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Application of Capillary Zone Electrophoresis Methods for the Investigation of *In Vitro* Drug Metabolism.

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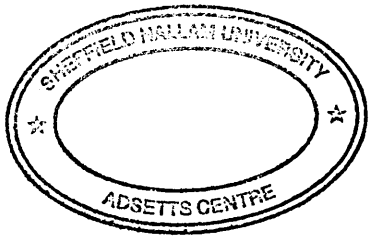
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Abstract

High performance liquid chromatography (HPLC) has for some time been the industry standard in pharmaceutical analysis. A diverse choice of column packing materials enables a wide range of analytical separations to be performed, including both normal and reversed phase as well as chiral chromatography. A variety of compatible detectors further increases the popularity of this instrument, allowing straightforward absorption/emission detection by ultraviolet light or fluorescence, in addition to the increased sensitivity of hyphenated technologies, with HPLC in conjunction with mass spectrometric detection and nuclear magnetic resonance spectroscopy.

Capillary zone electrophoresis (CZE) offers an alternative analytical technique to a similarly broad range of analytes and mechanisms of detection. The advantages of CZE are the reduced run times, higher efficiency separations and savings on solvent usage and disposal. The pharmaceutical industry has increasingly employed CZE for dissolution analysis and impurity assessments as well as main-component assays.

This thesis reports the potential of CZE as an analytical tool versus the more established technique of HPLC and their application to in vitro metabolism. In vitro metabolism looks at the metabolic fate of a drug, in this case by incubation with a suspension of the microsomal rat liver fraction. This method was chosen as it can generate a series of samples taken from a bulk incubation to provide a time profile over which the amount of parent compound present can be measured. In this way a series of compounds can be screened quickly and simply for their rate/extent of metabolism without the necessity for the more time consuming and expensive *in vivo* experimentation.

Analytical methods were developed or modified from published work for both a series of basic analogues including propranolol and an acidic compound, chlorogenic acid. A novel approach to the CZE method development for the acid was a statistical analysis technique called Factorial Experimental Design (FED), which allowed the time-saving, simultaneous evaluation of the major influential parameters involved. Once these assays were established, they were investigated for reliability, accuracy, reproducibility and linearity. A comparison was made between two different CZE instruments, a Beckman P/ACE and a Hewlett Packard HP^{3D}, and also between CZE and HPLC. The final investigation was to apply these two analytical techniques in the field of in vitro drug metabolism, using propranolol as a model compound.

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The theoretical basis for high performance liquid chromatography (HPLC) was laid down in the 1940s and although the major phase of its development as an analytical tool was not until the 1960s, it soon became an established and widely used technique in separation science in the 1970s. HPLC combined with ultraviolet (UV) spectral data, soon overtook gas chromatography (GC) and thin layer chromatography (TLC) to become the instrument of choice for the analysis of pharmaceuticals and the benchmark technique against which all others are compared. Reversed-phase-HPLC provided a quantum leap in various analytical fields and is still the industry standard for the separation and quantitation of drugs and drug metabolites extracted from urine, bile, plasma and faeces.

The last century has also documented the development of a variety of electrophoretic methods which have been applied to chemical and analytical fields; and more recently the last 40 years have seen their expansion into biochemical, biological and pharmaceutical sciences.

Electrophoresis is the movement of electrically charged species in a conductive medium under the influence of an electric field. Capillary electrophoresis (CE) is typically performed by filling a hollow silica capillary and two vials at either end (source vial and destination vial) with an electrolyte, or run buffer. The capillary inlet is placed into the sample vial, the sample introduced by voltage or pressure injection and the capillary inlet returned to the source vial. A potential difference is then applied across the capillary. The analytes migrate through the capillary and are measured at a detection window within the far end of the capillary to produce an electropherogram in which the analytes are separated according to their migration times.

Aims and Objectives

The aims and objectives of this thesis are report on the potential of CZE as a suitable analytical tool to be used by the drug metabolist alongside the established technique of HPLC and to compare their application for *in vitro* metabolism.

There are a number of different procedures for CE. These include capillary zone electrophoresis, the most simple form of CE, performed in an open tube filled with a run buffer where ionised solutes are separated according to their charge-to-size ratios. Other options have been developed to overcome various analytical problems: micellar capillary electrochromatography for the separation of neutral species via their hydrophobicities; capillary gel electrophoresis for the analysis

of proteins and DNA using size exclusion; capillary electrochromatography with solid phase packed capillaries which have the advantage of chromatographic as well as electrophoretic separation; and isotachophoresis and isoelectric focusing CE as a means of concentrating solutes for increased sensitivity. Each of these methodologies has its specific area of application, and in this case CZE was chosen for this piece of work as it is commonly used for small compounds which are easily capable of being ionised. This work in the thesis would compare the results for both acidic and basic compounds analysed by HPLC and CZE methods. Analytical methods would be developed or methods modified from published work for both a series of basic analogues to include propranolol and an acidic compound, chlorogenic acid. A comparison will be made between two different CZE instruments, a Beckman P/ACE and a Hewlett Packard HP^{3D}. This data would also allow discussion of the associated advantages and disadvantages of CZE and HPLC prior to its operation in *in vitro* testing.

In analytical method development for both biological testing during pre-clinical studies and quality control of the finished product, the speed of the development phase can affect the time to market. Method development is regularly based on a step-wise approach of varying one operating parameter at a time which can lead to very extended method development times. An alternative is to use a structured procedure which can quickly reach the global optima of choice of operating variables in a minimum number of experiments. One such approach is the statistical technique called Factorial Experimental Design (FED), which in addition to allowing time-saving through reduction in experiments, also allows for a simultaneous evaluation of the main influential parameters involved in the method development. In this programme, the method development on chlorogenic acid would be carried out using this method, and the investigation would be used to assess its value and its ease of use.

Once method development had been completed the method would be validated to include reliability, accuracy, reproducibility and linearity. This would be carried out as a comparison on two instruments, a Beckman P/ACE and a Hewlett Packard HP^{3D}.

A review of the published applications of CZE suggests that it has been reasonably widely used by the pharmaceutical industry for bulk drug, synthetic precursors and related substance assays, as well as the finished product, but their is limited documented methods on its application for the detection and measurement of drugs and their metabolites in biological samples. *In vitro*

metabolism looks at the metabolic fate of a drug, in this case by incubation with a suspension of the microsomal rat liver fraction. This method was chosen as it can generate a series of samples taken from a bulk incubation to provide a time profile over which the amount of parent compound present can be measured. In this way a series of compounds can be screened quickly and simply for their rate/extent of metabolism without the necessity for the more time consuming and expensive *in vivo* experimentation.

The lack of literature reports for CZE methods for *in vitro* metabolism is possibly due to a number of reasons. These are likely to include the difficulties of assays in the presence of the biological background and in particular the problems of detection of metabolites, which are often at the trace level.

The final objective of this project is to apply CZE and HPLC in the field of *in vitro* metabolism to obtain a consensus of information on aspects of their ease of use and applicability in biochemical analysis. For this work it is proposed to use propranolol as a model compound.

Therefore, in summary, the aims of this research programme are to:-

1) compare CZE and HPLC as a regular monitoring techniques for acidic and basic drugs in bulk and finished products, by using propranolol and its analogues and the acidic compound chlorogenic acid
2) assess the usefulness of factorial experimental design in the rapid method development for acidic and basic drugs
3) to compare CE instruments from different manufacturers in the work above
4) to apply the knowledge gained in the earlier part of the programme to *in vitro* metabolism studies.

1.1 A Brief History of Electrophoresis.

Various reviews[1-7,14] have chronicled the developments in electrophoresis over the last hundred and fifty years. It was Faraday who first instigated an interest in this field when he presented his laws of electrolysis in 1791. However, it was not until the mid-nineteenth century that Wiedermann formulated the initial theories of electrophoresis. It was noted that on applying an electric field

across the ends of a horizontal tube containing an aqueous ionic salt solution the inner surface of the tube acquired a negative charge. The solvated ions also took on an opposite charge which resulted in movement of the liquid in contact with the tube wall towards one of the electrodes. This phenomenon was termed electroosmosis by Helmholtz & Wiedermann in 1877. Further work by Kohlrausch in 1897 led to the development of displacement electrophoresis, which later became known as isotachophoresis. He also produced equations describing the order of electrophoretic migration of ions and the formation of moving boundaries with sharp fronts; the latter of which became very important for large ions and proteins.

Nobel prizes in this field have been awarded to Arrhenius for his dissolution theory of ions in water (1903); Svedberg (1926) for his work on proteins and ultracentrifugation and Tiselius (1948) for the development of the moving boundary method and chromatographic adsorption analysis during which he successfully identified four moving bands in serum corresponding to albumin and alpha, beta and gamma globulin. Other significant advances were made when Hardy discovered that the mobilities of proteins depend largely on the pH of the electrolyte solution in which they are present. By performing experiments at various pH values and measuring electrophoretic migration Michaelis found that enzymes could be characterised by their isoelectric points (pI).

Paper electrophoresis became very popular from 1950, especially as it was more simple to construct, cheaper and required much less bench space than Tiselius' boundary electrophoresis, the only other commercially available electrophoresis system, performed in a large open U-shaped tube. The amount of sample required for analysis was also reduced from many milligrams to less than a milligram for paper electrophoresis.

The use of gelatin and agar gels as supporting media in gel electrophoresis has been known for over a hundred years, but did not become commonplace until the introduction of polyacrylamide gels (PAG), which gave more reproducible results than the previously used starch gels. The incorporation of sodium dodecylsulphate (SDS) into the PAG enabled the determination of size to weight ratios of proteins and polypeptides depending on the number of SDS molecules adsorbed onto their surface (SDS-PAGE analysis).

In 1967 Hjerten[8] developed a method for free solution electrophoresis (FSE) in a revolving tube which could be used to separate not just proteins, amino acids and nucleic acids, but also viruses,

bacteria and both organic and inorganic ions. Further advances were made with the incorporation of ultra violet (UV) light absorption detectors in 1970 and conductivity detectors in 1972.

The first examples of the use of an electric field to generate an electroosmotic flow in LC were reported by Strain (1939) and Lecoq (1944), whilst Moulde and Synge (1952) applied this technique to thin layer chromatography. However, it was Pretorius (1974)[9] who performed the first separation by column chromatography using 75-125 μm i.d. particles in a 1 mm glass tube.

Capillary technology progressed from Tiselius' 1 mm U-shaped tube used in the late 1930s, to Mikker's[10] smaller diameter (200 μm i.d.) Teflon capillaries which significantly reduced the effects of diffusion and zone spreading from convection. Jorgenson and Lukacs'[11] Pyrex capillaries with an internal diameter of 75 μm further reduced Joule heating and enabled the use of higher voltages (30 kV). Efficiencies in excess of 400,000 theoretical plates are now achievable with capillaries constructed of fused silica which is transparent at lower wavelengths. These replaced the old glass and Pyrex versions which could only be used down to a wavelength of 280 nm.

The introduction of micellar electrokinetic capillary chromatography (MECC) by Terabe[12] in the mid 1980s, enabled the separation of neutral species by incorporating micelles which differentially separate compounds according to their hydrophobicity.

The next significant step came with the development of capillary electrochromatography (CEC), a cross between CE and high performance liquid chromatography (HPLC), which used capillaries filled with solid phase packing material. This technique had been reported by Jorgenson and Lukacs[13] in 1981 using 170 μm i.d. glass capillaries filled with 10μm packing material, but was not widely used until later in the 1980s. By increasing the voltages applied for separation and using narrow bore capillaries (<0.2 mm) the technique of high performance capillary electrophoresis (HPCE) was born, and with it came the ability to determine concentrations of various ionic substances e.g. water quality assays and monitoring for drugs and their metabolites. These methods offered advantages over gas-liquid chromatography techniques in which such substances generally required derivatisation to enable analysis.

1.2 System Overview

A CE instrument (Figure 1) comprises of a high voltage power supply; source and destination (inlet and outlet) vials; capillary; detector and a data capture device. There is a variety of commercially available CE systems with various different capabilities including methods of injection, capillary cooling, fraction collection, detectors and software.

Bench Operating Procedure

A new capillary should be conditioned prior to use in order to stabilise the capillary surface chemistry. The most common approach involves flushing a solution of 0.1 M sodium hydroxide under a pressure of 50 mbar through the capillary for approximately 10 minutes or 20-30 capillary volumes. A similar pressurised wash procedure is also incorporated between each sample, which comprises of a wash with 0.1 M sodium hydroxide for 1 minute, then water for 1 minute and run buffer for 4 minutes. Most instruments also have the option of buffer replenishment to ensure that there are no changes in the run buffer with time which could result in non-reproducibility of mobility of the analytes. It is suggested that the inlet and outlet vials and the capillary are rinsed and refilled with fresh run buffer to prevent any gradual changes in osmolarity due to a build-up of buffer and/or sample ions in the destination vial which would cause fluctuations in conductivity between the two vials and across the capillary. Recommendations for the care and maintenance of CE capillaries along with appropriate buffer/electrolyte preparation and solvent choice have been documented by Altria *et al.* [15-18].

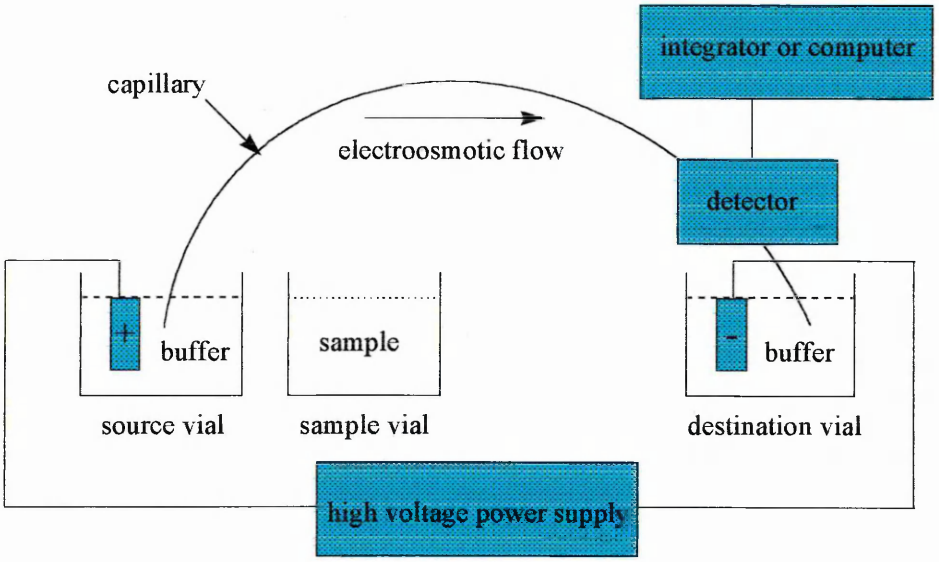


Figure 1 : A Schematic Diagram of a Capillary Electrophoresis Instrument

1.3 Electroosmotic Flow

When a buffer with a pH above 3 is placed in a silica capillary the inner surface of the capillary acquires a charge. This is due either to ionisation of the capillary surface or adsorption of ions from the buffer onto the capillary wall. In the case of fused silica capillaries, the surface silanol groups (Si-OH) are ionised to negatively charged silanoate groups ($Si\text{-}O^-$) at a pH above 3. This effect can be enhanced by first conditioning the capillary by flushing it through with a 1 M sodium hydroxide solution under a pressure of 50 mbar. The $Si\text{-}O^-$ groups attract positive ions from the buffer to form an inner fixed layer of cations on the capillary wall. However, the net charge is still negative and a secondary outer, mobile layer of cations is attached. These two layers form a diffuse double layer of cations, and when an electric field is applied, the mobile outer layer of cations is pulled towards the cathode. Since the cations are solvated they also pull the buffer solution with them, thereby causing an electroosmotic flow (EOF). In this way the run buffer moves through the capillary under the influence of the electrical field (the electroosmotic flow) from anode to cathode - positive to negative (Figure 2).

