9%) from Allergan Pharmaceuticals (Westport, Co. Mayo, Ireland) and analytical-reagent grade methanol from Lab Scan (Dublin, Ireland). All solutions were prepared with water purified in a Millipore Milli-Q water purification system. (AGB Scientific, Dublin, Ireland).

Apparatus

Experiments were carried out using a CES1 capillary electrophoresis system, (Dionex, UK) equipped with an on-capillary UV detector operated at 215 nm. Data were collected on a Dionex Model 4270 Integrator. Bare fused silica capillaries (50 μm id × 375 μm od) were also purchased from Dionex. A Metrohm Model 713 pH meter (Foss Electric, Dublin, Ireland) was used for measuring pH.

Gravity injection was employed, with the sample vials raised to a height of 100 mm for 30 s. A voltage of 20 kV (detector side cathodic) was applied. The detector was used in the reversed polarity mode. The capillary was rinsed with the run buffer for 120 s between injections. Optimum operating conditions were determined by investigating the effect of changes in the ionic strength of the buffer, temperature, pH and applied voltage.

Capillary preparation

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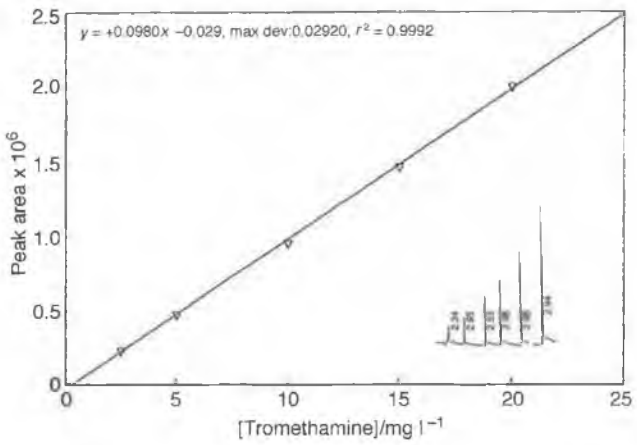

Fig. 3 Calibration curve of tromethamine concentration (2.5–25 mg l^{-1}) *versus* peak area; r^2 = 0.9992, slope = 0.098 absorbance l mg^{-1}. Electrolyte, 5 × 10^{-3} M DDP buffer (pH 6.5); UV detection at 215 nm. Electrophoretic conditions: capillary, 50 cm × 50 μm id; voltage, 20 kV (detector cathodic); gravity injection, 100 mm, 30 s.

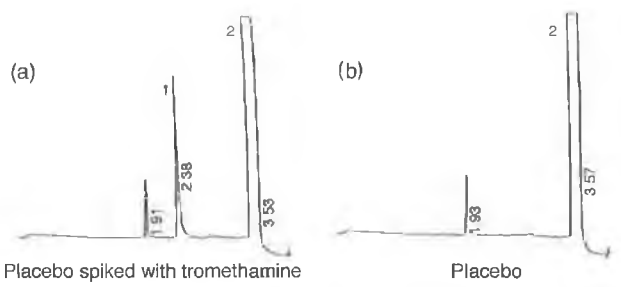

Fig. 4 Electropherograms of placebo containing TrisChem (polyhexamethylene biguanide), tyloxapol and disodium edetate, (a) with and (b) without tromethamine at 12 mg ml^{-1}. DDP buffer (pH 6.5) concentration, 5 × 10^{-3} M; UV detection at 215 nm. Electrophoretic conditions: capillary, 50 cm × 50 μm id; voltage, 20 kV (detector cathodic); gravity injection, 100 mm, 30 s. Peaks 1 = tromethamine; 2 = solvent.

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	0.9995	−3.25 × 10^{-2}	0.0987
Average ± s	0.9992 ± 2.9 × 10^{-2}	(−2.9 × 10^{-2}) ± 0.017	0.098 ± 0.002

Recovery studies over the concentration range 2.5–25 mg ml^{-1} were conducted by spiking (a) placebo Complete solutions and (b) de-ionised water with tromethamine. These solutions were then diluted 1 : 1000 v/v prior to injection on to the CE capillary. A mean recovery of 102.98 ± 2.59% was obtained. This relatively large variation may be due to the difference in the ionic strength of the sample solution and the distilled water standards. There was no significant difference in the migration times or peak shapes for the spiked placebo and the spiked distilled water standards.