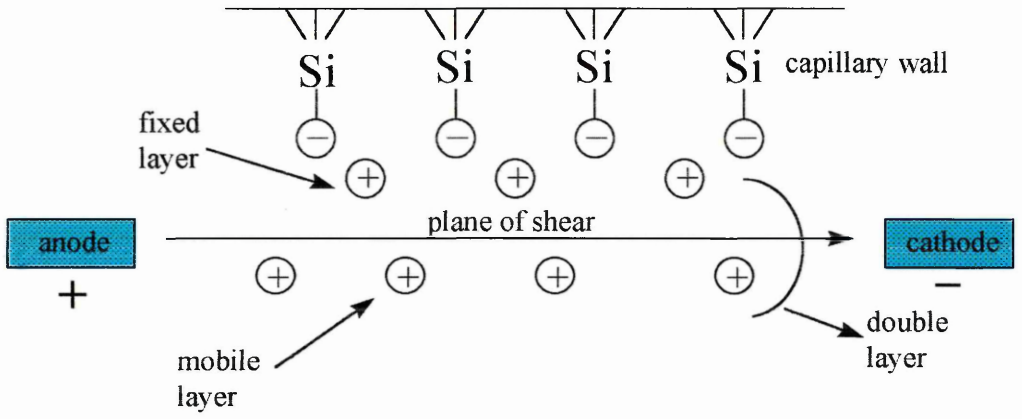


Figure 2 : Electroosmotic Flow

(as observed in CZE with a run buffer pH >3)

The potential difference across the two layers is termed the zeta potential, ζ. The EOF is proportional to the zeta potential which in turn is proportional to the thickness of the double layer. The thickness of the diffuse double layer is inversely proportional to the concentration of the buffer, with a 10 mM concentration giving a layer of approximately 1 nm.

$\zeta = 4\pi\delta e/\varepsilon$ where δ is the thickness of the double layer

e is the charge per unit surface area

ε is the dielectric constant of the buffer

There are major benefits in utilising electroosmotic flow. It carries solvated ions, whether positive, negative or neutral, through the capillary to the detector with the ability to analyse anions and cations in a single run without having to reverse the polarity of the system. Ions with quite large differences in their size-to-charge ratios can also be analysed together with reasonable run times. However, some highly charged anions can have an electrophoretic mobility greater than, and opposing the direction of, the EOF. These solutes will therefore not travel through to the detector, but remain in the capillary or migrate back into the source vial. For such analytes it will be necessary to reverse the polarity of the applied electric field and also to reverse the EOF by addition of a flow modifier (see below). The final advantage of EOF is its flat flow profile which

elutes components as narrow bands giving sharper peaks than the pumped or laminar flow profile seen with HPLC (Figure 3) or the turbulent flow of GC.

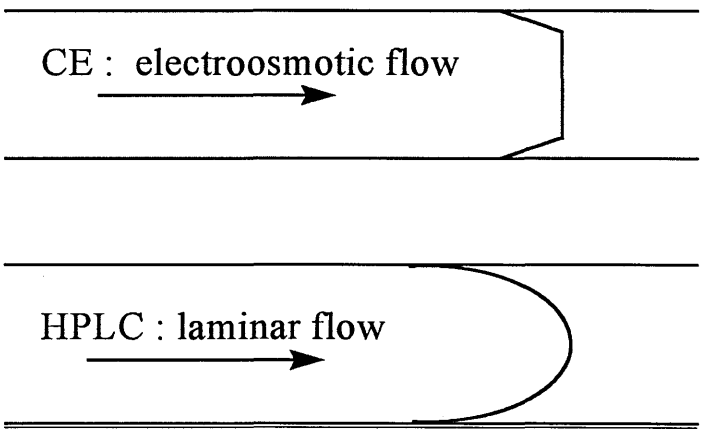


Figure 3 : Electroosmotic Flow of CE vs Laminar Flow of HPLC

In HPLC the pumped flow produces broader peaks with lower efficiencies as the solutes in the center of the column move significantly faster than those at the walls. In CE the frictional drag experienced near the walls of the capillary is small and detracts little from the overall flat profile giving higher efficiencies with less zone spreading. It is important, however, to maintain a constant EOF to avoid variable migration times and errors in peak identification and quantitation.

A faster EOF results in shorter migration times, giving sharper, narrower peaks with higher efficiencies. However, a shorter separation time may cause incomplete resolution of the solutes of interest. A compromise must therefore be reached between the EOF and separation time.

1.3.1 Reversing the EOF

When analysing solely for anions, especially if highly charged, they can be eluted quicker and run times reduced by reversing both the direction of the EOF and the polarity of the applied electric field. The simplest way to reverse the actual direction of the EOF is by addition of a flow modifier, e.g. a quaternary amine such as an alkyl ammonium salt, to the run buffer.

e.g. cetyltrimethylammonium bromide (CTAB)

diethylenetriamine (DETA)

hexamethonium bromide (diquaternary ammonium salt)

The quaternary amines bind in a similar double layer fashion, first forming ionic interactions with the silanoate groups on the capillary wall, then hydrophobic interactions with other quaternary amines of the opposite orientation such that the interior charge on the wall is positive, as shown below (Figure 4). Anionic solutes are attracted to these protruding positively charged amines and are pulled towards the positive electrode, resulting in a reversal in the direction of the EOF.

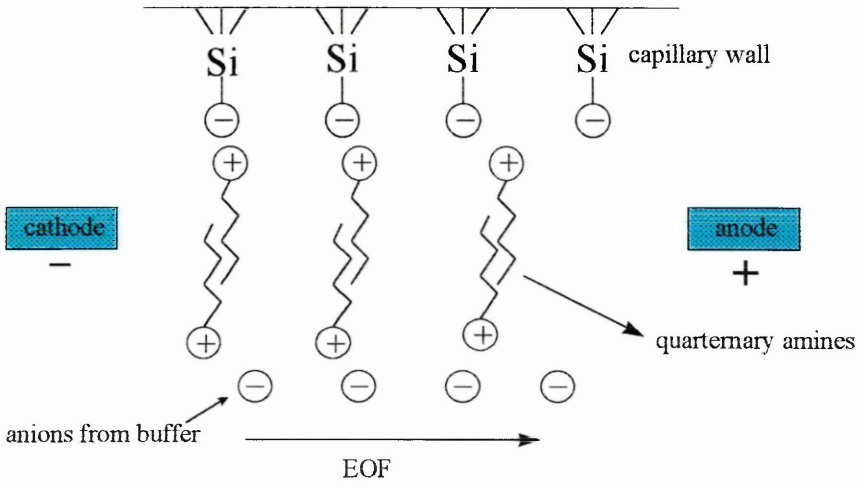


Figure 4 : Reversed Electroosmotic Flow

(Reversed EOF as a result of binding quaternary amines onto the capillary surface)

Amphoteric proteins such as alpha-lactalbumin can also be bonded to the capillary wall which allows both the magnitude and direction of the EOF to be modified by altering the pH of the run buffer. The isoelectric point (pI) for alpha-lactalbumin is pH 4.3 at which it is neutral. Raise the pH above 4.1 and it becomes cationic with a "normal" EOF, anode to cathode; lower the pH however, and it displays anionic characteristics with a reversed EOF from cathode to anode.

1.3.2 Changing EOF

The velocity of the EOF (v_{EOF}) is described by the equation below:

$$v_{EOF} = \varepsilon\zeta E/4\pi\eta$$ where E is the applied electric field in volts.cm^{-1}

η is the buffer viscosity.

The electroosmotic mobility, μ_{EOF} is described as:

$$\mu_{EOF} = \varepsilon\zeta/4\pi\eta$$

From these equations it can be seen that the electroosmotic mobility is dependent on the buffer characteristics, the dielectric constant and the viscosity of the buffer, which affect the zeta potential, but independent of the applied electric field strength.

1.3.3 Effect of Applied Voltage.

The easiest and most efficient way to modify the EOF is to change the voltage which directly affects the electric field. From the two equations above

$$v_{EOF} = \mu_{EOF} \times E$$

An increase in the applied voltage increases the EOF and decreases migration times giving higher efficiencies. However, this may also cause an increase in Joule heating which leads to peak broadening, non-reproducible migration times and possible sample decomposition. It is therefore desirable to choose the maximum voltage for efficiency whilst ensuring heat dissipation, if necessary by choosing a smaller internal diameter capillary or using a thermostated capillary compartment.

Using Ohm's law : $E = IR$ where E = electric field

I = current

R = resistance

and $E = V/l$ where E = electric field

V = voltage

l = capillary length

If the capillary length is kept constant, then voltage (V, in volts.cm^{-1}) may be substituted for E. A graph of I against V can then be plotted which should be linear with a zero intercept and a slope of 1/R (Figure 5). The maximum voltage can be read from the graph.

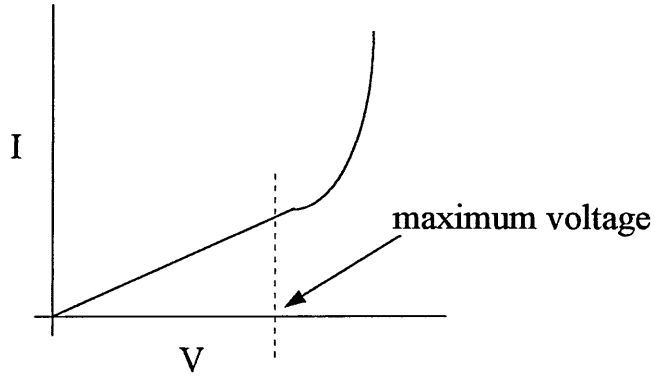


Figure 5 : Ohm's Law Plot Indicating Maximum Voltage

1.3.4 Effect of Capillary Length.

The capillary length is usually fixed by the instrument. However, if the capillary length can be varied by coiling it in a cassette the following rules apply. A longer capillary has a higher electrical resistance which reduces Joule heating and also has a greater surface area for heat dissipation. Since the applied electric field, E, is measured in volts.cm^{-1} ($E = V/l$), if the capillary length is halved then E is doubled, R is lowered and therefore I increases with a further increase in heat production. However, this can be lowered by decreasing the capillary diameter which reduces the current produced and Joule heating effects. If the capillary length is doubled, R increases and I decreases allowing a higher maximum voltage to be used. It is common to see separation conditions given as a voltage term, for example a 30 kV separation, whereas it would be more precise to quote the applied electric field in volts.cm^{-1} so that the parameters may be modified for instruments with different fixed length capillaries or restricted electrical settings.

1.3.5 Effect of pH.

A change in pH has a significant effect on the EOF as it affects the zeta potential. An increase in pH causes an increase in EOF, up to a pH of about 9, due to increased dissociation of the silanol groups on the inner capillary surface which becomes more highly charged. Almost no EOF exists below a pH of 2 as nearly all of the silanols are undissociated. A change in buffer pH also affects the ionisation of the solutes and their electrophoretic mobilities. As a rule of thumb an appropriate buffer pH is a minimum of 2 units above or below the solute pKa for the solute to be ionised.

1.3.6 Effect of Buffer Concentration.

Increasing the buffer concentration lowers the zeta potential and therefore decreases the EOF. Although a low buffer concentration gives shorter analysis times, if too low it can lead to broad, asymmetrical peak shape as a result of conductivity differences between the solute and the surrounding buffer causing a distortion in the electric field and an increase in current and Joule heating. Buffer concentrations should be at least 100 times that of the sample and are typically 10 mM to 100 mM to avoid problems of peak broadening due to disturbances in the EOF as described above. The use of organic buffers with a lower ion number, such as a tris buffer ([tris(hydroxymethyl)aminomethane] and tris hydrochloride), enables a higher molar concentration buffer to be used with a lesser degree of Joule heating than that associated with the equivalent concentration of an inorganic buffer.

1.3.7 Effect of Temperature.

A rise in temperature causes an increase in EOF because it reduces the viscosity of the run buffer, for example an increase of 1°C from 20 to 21°C reduces the viscosity of water by 2.4%. This reduction in viscosity is greater than the concurrent decrease in the dielectric constant (0.5% per °C for water).

1.3.8 Effect of Organic Solvent Additives.

These effects are more difficult to predict as they affect many variables including viscosity, dielectric constant and the zeta potential. An increase of 0-50% v/v methanol in the run buffer increases the viscosity of the solution, but a further increase from 50-100% v/v methanol reduces viscosity. In contrast, addition of acetonitrile from 0 up to 100% causes a decrease in viscosity.

1.3.9 Chemical Modification of the Capillary Wall.

The EOF may be greatly reduced or even eliminated by blocking the charges on the capillary wall which in turn reduces the zeta potential. This is achieved by dissolving chemical surfactants in the run buffer to give a dynamic coating of the capillary wall; or by the covalent bonding of polymers to the capillary wall.

Surfactants may be cationic, non-ionic or zwiterionic, for example cetyltrimethylammonium bromide (CTAB), polyoxyethylenesorbitan (TWEEN) and tris(hydroxymethyl)aminomethane (TRIS) respectively. Typically used polymers include polyacrylamide, poly(vinylalcohol) and poly(vinylpyrrolidone).

1.4 Electrophoretic Mobility

Electroosmotic flow, as described earlier, is a phenomenon which occurs when a potential difference is applied across the capillary. The EOF carries solutes through the capillary towards the detector. However, the basis of separation in capillary zone electrophoresis (CZE) is due to the electrophoretic mobility of the analytes. In the presence of an electric field, charged particles migrate, according to their electrophoretic mobility, towards the electrode of the opposite charge. Under these circumstances negative ions (anions) would migrate towards the positive electrode (anode) and back into the source vial without passing through the detection window in the capillary. However, the EOF is usually greater than the electrophoretic mobilities of the negatively charged solutes and they are carried with the buffer through the capillary and pass the detector at a net rate slower than that of the EOF. Cations, being positively charged, will migrate with the EOF towards the cathode as a result of their electrophoretic mobilities at a combined rate greater than

that of the electroosmotic flow alone. The rate at which the ions migrate depends upon their charge-to-size ratio. A smaller ion will migrate faster than a larger one of the same charge. An ion with a higher charge will also migrate quicker than a lower charged ion of the same size. In this way many negative ions will have a net movement, under the stronger influence of the electroosmotic flow, towards the cathode; neutral molecules travel together, unseparated, at the same rate as the EOF and cations migrate quickest of all. The overall order in which the analytes pass through the detector is therefore cations, neutrals, anions as shown in Figure 6.

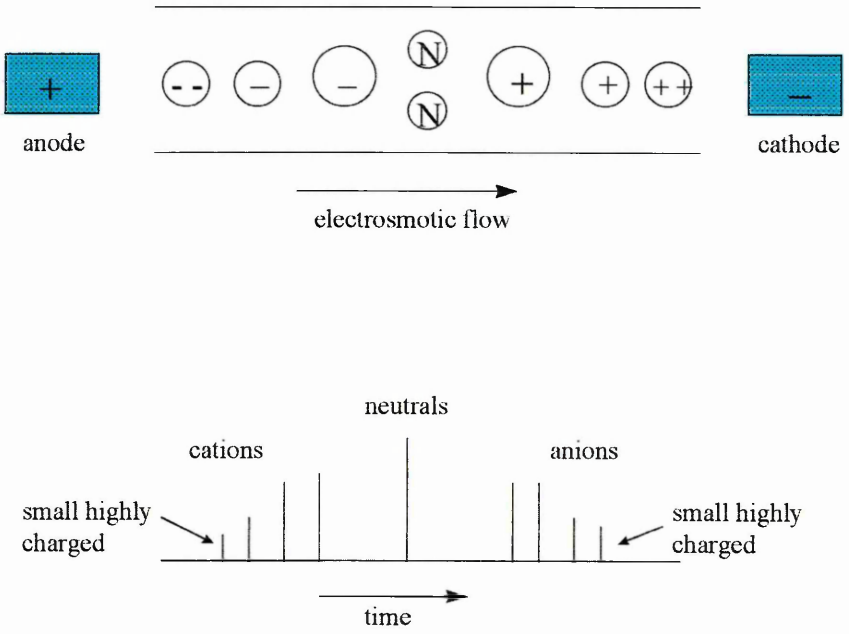


Figure 6 : Migration Order in Capillary Electrophoresis

Electrophoretic mobility is analogous to the electroosmotic mobility, μ_{EOF}, and has the same units, cm^2.volts.s. Just as an increase in buffer viscosity causes a reduction in EOF it also causes a reduction in electrophoretic mobility. Separation occurs because solutes migrate through the capillary at different velocities (ν_{EP}) according to the equation below.

$$\nu_{EP} = E(q/6\pi\eta r)$$

q = charge of the ionised solute

η = buffer viscosity

r = solute radius

The greater the charge-to-size ratio (q/r) the higher the electrophoretic mobility; and if the buffer strength and applied electric field remain constant, the higher the electrophoretic velocity also. Small highly charged molecules move through the capillary fastest; large molecules with a lower charge move slower, while neutral molecules have no charge (q) and therefore an electrophoretic mobility of zero.

An electrically charged solute will also migrate through a buffer under the influence of an electric field with an electrophoretic velocity in centimeters per second ($cm.s^{-1}$) - giving a total velocity of a combination of the solute's electrophoretic mobility and the applied electric field.

$\upsilon_{EP} = \mu_{EP}E$

υ_{EP} = electrophoretic velocity

μ_{EP} = electrophoretic mobility (= $q/6\pi\zeta r$)

E = applied electric field

It can therefore be seen that electrophoretic velocity is dependent on both mobility and electric field, whereas electrophoretic mobility is dependent on the size and charge of the solute and the buffer properties and is independent of an applied electric field.

It is in this way that mixtures of charged solutes can be separated, according to their charge-to-size ratios, by CZE. A solute's observed velocity is influenced by its electrophoretic mobility and the EOF:-

$\upsilon_{OBS} = \upsilon_{EP} + \upsilon_{EOF}$

Electrophoretic velocity and electrophoretic mobility can be measured from experimental parameters:

$\upsilon_{EP} = l/t_m - l/t_{nm}$

l = effective capillary length (from inlet to detector)

t_m = migration time of solute

t_{nm} = migration time of neutral marker e.g. mesityl oxide

$\mu_{EP} = (1/t_m - 1/t_{nm})(L/V)$ L = total capillary length

V = voltage

1.4.1 Factors which affect Electrophoretic Mobility

Factors that affect the size and/or charge of the solute or the buffer viscosity will affect electrophoretic mobility. They may also affect the EOF. Addition of a solvent modifier to the run buffer or a change in temperature will also lead to a change in electrophoretic mobility and EOF due to their effects on buffer viscosity. Buffer pH is very important in determining the degree of ionisation of the analytes. The mobility of anionic solutes increases with an increase in pH due to the resulting increase in negative charge around their pKa value. As pH decreases more of the negative charge is neutralised and mobility decreases. For cations, mobility is increased as the pH is lowered around their pKa value. The EOF is also increased at higher pH values, therefore changes are more easily observed using a coated capillary to eliminate EOF so migration times are only due to solute mobility.

1.5 Buffer Choice

The functionality and separation power of CE depend to some extent on the nature of the buffer chosen (Table 1) and upon the addition of any modifiers (Table 2). A buffer is most effective when within two units above or below the pKa of the solute under investigation. Zwitterionic buffers are particularly useful for the separation of proteins due to their low conductivity at their pI which reduces current draw and Joule heating.

Table 1 : Buffers for CE - pH

Buffer	Useful pH range
Phosphate	1.14 - 3.14
Citrate	3.06 - 4.76
Acetate	3.76 - 5.76
Phosphate	6.20 - 8.20
Borate	8.14 - 10.14
Zwitterionic Buffers	
MES	5.15 - 7.15
PIPES	5.80 - 7.80
HEPES	6.55 - 8.55
Tricine	7.15 - 9.15
Tris	7.30 - 9.30

MES = 2[N-morpholino]-ethanesulphonic acid

PIPES = Piperazine-N,N'-bis-[ethanesulphonic acid]

HEPES = N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]

The nature or properties of a buffer can be modified by the addition of various substances to improve selectivity and/or separation. Some examples are given in the Table 2 below.

Table 2 : Buffers additives for CE and their function.

Additive	Function
Inorganic salts	Protein conformational changes
Organic solvents	Solubiliser, EOF modifier
Urea	Solubilises proteins, denatures nucleotides
Sulphonic acids	Ion pairing agent, hydrophobic interaction
Cationic surfactants	Charge reversal of capillary wall
Cellulose derivatives	Reduces EOF, sieving medium
Amines	Covers free silanol groups.

1.6 Sample Injection

Strictly speaking sample introduction would be more technically correct phraseology for CE, however, the term "injection" has been adopted for common usage. Whereas a sample for HPLC or GC is injected into a moving stream of liquid or gas via a loop, valve or syringe; in CE the sample is introduced into the capillary while there is no buffer flow. Injection volumes are generally much smaller in CE (CZE, MECC: 1 to 50 nl) than in HPLC (10 to 250 μl); the exceptions being capillary isoelectric focusing (CIEF) where the whole capillary is filled with sample and capillary isotachophoresis (ITP) which can cope with volumes of up to 50% of the capillary. One reason why injection volumes are so small in CZE and MECC is that the capillary volumes are small; typically about 1 μl for a 50 cm by 50 μm internal diameter capillary, hence 1 to 50 nl sample sizes are common in CE. The length of the injected sample plug is kept to a minimum in CE to minimise zone spreading and the associated losses in efficiency and resolution. Samples may be introduced into the capillary by either hydrodynamic or electrokinetic injection.

1.6.1 Hydrodynamic Injection : This is achieved using either pressure or gravity.

Pressure - The capillary inlet is placed into the sample vial and pressure is then applied to the sample vial to force a small amount into the capillary before it is returned to the source vial. Alternatively a vacuum may be applied to the destination vial to draw the sample into the capillary. It should be noted however, that it is important to ensure that the buffer levels in the source and destination vials are kept constant to prevent siphoning effects. The volume of sample injected, V_i, is described by the Poiseulle equation:

$$V_i = \Delta P r^4 \pi t / 8 \eta L$$

where ΔP = pressure across the capillary

r = capillary inner radius

t = time the pressure is applied

η = sample viscosity

L = total capillary length

Gravity - Gravity or siphoning injection is accomplished by placing the capillary inlet into the sample vial and raising the sample vial so that it is higher than the destination vial. The injection volume by gravity can be calculated using:

$V_i = 2.84\times10^{-8}Htd^4/L$ where H = height the sample is raised

t = time sample is raised

d = capillary inner diameter

L = total capillary length

Hydrodynamic injection is reproducible provided that the variables are kept constant. This injection method is not suitable for capillary electrochromatography or capillary gel electrophoresis as the solid phase packing material or PAG produce too much resistance for gravity injection and may be extruded from the capillary on the application of pressure.

1.6.2 Electrokinetic Injection : In electrokinetic injection both the capillary inlet and the anode are placed into the sample vial and a voltage is applied to the sample vial for a given period of time; the anode and capillary are then returned to the source vial. Sample ions migrate into the capillary due to electroosmosis and electrophoretic mobility as they do during the separation. The quantity injected, Q_{inj}, is given by:

$Q_{inj} = V\pi ctr^2(\mu_{EP} + \mu_{EOF})/L$ where V = voltage

c = sample concentration

t = time voltage is applied

r = capillary radius

μ_{EP} = solute electrophoretic mobility

μ_{EOF} = electroosmotic mobility

This method can be prone to sampling bias in favour of higher charged, smaller cations which may become depleted on repeat injections from the same sample vial. Sample bias can be minimised by diluting the samples in a large volume of run buffer or by adding a small amount of a concentrated, non-detected ion to give all samples similar conductivities.

Gravity provides the simplest method of injection conceptually and is independent of sample matrix, giving good reproducibility in the event of differences in sample composition or pH, but

requires quite complex instrumentation. Pressure injection has all the advantages of gravity injection but requires more complex equipment with tight seals to provide a constant pressure/vacuum. Electrokinetic injection is the simplest method instrumentally with the only physical restriction being the ability to get both the anode and the capillary into the sample vial. Reproducibility is good provided that the sample matrix remains the same, but sample bias may be experienced.

1.6.3 Sample Stacking

The advantage of CE is its high efficiency separations for a wide variety of samples, but its main limitation is the lack of detection sensitivity, between one tenth and one hundredth that of HPLC when UV detection is used, due mainly to the restriction in sample volume. Sample stacking is one way of increasing sensitivity in CE as this concentrates the solute prior to electrophoresis. It is achieved by dissolving the sample in a buffer with a lower ion content than that of the run buffer and injecting hydrodynamically. The sample therefore has a lower conductivity so the solute ions migrate more rapidly through the injection plug until they reach the "barrier" with the run buffer, forming a "stacked" zone. Under normal CE running conditions cations will concentrate to the front of the plug, while anions are stacked at the rear - neutrals cannot be stacked. However, too low a concentration of buffer ions, or water, produces a laminar flow phenomenon due to the change in electroosmotic flow velocity resulting in peak broadening. A compromise must therefore be reached, for example, preparation of the sample in buffer one hundred-fold less concentrated than the run buffer

1.6.4 Field Amplification Sample Injection

This occurs when a sample in dilute buffer is injected electrokinetically into a capillary so that there is a higher electric field strength in the sample than in the run buffer. On application of a voltage the solute ions migrate more rapidly through the higher electric field of the sample plug until they reach the boundary with the lower electric field of the run buffer where they slow down forming a concentrated zone. The injection time can be reduced when using field amplification as more ions enter the capillary by electrokinetic injection in a more dilute buffer compared to one of a higher

concentration. This has the advantage of reducing problems associated with large injection volumes of low concentrations of buffer which causes changes in the electroosmotic velocity and the production of localised laminar flow as seen in sample stacking. Peak heights may also be increased by introducing a plug of water in front of the sample on the capillary to ensure an amplified field.

1.6.5 Sample Concentration by Isotachophoresis (ITP)

Large volumes of dilute samples can also be concentrated, or stacked, by sandwiching the sample of interest between electrolytes of high mobility (leading) and low mobility (terminating). Anions and cations must be separated in different runs using ITP. Generally the electroosmotic flow is eliminated so that the buffer and solute ions migrate, on application of an electric field, according to their electrophoretic mobilities alone, which may also be measured by the inclusion of "spacers" with known mobilities. The solute and buffer ions distribute themselves in the capillary until an equilibrium is reached where each component forms a discrete zone for its mobility and electric field. This process can be done on-column, termed transient ITP, where the buffer system can be changed after the bands have formed so that separation then occurs by CZE; or using dual capillaries - the first column on which the bands are concentrated, followed by electrophoretic separation on the second. On applying a constant current to the capillary, the zones move at a constant velocity with sharp boundaries through the detector, hence ITP is sometimes termed moving boundary or displacement capillary electrophoresis.

1.7 Capillary Choice

The first capillaries used were constructed from glass, Teflon or Pyrex. However, Pyrex is not transparent to ultraviolet light of short wavelengths so cannot be used below 280 nm and Teflon capillaries are less efficient at dissipating heat. Fused silica capillaries are far more popular now as they exhibit neither of these problems and are also easier to handle, being more flexible and easier to cut. Typically capillaries have an internal diameter (i.d.) of 50 to 100 μm: a compromise between a wider optical path diameter for greater detection sensitivity by UV/vis detection and a narrower i.d. which keeps Joule heating to a minimum. The capillary length affects separation time

and peak spacing - the longer the capillary the longer the separation time and the wider the peak spacing, but with the advantage of greater electrical resistance so Joule heating is reduced and a larger surface area improves heat dissipation enabling a higher voltage to be used. However, a longer capillary also suffers from greater axial diffusion which reduces efficiency. A shorter capillary creates more Joule heating for the same applied voltage and is less efficient at dissipating this heat so a compromise must be reached. It is therefore desirable to use the shortest capillary which still gives good separation of the analytes.

1.8 Diffusion and Joule Heating

Theoretically, a compound in solution introduced at one end of the capillary will form a "zone" and move unchanged through the capillary - hence capillary zone electrophoresis (CZE). In reality the solution will diffuse slightly into the surrounding run buffer by both longitudinal diffusion (parallel to the direction of migration) and radial diffusion (perpendicular to the direction of migration). This diffusion coefficient, D, has an effect on the spreading of the zone of the solute. However, this is minimal compared to the zone spreading caused by thermal, or convective, diffusion produced by passing an electric current through a conductive medium resulting in Joule heating. Joule heating warms the solution and causes convective diffusion. In free solution ion mobility increases by 2% per degree centigrade in the warmer centre of the capillary, resulting in poor separation due to zone spreading. Convective diffusion can be minimised by reducing the amount of heat generated and also by dissipating any heat that is generated. This is achieved by reducing the capillary diameter, rotating the capillary, adding a stabilising medium to the buffer, for example a gel or using a thermostated capillary compartment.

1.9 Methods of Detection.

In CE as in HPLC the solutes to be detected are in a liquid flow, therefore the detectors used are chosen using the same criteria i.e. sensitivity, linear dynamic range, selectivity and the ability to produce quantitative data. Commonly used detectors in CE include UV/vis absorbance - direct and indirect; fluorescence - direct and indirect; laser induced fluorescence; mass spectrometry; conductivity and refractive index detection. Indirect methods of UV or fluorescence detection are

used when the analyte has little or no natural chromophore/fluorophore. A background ion is incorporated into the run buffer which has a high UV absorbance/fluorescence, then, when the component of interest passes through the detection window its lack of absorbance/fluorescence results in a dip in the baseline which appears as a negative peak on the electropherogram. The major problem associated with on-column UV/vis detection is its poor sensitivity due to the very small amount of the solute present in the short, narrow detection window. Sensitivity can be enhanced by using a bubble cell or a "Z" cell to increase the path length and the volume passing through the detector, or using sample pre-concentration or sample stacking techniques, as described earlier.