The method developed represents a simple analytical procedure for the determination of tromethamine in an eye-care pharmaceutical preparation. This method compares favourably with other reported methods for tromethamine determination. Previously reported methods using HPLC[16,17] or GC[19] involve lengthy sample and standard preparation, involving derivatisation of tromethamine. The CE method developed here only requires dilution of the sample. Also, the previously reported chromatographic methods have run times of 12–15 min for HPLC,[16,17] 5.4 min for IC[18] and 3.6 min for GC.[19] The proposed CE method has a shorter run time of 2.38 min, but it does require a capillary rinse time of 2 min between injections. The instrument set-up and conditioning times are similar for both HPLC and CE. The total analysis time, including sample and standard preparation and run time, is significantly less with CE than with the previously reported chromatographic methods. Overall, CE uses smaller volumes of buffer and sample than HPLC, the capillaries are cheaper than HPLC columns and, coupled with the simple sample preparation required, the measurement of tromethamine concentrations using the method detailed in this paper would be more cost effective than using a chromatographic method.

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The Analyst

Determination of tromethamine in an eye-care pharmaceutical by capillary electrophoresis

Fiona A. McArdle* and Catherine J. Meehan

Department of Applied Science, Institute of Technology, Sligo, Ireland

Tromethamine [tris(hydroxymethyl)aminomethane] is used as an emulsifying agent, alkaliser and buffering agent in pharmaceutical preparations such as eye-care solutions. A new method for the determination of this compound in an eye-care preparation was developed using capillary zone electrophoresis with indirect UV detection. The method displayed linearity between 2.5 and 25 mg l^{-1} with a correlation coefficient of 0.9992. The limit of detection was 2.5 mg l^{-1}. Precision and accuracy were determined as relative standard deviation and % deviation, and were found to be 0.42% and 1.62%, respectively. Recovery studies of tromethamine in the pharmaceutical preparation gave a 102.98% recovery.

Keywords: *Capillary electrophoresis; eye-care pharmaceutical; indirect UV detection; tromethamine*

Capillary electrophoresis (CE) is gaining widespread acceptance as a standard analytical technique in pharmaceutical analysis. It has been employed in the determination of a variety of compounds including pharmaceuticals,[1–4] proteins,[5–7] polymers,[8] amines[9] and food constituents.[10–12] The eye-care industry has used CE to determine the constituents of contact lens cleaning fluids and ionic components in tears.[13] The main limitation of CE in pharmaceutical analysis is its lack of sensitivity and this is particularly true when determining trace chemicals in complex matrices.[14] However for the analysis of bulk pharmaceuticals or where low limits of detection are not required, this is not nesscessarily a problem.

In this study, CE was used to determine tromethamine [tris(hydroxymethyl)aminomethane]. Tromethamine is a weak amine base which is readily soluble in water. It is easily ionised and has a pK_b of 7.8 at body temperature.[15] It is commonly used as a buffering agent, alkaliser and emulsifying agent in pharmaceutical and cosmetic products. This work was focused on determining tromethamine in a contact lens cleaning solution.

Tromethamine has been determined by high-performance liquid chromatography (HPLC) with fluorescence[16] and UV[17] detection with retention times of 12 and 15 min, respectively. It has also been determined by ion chromatography (IC) with conductivity detection[18] and by gas chromatography (GC) with flame ionisation detection[19] with retention times of 5.4 and 3.6 min, respectively. For fluorescence, UV and flame ionisation detection, derivatisation of tromethamine is required, which leads to lengthy sample preparation times.