1.10 Efficiency and Resolution

Separation in electrophoresis is driven primarily by efficiency, rather than selectivity (which is generally the case in chromatography), due to the very short, sharp solute zones achievable with electrically driven flow. Resolution, (R_s), in CZE is the result of differences between the mobilities of the analytes and can be described by the expression :

$$R_s = \frac{2(t_2 - t_1)}{(w_1 + w_2)}$$

where t = migration time

w = baseline peak width.

The difference necessary to resolve two zones is dependent on the length of the zones, which in turn is determined by dispersion forces acting on those zones. Dispersion therefore controls both zone length and the mobility difference required for separation to be achieved. Dispersion is the result of differing solute velocities within a zone, defined by the baseline peak width, w_b. This can be measure for a Gaussian peak by:

$$w_b = 4\sigma$$

where σ = standard deviation of the peak (in time, length or volume).

The efficiency, or number of theoretical plates, N, can be calculated by:

$$N = \left(\frac{l}{\sigma}\right)^2$$ where l = capillary length

to give available plates per metre, or calculated directly from an electropherogram by:

$$N = 5.54\left(\frac{t}{w_{1/2}}\right)^2 \quad \text{or} \quad N = 16\left(\frac{t}{w}\right)^2$$ where t = migration time

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

Efficiency in CZE is independent of capillary length, provided that Joule heat can be dissipated - a shorter capillary has a smaller surface area to disperse heat. Therefore, the shortest capillary can be used which gives adequate separation, with no loss in efficiency and a shorter analysis time. This is the reverse of HPLC, where efficiency is proportional to column length.

The resolution of two components can also be described with respect to efficiency by:

$$R_s = \left(\frac{1}{4}\right)\left(\frac{\Delta\mu_{ep}}{\overline{\mu}_{ep}}\right)\sqrt{N}$$ where $\Delta\mu_{ep} = \mu_{ep2} - \mu_{ep1}$ (difference in mobility)

$\overline{\mu}_{ep} = \frac{\mu_{ep2} + \mu_{ep1}}{2}$ (average mobility)

However, unlike efficiency, which increases in a linear fashion with applied voltage, a similar, proportional increase in resolution is not observed. This is because of the square root term, which means a doubling in resolution requires a quadruple increase in voltage. Benefits in resolution from increasing the voltage are also countered by an increase in Joule heating. The key to improved resolution is to increase $\Delta\mu_{ep}$, the difference in mobilities between the two species, best achieved by selection of the correct mode of CE and appropriate buffer choice to give optimum selectivity. The operational parameters should be selected such that there is a balance between resolution and analysis time.

1.11 Modes of Capillary Electrophoresis

1.11.1 Capillary Zone Electrophoresis (CZE)

The simplest form of capillary electrophoresis is free solution electrophoresis (FSCE) or capillary zone electrophoresis (CZE) performed using a fused silica capillary filled with buffer. Separation of the analytes is based on differences in their charge-to-size ratio. The net charge of most organic analytes is pH dependent and determines their electrophoretic mobilities. Inorganic anions/cations may be fully dissociated across the pH range in which case their net charge will remain constant and separation is defined predominantly by size.

1.11.2 Isoelectric Focusing (IEF)

Isoelectric focusing relies on the fact that a molecule will migrate as long as it is charged. IEF operates in a pH gradient using zwitterionic chemicals called carrier ampholytes with a low pH at the anode and a high pH at the cathode. When a voltage is applied the ampholytes will migrate to the oppositely charged end of the capillary until they reach the point at which their net charge is zero (their isoelectric point, pI), where they then stop. This "focusing" of the sample reduces band broadening by diffusion resulting in sharper peaks. Samples are loaded, focused, then mobilised for detection by applying a low pressure with the voltage still on. To be effective the EOF must be suppressed in IEF. This is achieved by coating the capillary walls with methylcellulose or polyacrylamide. Applications include determination of pI values of proteins and separation of immunoglobulins, haemoglobin variants and recombinant proteins. Urea is often incorporated into the buffer to maintain solubility of the proteins to prevent precipitation at their pI values.

1.11.3 Isotachophoresis (ITP)

ITP can separate anions and cations, but not in the same run. A zero EOF is also essential in isotachophoresis along with a heterogeneous buffer system. Sample ions separate, according to their electrophoretic mobilities in the presence of an electric field, between a leading electrolyte (higher mobility) and a terminating electrolyte (lower mobility), as described in section 1.6.5.

1.11.4 Micellar Electrokinetic Capillary Chromatography (MECC)

In contrast to ITP, MECC requires a robust and controllable EOF and utilizes micelle-forming surfactants. These are long-chain organic molecules with a hydrophobic tail and a hydrophilic head. Conditions can be chosen such that the direction of anionic micelle migration opposes that of the EOF, but that the velocity of the EOF is still sufficient to sweep the micelles through the capillary and past the detector. MECC is a combination of electrophoretic and chromatographic separation. Above the critical micelle concentration (CMC) these molecules come together to form aggregates, with their tails inward and heads on the outside (Figure 7). MECC allows the separation of neutral species by their hydrophobicity as they freely diffuse in and out of the micelles. Surfactants may be natural (bile salts e.g. sodium taurocholate) or synthetic e.g. SDS or CTAB (cetyltrimethylammonium bromide); and fall into four classes: anionic, cationic, zwitterionic and nonionic.

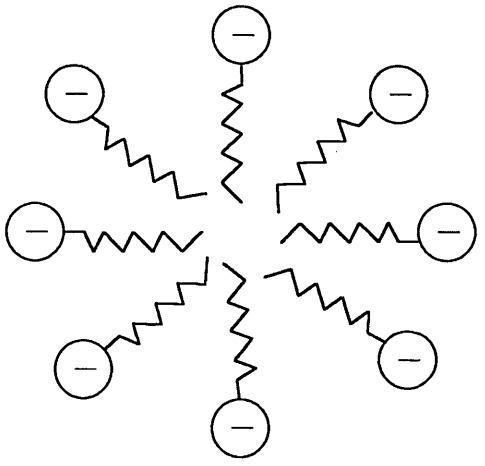


Figure 7 : Micelle Formation

(conformation of anionic micelle aggregation)

Separation is based on the partitioning of the neutral molecules between the buffer and the micelles. When a hydrophobic compound is added to an aqueous solution containing micelles it will partition into the hydrophobic portions of the micelles, effectively becoming solubilised, and will elute last from the capillary with the micelles. A highly water soluble compound will not partition into the micelles as it is not soluble in them; it therefore remains in the run buffer and is carried through the capillary to elute first at the same rate as the EOF. Molecules of intermediary hydrophobicity will partition in and out of the micelles and will elute from the capillary at a

migration time which is directly proportional to their hydrophobicities and the time they spend in the micelles.

1.11.5 Gel Electrophoresis (CGE)

Gel electrophoresis, either slab gel or capillary gel electrophoresis (CGE), uses polyacrylamide or agarose gels as anticonvective mediums for the analysis of proteins and deoxyribonucleic acids (DNA). Suppression of the EOF is very important for these separations. The proteins are denatured with 0.1% sodium dodecyl sulphate (SDS), which causes them to assume the same globular shape with a constant charge-to-size ratio. In this way DNA, proteins and oligonucleotides may be separated by size alone, as a result of the inherent molecular sieving properties of the gel and also by electromigration, and not charge-to-size ratios. Materials used may be physical gels for example hydroxypropylmethylcellulose (HPMC) which forms a porous structure by an entanglement of polymers; or chemical gels such as cross-linked PAG with urea or tris-borate-EDTA which forms covalent attachments to obtain a porous structure.

1.11.6 Capillary Electrochromatography (CEC)

CEC is a hybrid of CE and HPLC. An electric field is applied across a narrow bore capillary filled with a solid phase packing material so separation occurs as a result of both electrophoretic and chromatographic mechanisms. CEC is the most recent development in electrophoretic technology. More recent advances in CE techniques and equipment have improved CEC methods giving increased sensitivity and reproducibility with repeated injections of low nl injection volumes.

1.12 Applications

CE is applicable to the separation and/or analysis of a wide variety of compounds ranging from simple inorganic ions to complex biomolecules such as oligosaccharides, nucleic acids and proteins.

In theory any compounds which differ in their charge-to-size ratios can be separated by CZE. If molecules have the same charge-to-size ratios but are of different sizes, they may be separated by CGE. Compounds with no charge or which cannot be ionised but differ in their partitioning between a buffer and micelles can be separated by MECC. Also, compounds may be separated according to their isoelectric points by CIEF.

The applications of CEC combine those of both CE and HPLC: impurity analysis, chiral separations and main component assays as well as trace level detection. CEC can even be rationalised to be of universal importance as it takes advantage of the benefits of CE miniaturisation with highly efficient separations and the selectivity of HPLC. However, it is not without technical challenges - CE still offers the greatest promise in chiral separations whereas HPLC will continue to be the preferred option for preparative purposes.

It can therefore be appreciated that the various modes of CE can be applied in a wide variety of analytical fields to separate simple chemicals or complex mixtures whatever their physicochemical properties. Table 3 lists various substances and the possible CE techniques which could be used for their analysis in order of preference.

Anions: e.g. small inorganic anions such as chloride, nitrate, sulphate, phosphate etc. Traditionally these ions are separated by ion chromatography with suppressed conductivity detection. However, because these ions vary in their charge-to-size ratios they can also be separated by CZE with either indirect UV/fluorescence detection or conductivity detection. The above method is also applicable to small organic anions such as formate or acetate and has been used to detect organic acid drug counter-ions such as succinate and maleate[19], although it may be necessary to add flow modifiers to reverse the direction of the EOF.

Cations: e.g. alkaline earth and transition metals. These have traditionally been separated by cation exchange chromatography, but can also be analysed by CZE due to differences in their charge-to-size ratios. Cations are simpler to analyse than anions as they migrate in the same direction as the EOF therefore there is no need to add flow modifiers. Assi *et al.*[20] have successfully developed an assay for the simultaneous determination of basic drugs and their acidic counter-ions by CZE.

Proteins[21], Nucleic acids[22] and DNA[23] have traditionally been analysed using slab gel electrophoresis (PAGE) as their electrophoretic behaviour is fairly well understood. CE is

particularly well suited for these separations, using different modes of electrophoresis depending on the properties of the substance under investigation. CZE, CGE and MECC with chiral selectors have been used for peptide isolation; characterisation of peptide-binding reactions; purity determinations and mapping of protein digests. Serum proteins, glycoproteins, lipoproteins, antibodies, haemoglobins, food proteins and rec-proteins have all been successfully separated by CE. The development of hyphenated techniques such as CE-MS has provided high resolution separations with increased sensitivity for direct analysis from biofluids and the capability for enzymology and immunological studies using affinity CE to study protein structure. The rapid, high efficiency separations of CE have also made high throughput DNA sequencing cost-effective. Multiplexed CE has been used in sequencing the human genome to eliminate the bottleneck process of separating Sanger fragments and reading fluorescent dye labels.

Table 3 : CE Mode Selection (appropriate choices for various applications)

Analyte	← Order of choice				
Small Ions	CZE	ITP			
Small Molecules	MECC	CZE	ITP		
Peptides	CZE	MECC	IEF	CGE	ITP
Proteins	CZE	CGE	IEF	ITP	
Oligonucleotides	CGE	MECC			
DNA	CGE				
Pharmaceuticals	CZE	MECC	CEC		

1.12.1 Pharmaceutical Analysis[24]

The use of capillary electrophoresis in the pharmaceutical industry has greatly increased in recent years and such methods of analysis are now routinely accepted by regulatory authorities. CE offers

rapid, simple method development with reduced operating costs as well as additional and different separation mechanisms compared to HPLC. CE has been utilised for dissolution analysis to monitor the rates of dissolution of drugs from their formulations/tablets[24-27]; excipient analysis to measure the formulation ingredients such as sugars and lactose cyclodextrins[28], or the inorganic ion content in other raw materials e.g. buffers, sweeteners and electrolytes and provides excellent cross-correlation purity data with HPLC, with accurate impurity determinations down to 0.05% m/m[29,30]; as well as its most frequent application in main-component assays. The majority of acidic drugs, such as warfarin[31] and related substances, can be separated using a borate buffer with an approximate pH of 9, whilst a low pH phosphate buffer is suitable for most basic drugs, for example propranolol[32]. Another option for the analysis of basic drugs is short-end injection, where the sample is introduced into the end of the capillary nearest to the detector and a reversed polarity is applied[33]. This method reduces analysis times, increases sensitivity due to increased stacking effects, allows the use of greater electrolyte strengths and higher voltages which improve resolution and reduces peak tailing and can overcome problems with analysis of samples with high salt contents.

A vast number of CZE and MECC assays exist for the analysis of a variety of pharmaceutical products including amphetamines, anesthetics, antibiotics, anti-inflammatories, barbiturates, steroids, heart drugs, neurological agents and vitamins, comprehensive lists of which have been documented in recent reviews[5, 34, 35]. Many pharmaceutical assays also take advantage of the increased selectivity offered by chiral CE[35] for enantiomeric separations. Chiral CE method development has been facilitated by existing knowledge in chiral HPLC and is highly efficient, simple, fast and convenient. There are now a wide variety of chiral selectors[37-41]available for both aqueous and non-aqueous CE, including cyclodextrins, crown ethers, antibiotics, polysaccharides, proteins and chiral surfactants. The microsize nature of CE allows the investigation of more numerous, exotic and expensive reagents, often aided by a factorial experimental design to optimise operating parameters. Another factor in the increasing interest in CE is the introduction of hyphenated techniques to enhance detection and provide structural information using CE-MS[42] and CE-NMR[43], although the nanolitre injection volumes of CE can be a limiting factor. A further advancement lies in the arena of CEC, a technique which couples the miniaturisation and electrically driven plug flow profile of CE with the added separation of a solid phase packing material. Euerby *et al.*[44]describe the separations of both acidic and basic drugs, including diastereoisomeric steroids, barbiturates and non-steroidal anti-inflammatories, using standard, full

length capillary and short-end injection techniques and incorporating isocratic and step-gradient elution.

Whilst the use of CE in pharmaceutical analysis is now becoming an established technique, its further application in drug metabolism studies is still relatively novel. Lanz and Thormann[45] describe a chiral CE method, using a pH 3 phosphate buffer with hydroxypropyl-β-cyclodextrin, for the characterisation of the stereoselective *in vivo* metabolism of methadone and its primary metabolite in human urine. Similarly, Thormann *et al.* [46] have reported a successful assay for the stereoselective *in vivo* metabolism of thiopental and its metabolite pentobarbital via analysis of their enantiomers in human plasma using chiral recycling isotachophoresis with a pH 8.5 phosphate buffer and hydroxypropyl-γ-cyclodextrin. In both cases chiral CE proved to be a simpler and less expensive method than HPLC with an enantioselective column, or GC with stable isotope labelling or sample derivatisation; although solid phase/liquid-liquid extraction was recommended for sample clean-up prior to analysis.

In vivo metabolism studies are time consuming and costly. These factors can be reduced by employing *in vitro* metabolism techniques using, most commonly, liver and kidney homogenates to evaluate the *in vitro* clearance and metabolic stability of potential drug candidates. HPLC has been the traditional analytical technique for such studies, although some CE methods have been reported. A comprehensive review of the application of CE and related techniques in both *in vivo* and *in vitro* metabolism studies has been published by Naylor *et al.*[47]. This paper describes the importance and difficulties of analysing biological samples in order to characterise and identify metabolically transformed products; including a CZE- MS analysis method for the *in vitro* metabolism of HAL, (4-(4-chlorophenyl)-1-[4-(4-fluorophenyl)-4-oxobutyl]-4-piperidinol), in guinea pig microsomes. Blaschke *et al.* compared both chiral and achiral HPLC methods against chiral CZE analysis for the stereoselective *in vitro* metabolism of the racemic antiasthmatic/antiallergic drug flezelastine in human, rat, bovine and porcine liver microsomes. Once again, the greater efficiencies of CZE were found to provide a more suitable assay for the enantiomers, with improved separation over HPLC.

With the advantages of simple, rapid method development, short analysis times and the high efficiencies giving improved separations, along with further advances in non-aqueous CE and CEC it is proposed that we will see capillary electrophoretic techniques increasingly becoming methods of choice in this field.

Method development from first principles can be a daunting task, especially in CZE with the numerous parameters which can be altered to affect migration. This process can be made easier and quicker using one of several statistical experimental design techniques. These include simple screening designs to establish the key factors, central composite studies and overlapping resolution mapping which give response surfaces to predict areas of optimal performance and factorial experimental design (FED) which provides the most detailed information for both inter and intra-parameter interactions. In FED the maximum amount of information can be gleaned from the minimum number of experimental runs, either using a full factorial design if only a few parameters and levels are to be evaluated, or using a fractional factorial design to reduce the number of runs when many levels and or parameters are examined. It is important to remember in a full FED to include every possible combination of factors to obtain a complete, unbiased result.

The simplest form of FED is to investigate 2 factors (x and y) at 2 levels (x_1, x_2, y_1 and y_2). In this case only one function can be fitted to the data - a straight line. The effect of changing each variable may be additive (Figure 8) or interactive (Figure 9).

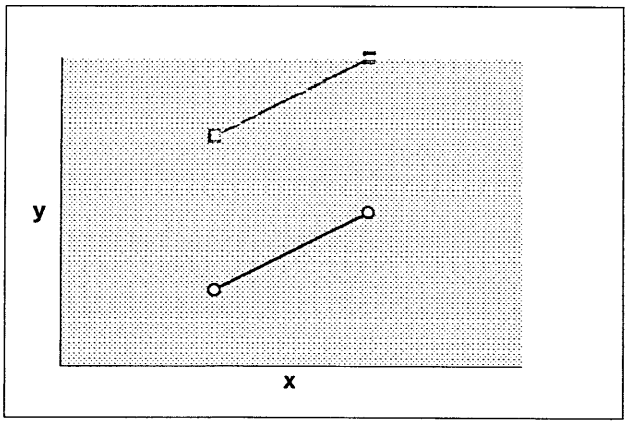


Figure 8 : FED - Additive Relationship

(2 factors examined at 2 levels showing an additive effect)

The main effect for each factor can be estimated by calculating the average difference between the results.

$$\frac{(x_1,y_1 - x_1,y_2) + (x_2,y_1 - x_2,y_2)}{2} = \text{Main Effect}$$

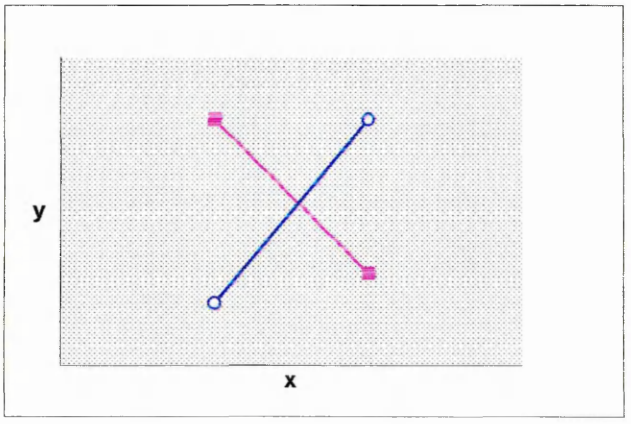


Figure 9 : FED - Interactive Relationship

The interactive effect for each factor can be estimated by calculating half the difference between the results.

$$\frac{(x_1,y_2 - x_1,y_1) - (x_2,y_1 - x_2,y_2)}{2} = \text{Interactive Effect}$$

It can be seen, therefore, that it is important to establish whether the factors have a main or interactive effect. Factorial design is a good method for obtaining this information.

The minimal 2 factor, or 2^f, design is very popular; requires few experimental runs; is easy to interpret and can give an indication of major trends. It also forms the basis of FED which can be augmented to give a greater quantity and quality of data. A 3^f design provides more information

on the effects and interactions of the factors in which main (linear) and quadratic (curved) effects can be separated. By including more experimental points the fit of the line may be improved. While a linear fit is the only functional available for 2 data points, the inclusion of a third data point gives the option of curvature as described by a quadratic.

The two important decisions in FED are which factors to choose and what data points to describe them. All permutations of the factors must be examined over a range which is neither too wide nor too narrow to accurately describe the curvature of a quadratic effect.

The display of levels to be investigated for each factor is called a design matrix (Table 4 = 2^f design and Table 5 = 3^f design) which may also be presented graphically in the form of a box diagram (Figures 10 (2^f) and 11 (3^f)). Traditionally used signs are - and +, or 0 and 1. - / 0 indicating without (qualitatively) or low values (quantitatively) and + / 1 indicating with (qualitatively) or high values (quantitatively). By filling in the results obtained at the corners of a square (2^f) or a cube (3^f) the general trends become evident for the effects of combining each and every variable.

Table 4 : FED - 2^f Design Matrix.

(this table details the 4 experimental runs required to fulfill a 2^f Design Matrix)

Run number	Time (min)	Temperature (°C)	Response (mg product)
1	30 (-)	20 (-)	60
2	30 (-)	37 (+)	76
3	60 (+)	20 (-)	73
4	60 (+)	37 (+)	93

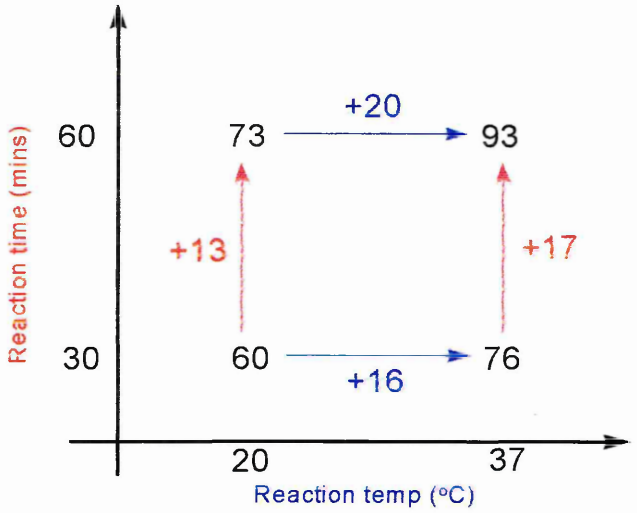


Figure 10 : FED - 2^f Box Diagram

(results from Table 4 represented graphically)

Table 5 : FED - 3^f Design Matrix

(this table details the 8 experimental runs required to fulfill a 3^f Design Matrix)

Run	Time (min)	Temperature (°C)	Buffer A (-) or Buffer B (+)	Response (mg product)
1	-	-	-	63
2	+	-	-	43
3	-	+	-	75
4	+	+	-	58
5	-	-	+	65
6	+	-	+	52
7	-	+	+	62
8	+	+	+	80

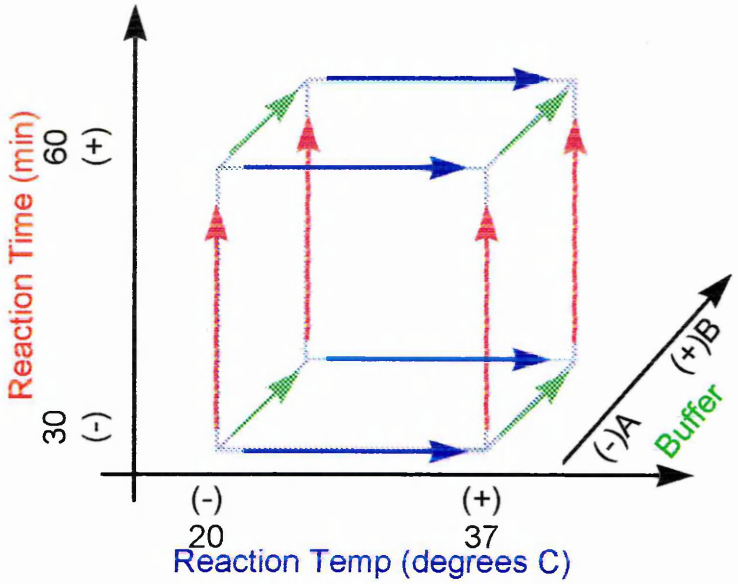


Figure 11 : FED - 3^f Box Diagram

(results from Table 5 represented graphically)

For optimisation of conditions the variables should be plotted in pairs on 2 dimensional linear axes, joining high and low values - optimal results will be obtained where the lines cross in Figure 12 below.

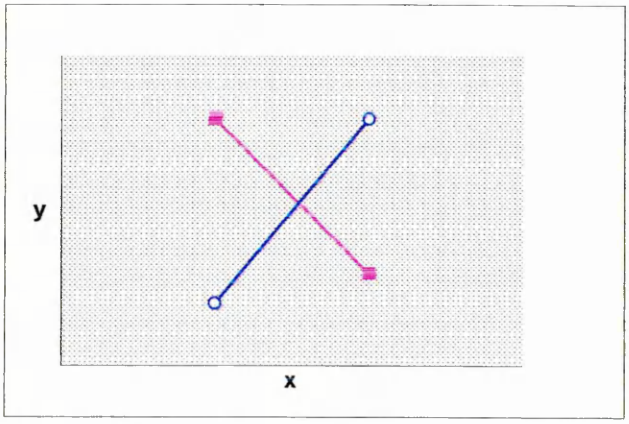


Figure 12 : FED - Optimal Parameters as Indicated by an Interactive Relationship

In a 3^f design each effect can be examined individually by calculating the mean differences for each opposing side of the cube (Figure 13).

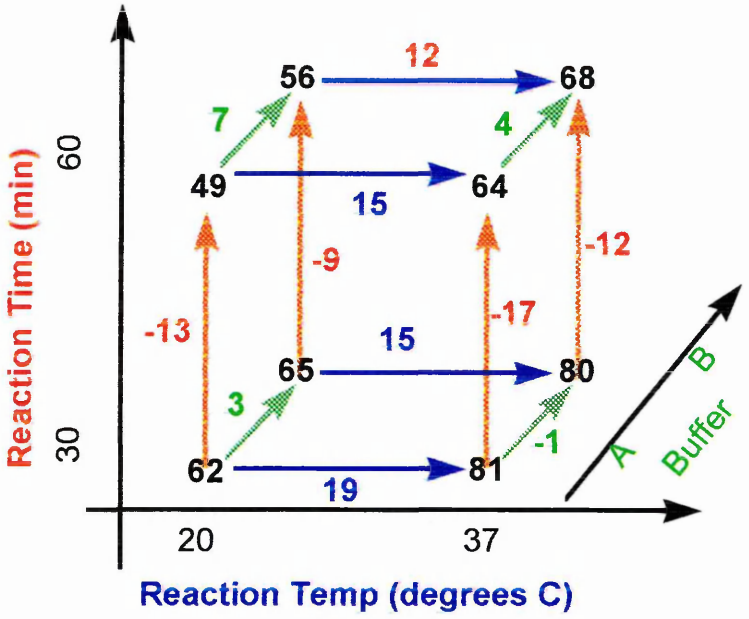


Figure 13 : FED - A 3D Box Diagram Depicting Mean Results

The major advantage of factorial design is the vast reduction in both experimental samples and experimental runs and correspondingly in time and effort. This may also mean that all of the variables may be examined on the same day under the same conditions, e.g. equipment, test solutions, buffers, ambient temperature etc., which also reduces variability introduced by separate experiments performed on different days. Results obtained in this way are more accurate and reliable than the alternative of duplicate sampling, for the same degree of precision, which also may not indicate the possibility of an interaction between the factors under investigation. A further advantage of FED is that if the conditions chosen initially were not optimal, then analysis of the values obtained at the corners of the square/cube will point towards conditions for the best results.

The disadvantages of FED become evident if all possible combinations of the variables are not represented which can lead to poor or biased estimates of effects. Also, if many levels for each of

the factors are being co-considered then the numbers of samples increases dramatically, for example an experiment with 3 factors at 3 levels (3^3) would require 27 runs if all permutations were to be covered.

FED can be applied advantageously in CE method development, especially with the advent of programmable instruments whereby a series of experiments can be set up to examine different operating temperatures, buffer concentrations, pH values and applied voltages in a single run left overnight with no additional input required from the analyst. In this way the predominant factor(s) affecting migration / separation / resolution can be identified easily and at reduced cost in both time and materials. The incorporation of FED in general CE method development has been reviewed by Altria *et al.*[50], whilst specific applications in pharmaceutical analysis have been documented by Clark *et al*[51], Pokorna *et al*,[52] and Vargas *et al*[53].

3.1 Method Development.

It is good practice to collate as much information as possible on the component(s) to be analysed in order that the most appropriate mode of CE may be chosen and the parameters therein. It may be that a method has already been reported, as was the case with propranolol, which can be modified to suit your needs, or it may be necessary to start from first principles, as with chlorogenic acid. Once a suitable method has been developed it can be optimised to give a better separation. Traditionally this has meant a long and tedious set of experiments, adjusting each of the many variables (temperature, buffer type, pH and concentration etc.) one at a time to be sure that any effect on migration, selectivity and separation could be attributed to the correct parameter. Now, however, it is possible to reduce the number of experiments necessary and the time and effort involved to perform them by using a structured method design procedure such as factorial experimental design (FED) explained earlier. Not only was it expected that FED would indicate the variable responsible for improved resolution and selectivity, but also the effects of combinations of the various parameters and the direction of change which could be beneficial to the separation. Once a method has been optimised it should be tested by repeated injections for accuracy and precision to ensure that it will be reliable, robust and reproducible. If the method was to be quantitative, then the limit of detection and linearity should be calculated by running a series of calibration standards. The limit of detection is generally taken to be the lowest concentration which can be measured reproducibly and is at least twice the height/area of the baseline noise of the system. Further analytical parameters which may also be investigated include the specificity and selectivity of the method to ensure there are no endogenous components which interfere with or influence the peaks of interest.

Points to Consider

When beginning CE method development from first principles there are several aspects which should be born in mind to increase the chances of success. The component of interest should be evaluated for solubility, stability and the most appropriate method of detection, whilst also considering the optimum buffer choice (pH, ionic strength, any additives) and also the operating temperature.

A number of questions should be addressed in each of the following areas (i-iv) :-

i) Compound solubility :

Is the compound of interest soluble at 1mg/ml in water?

Is this true across a range of pH values?

If not a small quantity, up to 25%, of organic modifier such as methanol or acetonitrile may be added. For small, insoluble, hydrophobic molecules the addition of sodium dodecylsulphate (SDS) forms micelles which effectively "solubilise" the compound in their hydrophobic portions. Proteins can be solubilised by including urea in the run buffer or a dispersing agent such as ethylene glycol.

ii) Compound stability :

Is the analyte unstable at any particular pH or pH range?

Is it thermally labile?