Tromethamine in its ionised state possesses electrophoretic mobility and, although it cannot be detected by direct UV detection, indirect UV detection was used in this study. Indirect UV detection has been used previously for the detection of inorganic ions by CE.[20]

Dimethyldiphenylphosphonium hydroxide (DDP) was chosen as the UV-absorbing species in this study as it possesses the same charge as the analyte under the conditions used. It is a highly mobile ion and can act as both electrolyte, facilitating electroosmotic flow, and absorbing species, allowing indirect UV detection. It is obtained by passing dimethyldiphenylphosphonium iodide (DDPI) through an anion-exchange cartridge. The UV detector displays a decrease in absorbance as the non-absorbing analyte passes through the detection cell. The polarity of the output signal is reversed to allow positive peaks to be observed.

This paper presents a validated method for the determination of tromethamine in a contact lens cleaning solution by capillary zone electrophoresis (CZE). The method uses indirect UV detection and requires minimal sample preparation.

Experimental

Reagents

DDPI and α-hydroxyisobutyric acid were purchased from Sigma–Aldrich (Dublin, Ireland), anion-exchange solid-phase extraction cartridges from Dionex UK, Camberely, Surrey, UK), tromethamine (ultra-pure grade, 99.9%) from Allergan Pharmaceuticals (Westport, Co. Mayo, Ireland) and analytical-reagent grade methanol from Lab Scan (Dublin, Ireland). All solutions were prepared with water purified in a Millipore Milli-Q water purification system. (AGB Scientific, Dublin, Ireland).

Apparatus

Experiments were carried out using a CES1 capillary electrophoresis system, (Dionex, UK) equipped with an on-capillary UV detector operated at 215 nm. Data were collected on a Dionex Model 4270 Integrator. Bare fused silica capillaries (50 μm id × 375 μm od) were also purchased from Dionex. A Metrohm Model 713 pH meter (Foss Electric, Dublin, Ireland) was used for measuring pH.

Gravity injection was employed, with the sample vials raised to a height of 100 mm for 30 s. A voltage of 20 kV (detector side cathodic) was applied. The detector was used in the reversed polarity mode. The capillary was rinsed with the run buffer for 120 s between injections. Optimum operating conditions were determined by investigating the effect of changes in the ionic strength of the buffer, temperature, pH and applied voltage.

Capillary preparation

Fused silica capillaries, 50 cm long, were prepared in accordance with the instrument manufacturer's instructions.[20] The capillary was conditioned for use by flushing with 1 M NaOH for 45 min, rinsing with de-ionised water for 30 min followed by conditioning with buffer for 1 h to equilibrate the capillary sufficiently.

Buffer preparation

A DDPI solution was prepared by diluting the appropriate amount with de-ionised water to give a final concentration of 25×10^{-3} M. It was necessary to heat this solution to approximately 80 °C to ensure complete dissolution of the DDPI. This solution, when cool, was passed through an anion exchange solid phase extraction cartridge, at a rate of 2

ml min^{-1}. The first 2 ml of eluate were discarded. A 20 ml volume of the eluate was diluted to 100 ml and the ratio of eluate to α-hydroxyisobutyric acid was varied to optimise the separation and run time. All electrolyte solutions were vacuum filtered through a 0.45 μm nylon 6,6 filter before use.

Standards and samples

A 100 mg l^{-1} stock standard solution of tromethamine was used as the primary standard. This was prepared fresh daily. Working standard solutions in the range 5–25 mg l^{-1} were prepared from the primary standard.

Samples of an eye-care solution, Complete, were supplied by Allergan Pharmaceuticals. This sample was stated to have a tromethamine concentration of 12 mg ml^{-1}. The sample preparation involved dilution of the sample 1 : 1000 v/v with deionised water. Samples of Complete containing all constituents except tromethamine (placebo) were also supplied and these were used in validation and recovery studies. All standard and samples were passed through a 0.45 μm nylon 6,6 filter to remove any particulate matter present before injection on to the capillary.

Results and discussion

Optimisation of CE conditions

As tromethamine does not contain an absorbing species, its determination was attained by using indirect UV detection. The absorbing species used was DDP. In spectrometric studies DDP was found have a molar absorptivity of 1.65×10^5 mol^{-1} dm^2 and to obey the Beer–Lambert law up to a concentration of 1.4×10^{-2} M at a wavelength of 215 nm. A linear regression of 0.9984 was obtained for DDP concentration *versus* absorption. As DDP is a cation and contributes to the electroosmotic flow (EOF), varying its concentration affects the EOF in CZE. The DDP concentration was varied between 0.32×10^{-3} and 5.0×10^{-3} M and it was found that increasing the concentration caused a decrease in the migration time of the tromethamine (Fig. 1). This is largely due to the increase in EOF at higher DDP concentrations. However, at high electrolyte concentrations the current generated upon application of the voltage exceeded the manufacturer's recommended limit and caused the instrument to cut out. For this reason, the concentration of DDP used in the electrolyte for CZE was 5×10^{-3} M.

Using a DDP concentration of 5×10^{-3} M, the pH of the electrolyte was varied by altering the amount of α-hydroxyisobutyric acid and this caused a change in the EOF and electrophoretic mobility of tromethamine (Fig. 2). The EOF was taken as the retention of the solvent in which the samples and standards were dissolved. This solvent did not contain UV absorbing species and therefore a peak was observed as it eluted. At pH < 3.0, the EOF was very slow, owing to the suppressed ionisation of the silanoate residues on the internal capillary wall, thus reducing the zeta potential. At pH > 7, tromethamine eluted with the EOF, indicating that at this pH tromethamine has no charge. In all subsequent work, pH 6.5 was used.

The applied voltage was varied in the range 15–25 kV. The migration time of tromethamine decreased with increasing applied voltage, as expected. It was decided to use an applied voltage of 20 kV in all subsequent work, because high applied voltages can result in Joule heating[21] and the instrument employed did not have a thermostat facility.

Day-to-day temperature variations inside the capillary housing from 15 to 20 °C were noted, but this did not have a significant effect on the tromethamine peak shape or migration time. To ensure minimum temperature fluctuations, a cooling jacket was placed on the capillary at all times.

Validation

Using the DDP buffer, at a concentration of 5×10^{-3} M and with a pH of 6.5, a linear signal was obtained from standard tromethamine solutions in the range 2.5–25 mg l^{-1} and gave a regression coefficient of 0.9992 (Fig. 3). The sensitivity of the system was evaluated from the slope of the linear regression line and was found to be 0.098 absorbance l mg^{-1}.

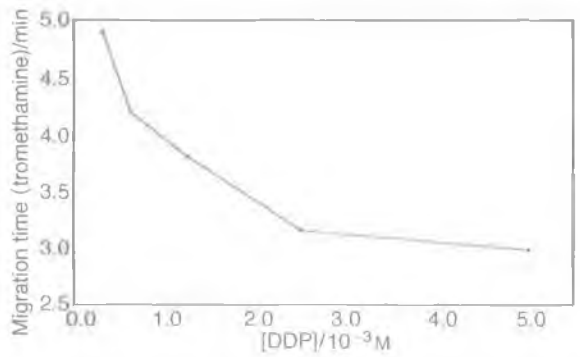


Fig. 1 Variation of migration time of tromethamine (12 mg l^{-1}) with DDP buffer (pH 6.5) concentration in the range 0.32×10^{-3}–5×10^{-3} M. UV detection at 215 nm. Electrophoretic conditions: capillary, 50 cm × 50 μm id; voltage, 20 kV (detector cathodic); gravity injection, 100 mm, 30 s.