Problems such as these could restrict the operating parameters and require further method optimisation to avoid confusing results from the inherent physicochemical properties of the compound leading to its degradation during analysis.

iii) Detection :

What is the UV wavelength which gives maximum absorption?

What concentrations are you expecting to be working at?

Will sensitivity be a problem?

Is mass spectrometry an alternative method of detection?

If a mixture of components is to be analysed, know how many in order to ensure resolution of all the components.

iv) Buffer choice :

As a general rule, a buffer is most effective within one or two pH units of its pKa. It is best to work at a low buffer concentration to avoid non-specific ionic strength effects and to reduce Joule heating - 50 mM is a good, recommended starting point. If possible, prepare buffers at the temperature at which they will be used and re-check pH measurements after the addition of any modifiers as they may alter the pH. All buffers should be filtered prior to use (0.45 μm - 0.2 μm filter) and refrigerated to prevent microbial contamination.

The following starting conditions are suggested:-

50 cm, 75 μm i.d. fused silica capillary

temperature of 25°C

20 kV applied voltage

UV at 214 nm

50 mM buffer of an appropriate pH

5 second, 50 mbar pressure injection of 1 mg/ml solution.

To obtain increased efficiency the ionic strength of the run buffer may be increased and to improve separation the pH is adjusted.

3.2 Materials

The following section contains details of the materials used.

a) Compounds:

i) Propranolol (RMM = 259.34) was obtained from Sigma (Poole, UK.)

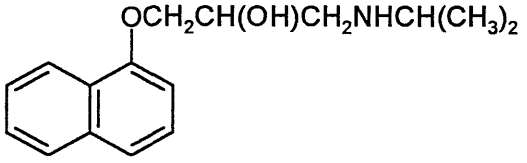


Figure 14 : Structure - Propranolol

ii) Chlorogenic acid (RMM = 354.3) was obtained from Sigma (Poole, UK.)

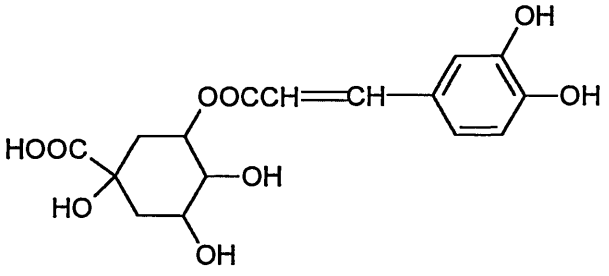


Figure 15 : Structure - Chlorogenic Acid

b) Reagents:

i) HPLC grade methanol was obtained from Fisher Scientific (Loughborough, UK.)

ii) Trifluoroacetic acid (TFA) was obtained from Fluka (Buckinghamshire, UK.)

iii) Ammonium acetate was obtained from Fisher Scientific (Loughborough, UK.)

iv) Lithium hydroxide was obtained from BDH (Poole, UK.)

v) Phosphoric acid was obtained from BDH (Poole, UK.)

vi) Sodium hydroxide (analytical reagent RMM = 40) was obtained from Fisons (Loughborough, UK) and prepared as a 0.1 M solution (4g in 1L deionised water).

vii) Sodium tetraborate ($Na_2B_4O_7.10H_2O$) was obtained from Sigma (Poole, UK.)

viii) β- Nicotinamide adenine dinucleotide phosphate (β-NADPH) reduced form was obtained from Sigma (Poole, UK.)

ix) Boric acid (H_3BO_3) was obtained from Sigma (Poole, UK.)

c) HPLC mobile phase:

i) Propranolol : 60:40:0.1:0.77 v/v/v/w methanol:deionised water:TFA:ammonium acetate

ii) Chlorogenic acid - a suitable system could not be developed.

d) CE buffers:

i) Propranolol : 50 mM lithium phosphate buffer

ii) lithium phosphate molecular weight = 41.96, i.e. 50 mM ≡ 210 mg in 100 ml purified water, pH to 2.5 with phosphoric acid.

iii) Chlorogenic acid : 50 mM borate buffer

iv) sodium tetraborate ($Na_2B_4O_7.10H_2O$) molecular weight = 381.4 which includes 180 water,
ie. 50 mM ≡ 252 mg in 100 ml purified water, measured pH = 9.2

All buffers were filtered using a 0.45 μm filter from Gelman Sciences (Northampton, UK) prior to use and stored at 4°C.

3.3 Instrumentation

The following apparatus were used in these experiments:

A Perkin Elmer HPLC system (Beaconsfield,UK) with a PeakPro data capture program (BBN Software Production Corporation, Cambridge, UK)

A Beckman P/ACE 2050 (High Wycombe, UK).

A Hewlett Packard HP^{3D} CE (Stevenage, UK).

The details of each instrument are outlined below:

HPLC

Perkin Elmer ISS 200 series autosampler
Perkin Elmer ISS 200 series gradient pump, isocratic flow at 1 ml/min
Shimadzu SPD-10 UV detector (Milton Keynes, UK)
Hichrom 5 μ C18 HiRPB column 25 cm × 4.6 mm (Reading, UK)

Beckman P/ACE

System 2050 with integral autosampler
UV filters for detection at 200 nm, 214 nm, 254 nm, 280 nm, 260 nm and 300 nm
(monitored at 200 nm - optimised against interference)
Fused silica capillary, eCAP, 375 μm o.d. x 75 μm i.d. x 67 cm length (50 cm effective length)
obtained from Beckman, High Wycombe, U.K.

Hewlett Packard HP^{3D}

Integral autosampler
UV/vis Diode array detector (190 nm - 600 nm)
(monitored at 214 nm - optimised against interference)
Fused silica capillary, eCAP, 375 μm o.d. x 75 μm i.d. x 67 cm length (50 cm effective length)
obtained from Beckman, High Wycombe, U.K.

3.4 Experiments

The methods used can be divided into two sections; those for HPLC and those for CE analysis, the experimental details are outlined below. The results were used to compare the reliability, reproducibility, accuracy, precision and general ease of use of the two analytical techniques. The use of both the Beckman P/ACE and the HP^{3D} gave a comparison of the two CE instruments.

Two compounds were chosen for investigation, a base and an acid; these were propranolol and chlorogenic acid respectively.

For propranolol analysis, existing assays for both HPLC and CE were modified to suit the equipment and conditions used. Various attempts were also made to further modify and optimise the propranolol CE method for use with a series of analogous compounds. However, they did not appear to run under these conditions and a separate method was developed for the analysis of this group of chemicals.

A CE method was developed from first principles for chlorogenic acid, using standard starting conditions. Attempts to develop a suitable HPLC method for chlorogenic acid were unsuccessful.

The method development and modifications performed are outlined in the experiments described below. These methods were then used to evaluate the various analytical techniques and assess their application to pharmaceutical science in the field of *in vitro* metabolism.

3.4.1 Experiments to reproduce the existing propranolol assays.

CE : An existing method[32] was adapted for the analysis of propranolol by CE. A hollow silica capillary with an internal diameter of 75 μm and 50 cm in length to the detection window was used with a low pH buffer system (50 mM lithium phosphate pH 2.5) to ionise the base. Propranolol was monitored at a wavelength of 214 nm. This method was run initially on the Beckman P/ACE, but also, at a later date on the HP^{3D} to give a direct comparison of the two CE instruments (Experiment 3.4.4).

HPLC : An existing reversed phase chromatography method[32a] was adapted for the HPLC analysis of propranolol using a methanol/water/ammonium acetate/trifluoroacetic acid mobile phase. A higher UV wavelength of 254 nm was chosen to avoid background absorbance from the methanol in the mobile phase.

The CE and HPLC conditions chosen are summarised below:-

P/ACE, HP^{3D} CE	Perkin Elmer HPLC
Fused silica capillary	Hichrom 5 μ C18 HiRPB column,
57 cm (50 cm to detection window)	150 × 4.6 mm
75 μm internal diameter (i.d.)	Mobile phase = 60:40:0.1:0.77
Temperature of 30 °C	methanol:water:TFA: ammonium acetate (v/v/v/w)
50 mM pH 2.5 lithium phosphate buffer	
30 kV applied voltage	Flow rate = 1 ml/min
UV detection at 214 nm.	UV detection at 254 nm.
5 second, 50 mbar pressure injection.	Injection volume 10 μl

Equipment set-up : The respective instruments were set up as outlined above. The HPLC column was washed through with 100% methanol to wet the silica then allowed to equilibrate with mobile phase for twenty minutes prior to use. Each new CE capillary was conditioned with 0.1 M sodium hydroxide for 10 minutes, then flushed through with water for 1 minute, then washed with the run buffer for 4 minutes prior to use. This conditioning routine was performed before each set of experimental work and also as a rinsing step, but with a 1 minute initial flush with the 0.1 M sodium hydroxide, between each sample injection. Silica is soluble in a basic solution, so the process of flushing the capillary with sodium hydroxide dissolves some of the outer silica leaving a "new" surface. The alkaline wash also removes any buffer ions or proteins which may have adsorbed onto the capillary wall. Organic impurities may be removed by incorporating an organic rinse with a solvent such as methanol. The following rinses with water and run buffer remove water-soluble salts and re-equilibrate the capillary to give reproducible migration times. An alternative conditioning procedure may use an acidic solution such as 0.1 M hydrochloric acid, however this would be more applicable to a method using an acidic run buffer to reduce equilibration times and improve reproducibility of migration times.

The conditions stated above for alkaline capillary conditioning and washing were found to be suitable for the investigations carried out for both propranolol and chlorogenic acid and were included as an integral part of each experiment - this information will not be repeated in subsequent sections.

Sample preparation : Propranolol was dissolved in methanol at 10 mg/ml. Subsequent dilutions were made with purified water to give analytical standards between 0.005 mg/ml and 2 mg/ml. The 2 mg/ml propranolol standard was chosen to give an adequately measurable UV absorbance by both HPLC and on the Beckman P/ACE.

Sample analysis : Six replicate analyses of the 2 mg/ml propranolol solution were made by HPLC and CE according to the protocols above to confirm suitability of the methods and the production of an adequate, measurable and reproducible response.

3.4.2 Method modification for CE analysis of the propranolol analogues

A group of 13 analogues similar in structure to propranolol were also chosen for analysis to assess the selectivity of CE using the HP^{3D}. The close resemblance of this set of compounds to propranolol gave the expectation that they would run under very similar conditions on both HPLC and CE. This proved to be the case for HPLC but not so for analysis by CE.

Propranolol analogues : See Table 6

Sample preparation : Each compound was dissolved in methanol to give a 0.1 mg/ml solution.

Sample analysis 1 : A single injection was performed for each compound on the HPLC and Beckman P/ACE instrument as described in 3.4.1 above.

Sample analysis 2 : Further method modification was required to run the propranolol analogues by CE as they were not detected with the 50 mM pH 2.5 lithium phosphate conditions; this result was confirmed by reanalysis following reconditioning of the capillary. A complete switch was then made to a basic 50 mM borate run buffer with a measured pH of 9.2 using the parameters detailed below:-

HP^{3D} CE

fused silica capillary

57 cm (50 cm to detection window)

75 μm internal diameter (i.d.)

temperature of 30°C

5 second, 50 mbar pressure injection

UV detection at 214 nm.

20/30 kV applied voltage

20/50 mM pH 9.2 sodium tetraborate buffer

Initially a 20 mM run buffer was prepared and used with an applied voltage of 30 kV; this was subsequently modified to a 50 mM run buffer with 30 kV and 20 kV applied voltages to optimise the run time and response.

3.4.3 A comparison of the propranolol analogues run on CE versus HPLC.

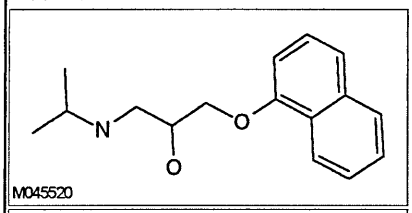
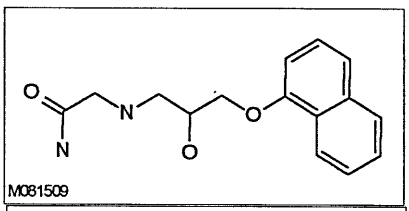
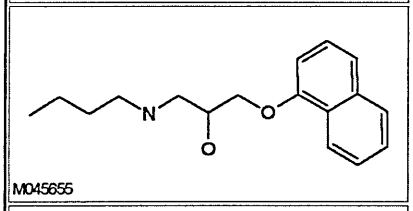
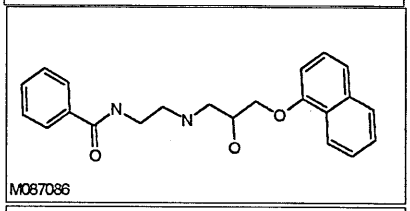
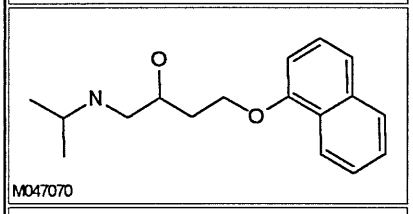
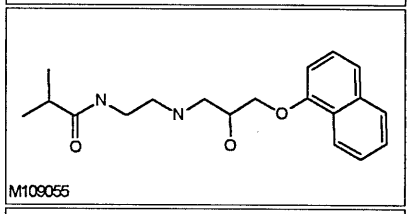
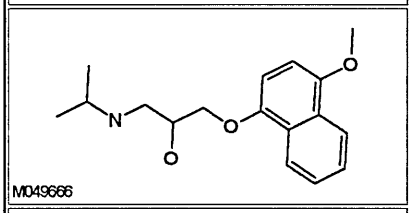
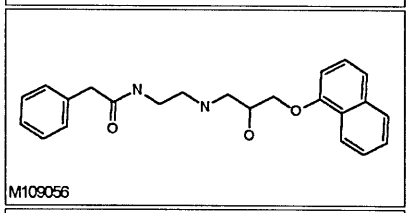
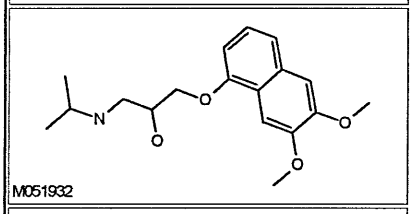
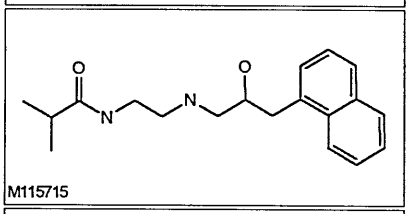
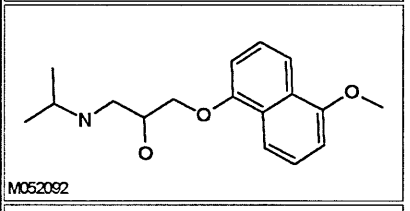
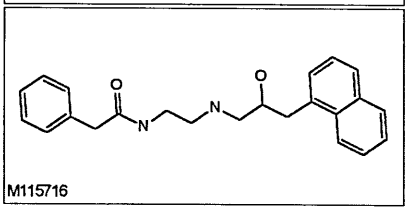
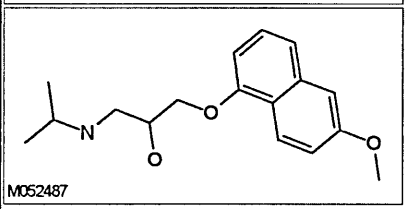
The same group of 13 propranolol analogues was investigated further to assess selectivity by HPLC. All compounds, including propranolol, were run under a single generic HPLC method, based on the propranolol assay conditions, to compare selectivity of HPLC versus CE. The methodologies for each are outlined overleaf:-

P/ACE, HP^{3D} CE	Perkin Elmer HPLC
Fused silica capillary	Hichrom 5 µ C18 HiRPB column,
57 cm (50 cm to detection window)	250 × 4.6 mm
75 µm internal diameter (i.d.)	Mobile phase = 60:40:0.1:0.77
Temperature of 30°C	methanol:water:TFA: ammonium
	acetate (v/v/v/w)
50 mM pH 9.2 borate buffer	
20 kV applied voltage	Flow rate = 1 ml/min
UV detection at 214 nm.	UV detection at 254 nm.
5 second, 50 mbar pressure injection.	Injection volume 10 µl

Sample preparation : Each compound was dissolved in methanol to give a 0.1 mg/ml solution. The solutions used were those prepared in experiment 3.4.2 above.

Sample analysis : A single injection was performed for each compound as described above. Once a suitable migration/retention time had been established for each compound the method was assessed for reproducibility, linearity and precision in Experiment 3.4.7.

Table 6 : Propranolol Analogues (NB. Hydrogen atoms are not shown)

Structure CHEMISTRY	Mol.Wt	Structure CHEMISTRY	Mol.Wt
 M045520	295	 M081509	274
 M045655	273	 M087086	364
 M047070	273	 M109055	330
 M049666	289	 M109056	378
 M051932	307	 M115715	314
 M052092	289	 M115716	362
 M052487	289		
	243		

3.4.4 A comparison of the Beckman P/ACE and the Hewlett Packard HP^{3D} CE instruments.

The 2 mg/ml methanolic analytical standard for propranolol prepared in experiment 3.4.1 was also run, using the same conditions as described previously, on the Hewlett Packard HP^{3D} to provide a direct comparison of the two sets of CE equipment.

A single set of calibration results was also obtained on the Beckman P/ACE as part of the *in vitro* experiment, details of which are given in 3.4.8.

These results were combined with the HPLC and Beckman P/ACE data for an overall comparison of the two CE instruments and the analytical power of CE versus HPLC. This information will be discussed jointly, with later results, in sections 4.4, 4.7 and 4.8.

3.4.5 The development of a CE assay for chlorogenic acid

A method for the analysis of propranolol by CE had already been established. The aim of this experiment was to develop and optimise an analytical CE method from first principles. Chlorogenic acid was chosen for this purpose (in contrast to propanolol, a base), in order to investigate the suitability of CE for acidic compounds. The method development for chlorogenic acid was performed on the HP^{3D} CE instrument, using a UV wavelength of 214 nm to monitor UV absorbance. To ensure that the solute, an acid, was ionised for CE analysis a 50 mM borate buffer system was chosen which had a measured pH of 9.2. Other parameters chosen were standard starting points for a CE assay[4], the complete protocol for which is given overleaf. As this method would be used for chlorogenic acid in a further experiment to examine reproducibility, accuracy and linearity of response, a reliable, robust method was required.

HP^{3D} CE

fused silica capillary

57 cm (50 cm to detection window)

75 μm internal diameter (i.d.)

temperature of 30°C

50 mM pH 9.2 borate buffer

5 second, 50 mbar pressure injection

30 kV applied voltage

UV detection at 214 nm.

Sample preparation : The compound, a white solid, was prepared as a 10 mg/ml solution in methanol and serial dilutions made in the run buffer to give analytical standards in the range of 0.005 mg/ml to 1 mg/ml. All solutions were filtered prior to use and stored at 4°C.

Concentrations of Chlorogenic acid : 0.005 mg/ml , 0.01 mg/ml , 0.025 mg/ml , 0.05 mg/ml, 0.1 mg/ml, 0.25 mg/ml, 0.5 mg/ml and 1 mg/ml.

Sample analysis : A single injection of the 0.1 mg/ml solution was performed to check for migration. These conditions proved successful and were used for experiment 3.4.6 and underwent subtle modifications in experiment 3.4.7.

3.4.6 Statistical analysis for method development of chlorogenic acid.

The method optimisation for propranolol and its analogue compounds was investigated on an individual basis, examining the effect of altering each variable one at a time. This was costly both in time and effort. An alternative approach is to use a factorial experimental design (FED)[49] as described in chapter 2.

The development and optimisation of the CE method for chlorogenic acid was assessed using a statistical program based on factorial experimental design. A standard set of CE conditions was

chosen (as described above in 3.4.5) which gave a suitable migration time for the compound, then subtle changes were made to the various parameters to investigate their effects on migration time. In the instance of developing a CE method for chlorogenic acid the variables, or factors involved included buffer pH, buffer concentration, applied voltage and capillary temperature (capillary type, length and internal diameter were predetermined by availability - a hollow silica 50 cm by 75 μm capillary; a borate buffer system was also chosen as a constant.) In order to reduce the variables to only 3 factors at a time, to be evaluated at 2 levels, it was decided to exclude temperature into a separate run (as an increase in temperature generally results in a reduction in migration time). The main and interactive effects of buffer concentration, pH and electric field strength (kV) were therefore examined in two separate experiments performed at 2 different temperatures (25 and 30°C) as laid out below:-

Factor	Level 1	Level 2
pH	9.2	8 (pH reduced to 8 by addition of boric acid)
Conc.	20 mM	50 mM
kV	20 kV	30 kV

Each permutation of these factors is represented in Table 7 overleaf:-

Table 7 : Factorial design for chlorogenic acid.

		Conc.	Applied Voltage	Temperature	
Run number	pH	mM	kV	°C	°C
1	9.2	50	30	25	30
2	9.2	50	20	25	30
3	9.2	20	30	25	30
4	9.2	20	20	25	30
5	8	50	30	25	30
6	8	50	20	25	30
7	8	20	30	25	30
8	8	20	20	25	30

Sample preparation : The 0.1 mg/ml methanolic solution of chlorogenic acid prepared in experiment 3.4.5 was used for this investigation.

Sample analysis : A single injection was made under each set of conditions as tabulated in Table 7. (Single injections only were performed as the methods developed had been tested and were considered to be robust and reliable.)

3.4.7 A comparison of HPLC versus CE for precision, linearity and reproducibility

Data were obtained for chlorogenic acid in order to investigate the robustness of the CE assay developed and to provide comparative results for an acidic compound against those obtained previously for propranolol, a base. Further results were also generated by HPLC for propranolol, again to demonstrate assay stability, but also to compare CE versus HPLC techniques for precision, linearity and reproducibility.

Sample preparation : A series of solutions was produced to cover a range of concentrations from 1 mg/ml to 0.005 mg/ml by serial dilutions of a 10 mg/ml methanolic solution of each compound into the appropriate, filtered run buffers for propranolol and chlorogenic acid (prepared in 3.4.5) respectively.

Concentrations: 0.005 mg/ml, 0.01 mg/ml, 0.025 mg/ml, 0.05 mg/ml, 0.1 mg/ml, 0.25 mg/ml, 0.5 mg/ml and 1 mg/ml.

Sample analysis : Six replicate injections were made at each concentration for propranolol and chlorogenic acid, using the methods outlined previously, to investigate accuracy and reproducibility of both injection volume and response. The combined results were also analysed by linear regression to produce a calibration line and correlation coefficient to assess linearity. These data were generated on the HP^{3D} and HPLC for propranolol and for chlorogenic acid using the HP^{3D} CE system only.

In addition to these samples, a single calibration was also produced as part of the *in vitro* incubation in experiment 3.4.8 run on the Beckman P/ACE and HPLC.

3.4.8 The application of capillary electrophoresis to *in vitro* metabolism.

Aim : Propranolol was incubated with rat hepatic microsomes to produce phase I metabolites in order to assess the application of HPLC and CE for "real" pharmaceutical samples in a biological matrix. These samples were analysed using the previously stated HPLC and CE methods to generate metabolic profiles using each instrument. The two analytical techniques were compared for suitability and ease of analysis.

Experimental Design : Propranolol was incubated at a final concentration of 25 μM (7.4 μg/ml) as a shaking suspension with rat hepatic microsomes (1 mg/ml protein) at 37°C for 2 hours. Aliquots of 1ml were removed into an equal volume of methanol to terminate the incubation at the following timepoints: 0 minutes, 15 minutes, 30 minutes, 60 minutes and 120 minutes (flask 1).

Three control flasks were also included to validate the experiment: a buffer control to demonstrate the stability of propranolol at 37°C, and ensure there is no inherent breakdown of the compound under the incubation conditions (flask 2); a control without NADPH to prove that metabolism occurs only when an energy source is supplied and there is no protein degradation of propranolol (flask 3) and finally a positive control using 7-ethoxycoumarin (7-Ec) which is known to undergo oxidative phase I metabolism to 7-hydroxycoumarin (7-Hc) to show that the microsomes were metabolically viable (flask 4).

Similar 1 ml samples were taken into methanol (1 ml) from flasks 2 and 3 at 0 and 120 min timepoints only, and from flask 4 at 0 min, 30 min, 60 min and 120 min timepoints.

Materials:

0.1 M Phosphate buffer pH 7.4.

Rat liver microsomes were prepared at 17.11 mg protein/ml on 20.2.96 using a differential sucrose gradient, diluted to 1.117 mg protein/ml in 0.1 M phosphate buffer, pH 7.4.

NADPH : Sigma N-1630 Lot 36H7125, prepared at 75 mg/ml in 0.1 M phosphate buffer pH 7.4

Propranolol : prepared as 100 times spiking solution i.e. 5 mM (1.475 mg/ml in methanol) for a final incubation concentration of 25 μM, in order to reduce the volume required to be added to each incubation and maintain enzyme viability.

Preparation:

NADPH : 126 mg + 1.68 ml buffer ≡ 75 mg/ml (keep on ice).

Propranolol solutions: 2.376 mg + 1.61 ml methanol

Microsomal dilution for protein mix : 3 x 0.9 ml x 17.11 mg = 46.2 mg protein
+ 38.66 ml buffer to give 1.117 mg protein/ml

Microsomal mixes : Each flask contains, per ml - 0.895 ml 1.117 mg protein/ml
100 µl NADPH solution/buffer
5 µl compound solution/methanol

Flask 1 - sufficient for 6 tubes: 5.37 ml protein mix
600 µl NADPH
30 µl propranolol spiking solution

Flask 2 - sufficient for 3 tubes: 2.685 ml buffer
300 µl NADPH
15 µl propranolol spiking solution

Flask 3 - sufficient for 3 tubes: 2.685 ml protein mix
300 µl buffer
15 µl propranolol spiking solution

Flask 4 - sufficient for 6 tubes: 5.37 ml protein mix
600 µl NADPH
15 µl 7-Ec spiking solution

Calibration details: A standard calibration curve for propranolol was also spiked, using the solutions prepared previously, to allow quantitation of the results. Eight tubes, containing 0.895 ml protein mix, 0.1 ml buffer and 1 ml methanol, were spiked with 0.005 ml of the propranolol stock solutions to contain a final concentration of 0.005 mg/ml, 0.01 mg/ml,

0.025 mg/ml, 0.05 mg/ml, 0.1 mg/ml, 0.25 mg/ml, 0.5 mg/ml and 1 mg/ml. The calibration samples were extracted in the same way as the incubation samples as described below.

Incubation : All flasks were spiked with the appropriate compound solution into either the protein mix or buffer as described above and transferred into the heated water bath to pre-incubate for 5 minutes. The incubation was initiated by the addition of NADPH/buffer into the various flasks according to the protocol. Aliquots of 1 ml were removed from the flasks into test tubes containing 1ml methanol to terminate the incubation at the timepoints stated above. All samples were stored at -20°C prior to analysis using the aforementioned methods. The HPLC conditions for 7-Ec are shown below.

Sample Treatment : All samples were vortex mixed then centrifuged at 2,000 rpm for 10 minutes to spin down the protein and buffer salts. The supernatants were decanted into fresh glass test tubes and reduced to dryness at 37°C under nitrogen.

Sample Resuspension : All samples were redissolved in 50 µl methanol, vortex mixed, then 50 µl of purified water was added and the samples vortex mixed again. Each sample was removed into the appropriate HPLC/CE vial for analysis on the various pieces of equipment under the assay conditions stated in previous experiments.

High performance liquid chromatography details for 7-Ec analysis.

Column type : Zorbax Rx-C8 250 x 4.6 mm, serial number : AU 16905

Eluent : A = acetonitrile, B = water 0.1% TFA, gradient system as outlined below:-

Time	% A	% B	% C	% D	Gradient
10	10	90			step
22	43	57			linear
10	55	45			linear
8	55	45			/

Flow rate : 0.75 ml/min

Detection system : LDC Analytical Fluorescence detector, excitation wavelength 325 nm, emission wavelength 460 nm

Injection volume : 20 µl

4.1 Experiments to Reproduce the Existing Propranolol Assays.

The aim of this piece of work was to reproduce, and modify if necessary, previous methods for the analysis of propranolol by HPLC and CE. Both methods were successful using the apparatus and conditions stated earlier (3.4.1). These conditions were found to be satisfactory, giving results typified by the chromatogram and electropherogram shown below in Figures 16 and 17 respectively.

Propranolol had a mean retention time of 4.2 minutes by HPLC (Figure 16) and a mean migration time of 6.3 minutes on the Beckman P/ACE (Figure 17) and 4.50 minutes on the HP^{3D} (see Experiment 4.4).

The individual retention/migration times and peak areas are presented and discussed in detail in section 4.4.

These methods were used for all future investigations.

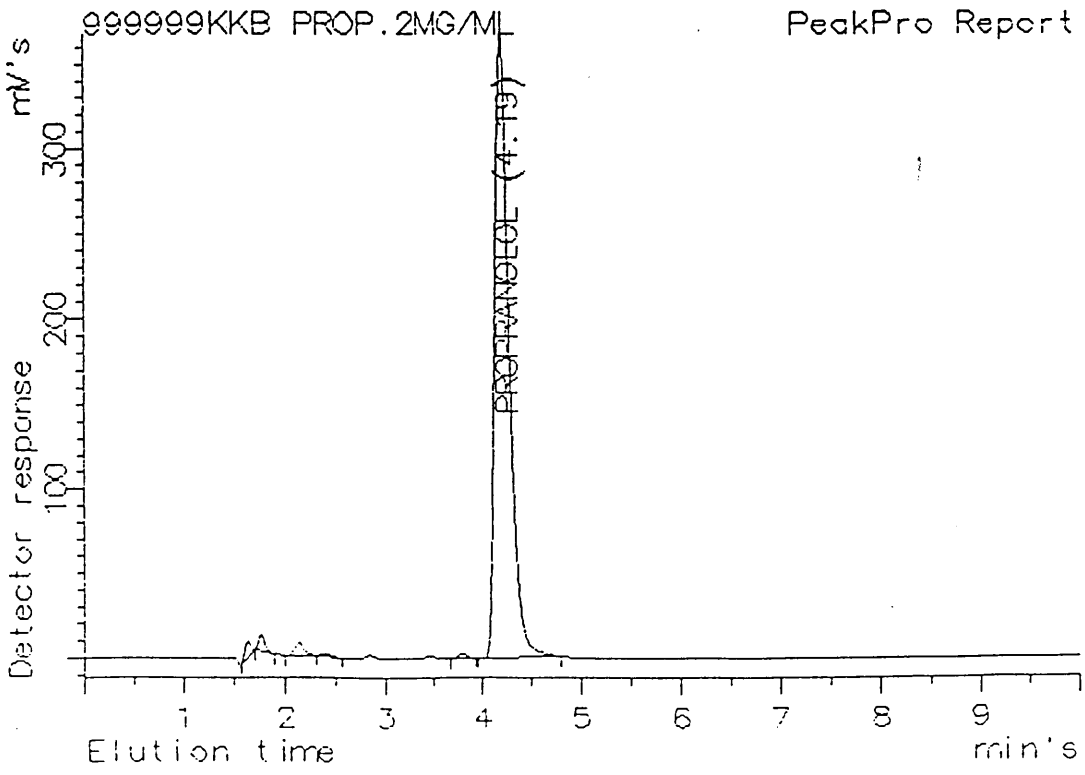


Figure 16 : HPLC Chromatogram of Propranolol.