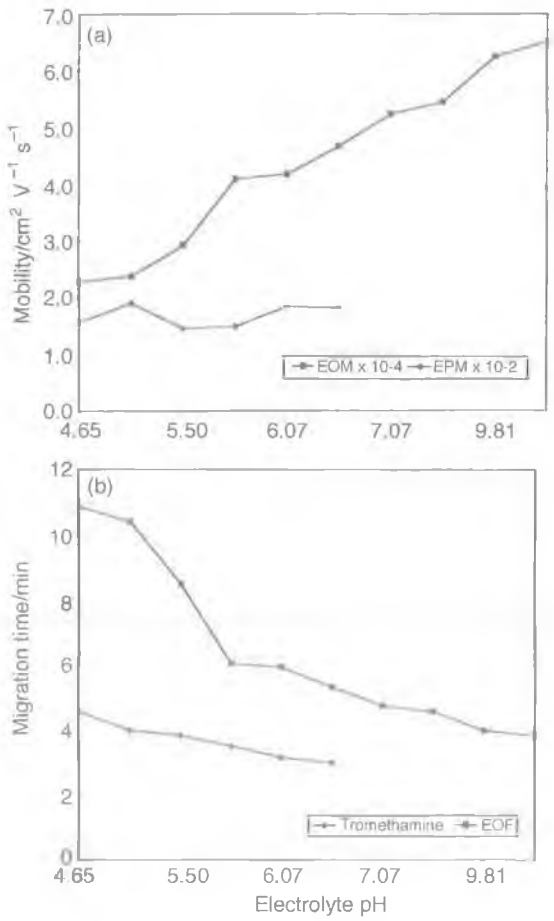


Fig. 2 (a) Electrolyte pH *versus* (■) electrophoretic mobility of tromethamine (20 mg l^{-1}) and (◆) mobility of solvent peak. (b) Electrolyte pH *versus* (◆) migration time of tromethamine (20 mg l^{-1}) and (■) migration time of solvent peak. UV detection at 215 nm. Electrophoretic conditions: capillary, 50 cm × 50 μm id; voltage, 20 kV (detector cathodic); gravity injection, 100 mm, 30 s.

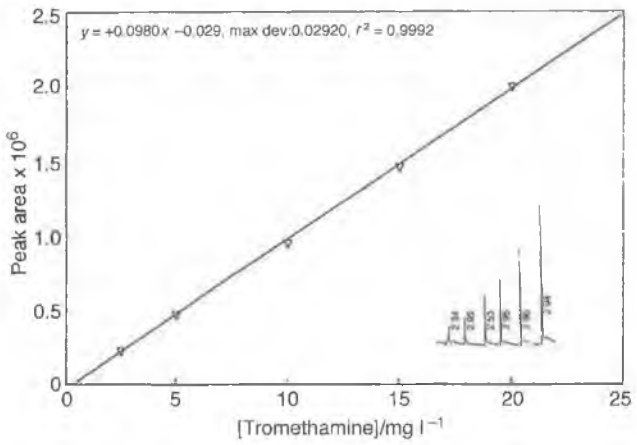
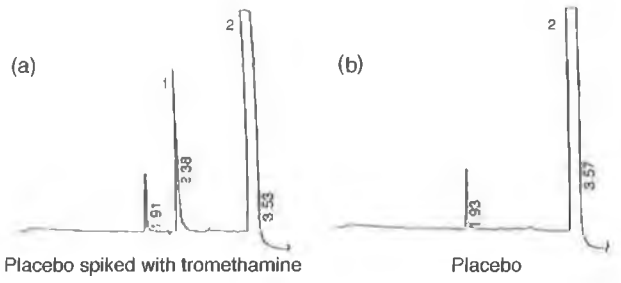


Fig. 3 Calibration curve of tromethamine concentration (2.5–25 mg l^{-1}) *versus* peak area; r^2 = 0.9992, slope = 0.098 absorbance 1 mg^{-1}. Electrolyte, 5 × 10^{-3} M DDP buffer (pH 6.5); UV detection at 215 nm. Electrophoretic conditions: capillary, 50 cm × 50 μm id; voltage, 20 kV (detector cathodic); gravity injection, 100 mm, 30 s.



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Recovery studies over the concentration range 2.5–25 mg ml^{-1} were conducted by spiking (a) placebo Complete solutions and (b) de-ionised water with tromethamine. These solutions were then diluted 1 : 1000 v/v prior to injection on to the CE capillary. A mean recovery of 102.98 ± 2.59% was obtained. This relatively large variation may be due to the difference in the ionic strength of the sample solution and the distilled water standards. There was no significant difference in the migration times or peak shapes for the spiked placebo and the spiked distilled water standards.

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