(for conditions used see section 3.4.1)

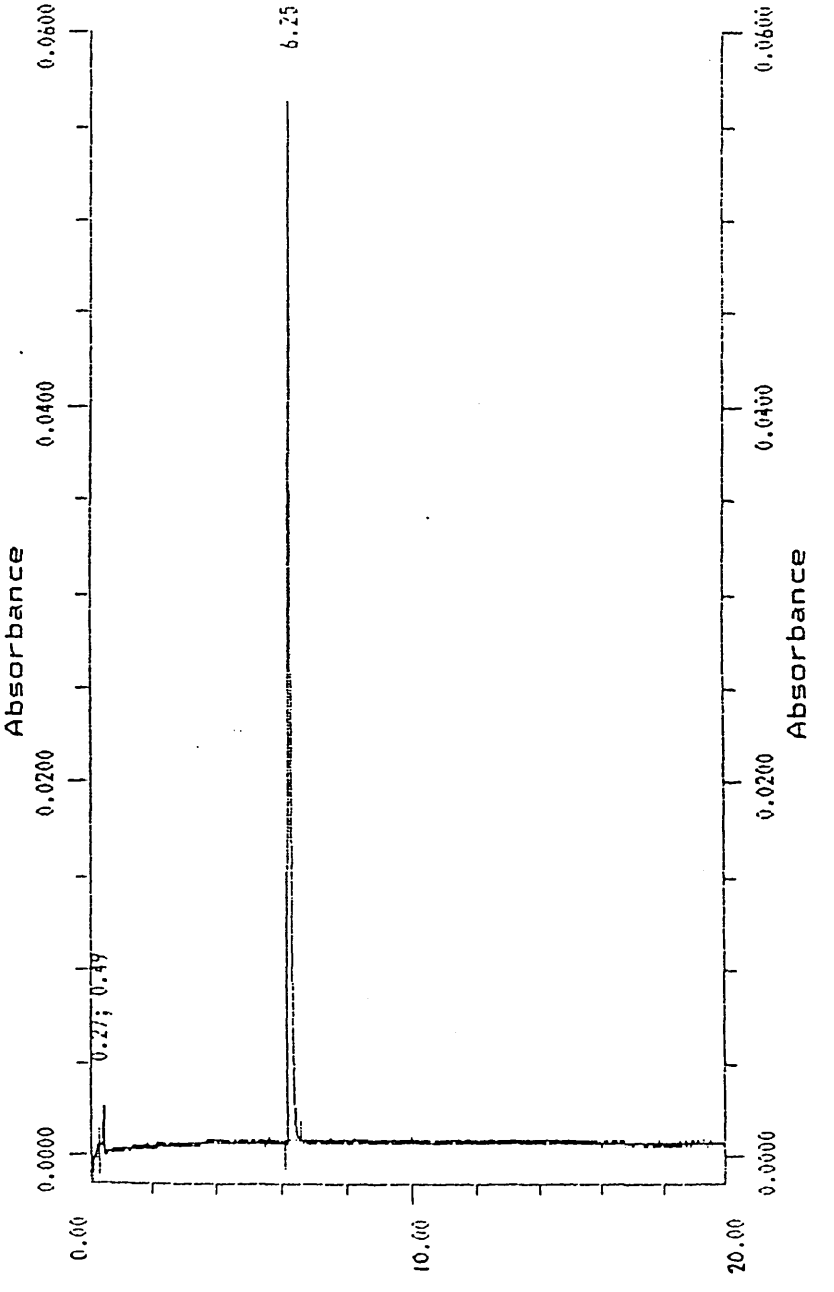


Figure 17 : CE Electropherogram of Propranolol

(for conditions used see section 3.4.1)

4.2 Method Modification for CE Analysis of the Propranolol Analogues.

The CE method employed in section 4.1 above was also used as a starting point for a generic assay to analyse a group of 13 compounds of similar structure to propranolol (see Table 8, section 4.3). None of the analogues were successfully run under these conditions; there was no evidence of any migration with no UV absorbing peaks in the electropherograms.

The equipment and buffer were demonstrated to be performing correctly by a repeat injection of propranolol which gave the expected migration time.

To address this problem, the first approach was to increase the ionic strength of the run buffer from 50 mM to 100 mM, 150 mM and 200 mM in order to decrease the EOF, but this had no affect. Increasing the applied voltage in order to increase the EOF was also unsuccessful. The estimated pKa values for the analogues are between 7 and 10.6 so all the compounds would be expected to be predominantly ionised at a pH of 2.5. However, an optimal run buffer pH is generally 2 units above/below the pKa of the component of interest which is some way outside the present operating conditions. At a pH of 2.5 almost all of the silanol groups on the inner surface of the capillary wall will be protonated which will strongly suppress the EOF. It was hypothesised that this could be the reason that the EOF could not be effectively modified by subtle changes in the applied voltage or ionic strength of the run buffer.

A complete switch was made to a 20 mM borate buffer with a measured pH of 9.2 and an applied voltage of 30 kV. Under these conditions every one of the analogues ran with migration times of between 1.49 minutes and 1.8 minutes (results "a" in Table 8). Further modification to a 50 mM run buffer and a 20 kV applied voltage increased migration times to between 2.9 and 3.94 minutes (results "b" in Table 8).

Propranolol failed to migrate under these modified conditions. The explanation for this is difficult to identify. The propranolol analogues could not be run on the propranolol system and vice versa, yet their physicochemical properties and structural chemistry are very similar. They all contain a naphthalene ring and an amino-alcohol moiety, share broadly similar pKa values (6 of the 13 the same as propranolol) and their relative molecular masses values span that of propranolol.

At pH 2.5, even if the EOF had been completely suppressed, the analogues should all have been ionised and would migrate according to their electrophoretic mobilities alone and therefore should

have produced a UV absorbing peak as they passed the detection window in the capillary, but none were apparent. Also, at a pH of 9.2, with propranolol in its unionised form, it should still have been evident in the electropherogram migrating with the EOF.

It was concluded that either the migration time of the ionised components or the elution time of the EOF was in excess of the period of data capture, even when this was extended to 20 minutes; or that there was complete adsorption of the analyte onto the capillary surface. Either way this was an unacceptably long run time for a CE assay and so the methodology was altered accordingly.

The revised CE method gave acceptable migration times once modified to give slightly improved selectivity. These results are presented (Table 8) and discussed further along with the next set of experimental data looking at the same group of analogues analysed by HPLC.

4.3 A Comparison of the Propranolol Analogues Run on CE versus HPLC.

The previous experiment described the problems encountered when attempting to analyse a set of compounds structurally very similar to propranolol using the same CE conditions. The methodology had to be completely revisited in order to successfully run the analogues.

However, each of the analogues as well as propranolol was successfully chromatographed using the propranolol HPLC method as a generic assay. The HPLC retention times for the analogues are given in Table 8 alongside their corresponding migration times by CE. Other information and physicochemical properties listed in this table include structures, relative molecular mass (RMM), pKa values, log P values (octanol:water partition coefficient) and log D values (octanol:pH 7.4 buffer partition coefficient) of the various compounds.

The HPLC method showed good selectivity between the analogues, with retention times in the range of 4.5 minutes to 12.2 minutes. The order of elution from the column was consistent with increasing RMM and lipophilicity of the compounds. The major factor influencing the retention times of these molecules by HPLC appeared to be the non-polar, reversed phase interactions of the lipophilic naphthalene rings with the C18 carbon chains of the packing material. Weaker, secondary, polar/cation exchange interactions between the hydroxyl moieties and the exposed, unmodified silanol groups on the silica surface can also be expected. This mechanism is especially important in the retention of basic compounds such as these. A higher pKa value indicates a

stronger base which will tend to experience increased retention via silanol interactions. However, the solid phase packing material in the column was base-deactivated so the majority of the silanol groups were masked, thereby reducing ionic interactions. Under these conditions the solutes will distribute between the two phases and elute according to their degree of interaction with the non-polar bonded stationary phase.

The compounds investigated fall into two broad categories, aminoalcohols with or without an amide group (Table 8a = aminoalcohols, Table 8b = aminoalcohols plus amide function). The aminoalcohol moiety increases polarity and will generally reduce retention times. In fact, M065318, the only compound to lack the alcohol, is less polar and has a higher log P and log D value with a corresponding increase in retention and a later elution time. Some structures also contain an O-methyl substituent on the aromatic end of the molecule which further increases polarity. The more polar groups present, the earlier their elution order from the column, as seen with M051932. Although this compound has a higher molecular weight than some of the others in the series, it also has the greatest number of polar groupings which reduce non-polar interactions with the stationary phase, thus causing it to distribute preferentially into the mobile phase and elute first from the column. This trend was further demonstrated by M052487, M052092 and M049666 which all contain a single O-methyl function in various orientations on the naphthalene rings and are less retained than M047070, M045655 and M065318 which lack this grouping. Propranolol, (M045520), contains the aminoalcohol moiety but no further polar substituents so eluted between these last two sets of compounds.

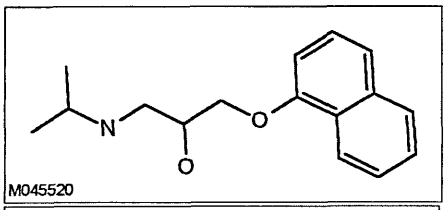
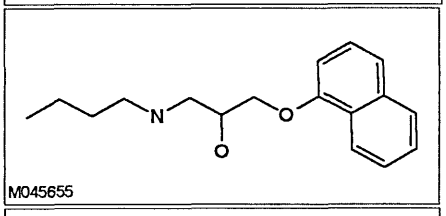
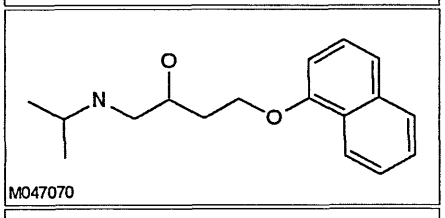
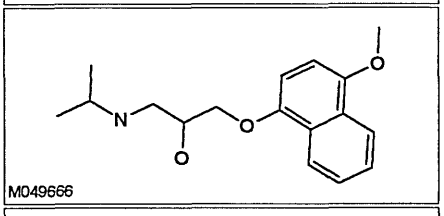
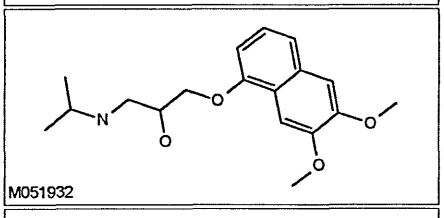
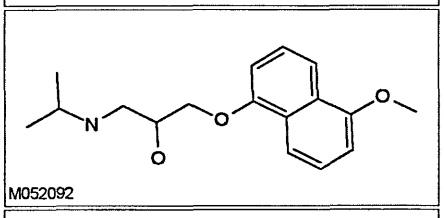
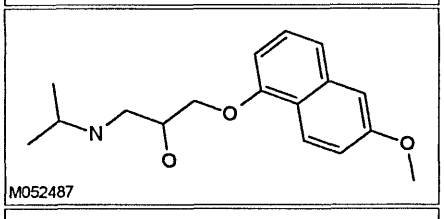
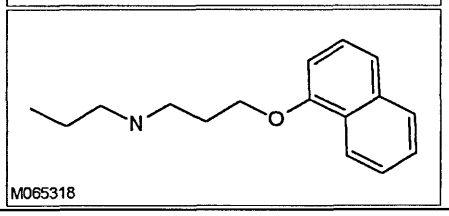
The amides, while still containing the functional aminoalcohol moiety, lack the additional O-methyl groups on the aromatic moeity, having instead an amide function in the aliphatic end of the molecule. The majority of the members of this subgroup are also larger molecules with higher molecular masses than those discussed above, due to longer side-chains, some of which also contain additional lipophilic benzene rings. These substituents increase retention via non-polar interactions with the bonded phase, resulting in much later elution times e.g. M115716, M087086 and M109056 which eluted in order of their increasing RMM. The exception in this group was M081509 which has one of the lowest molecular mass and is the most polar molecule, as indicated by its low log P and Log D values, which greatly reduces its stationary phase interactions preferring to remain in the mobile phase and elute much earlier from the column than other members of this sub-series.

The CE method demonstrated less selectivity for the propranolol analogues with migration times of between 1.49 minutes and 1.80 minutes using a 20 mM pH 9 borate run buffer (CE results column "a", Table 8a and Table 8b) and a 30 kV applied voltage (CE results column "b", Table 8a and Table 8b). These conditions were altered to a 50 mM buffer concentration and a 20 kV applied voltage to decrease migration times in an attempt to improve resolution. This was partially successful with modified migration times of between 2.93 minutes and 3.94 minutes. Given more time these conditions could have been further optimised by looking at slightly different pH run buffers to pull the analogues further apart and demonstrate the high quality of selectivity promised by CE with very sharp peak shape, high efficiencies and shorter analysis times compared to HPLC. The relative efficiencies of HPLC and CE for propranolol are calculated in the following experimental results section, 4.4, and further compared for precision, reproducibility and linearity in section 4.7.

The major determining factors for the migration times of these analytes by CE was thought to be the degree of ionisation of the solutes and the size of the molecule in relation to the number or proportion of ionisable groups present, the charge-to-size ratio. As described previously in the introduction, a small analyte will migrate quicker than a larger analyte of the same charge as it has a higher electrophoretic mobility and a more highly charged molecule will migrate faster than a less ionised molecule of the same size which will have a lower electrophoretic mobility. The degree of ionisation of the analyte and its electrophoretic velocity can be controlled by varying the operating pH of the run buffer. This will also affect the speed of the EOF - in a low pH environment the silanol groups on the silica surface of the capillary wall remain protonated, but raise the pH and more of the silanol groups become negatively charged silanoate groups which interact with the buffer ions and increase the EOF towards the cathode. The pH of the run buffer will also determine the degree of ionisation of the analytes according to their respective pKa values. The two subsets of analogues also fell into one of two bands in terms of their pKa values and migration times: the aminoalcohols with pKa values of between 9.5 and 10.6, and the amides with pKa values in the range of 7 to 8. Those compounds with a pKa closer to the operating pH of 9.2 (the aminoalcohols) should be predominantly in their ionised form and migrate quickly through the capillary; whereas those with a pKa below 9.2 (the amides) should be present mostly as an unionised species and have longer, slower migration times. This trend was reflected well by the data obtained for this series of compounds, with each of the aminoalcohols displaying higher electrophoretic velocities and migrating faster than the less ionised amides. M065318, an

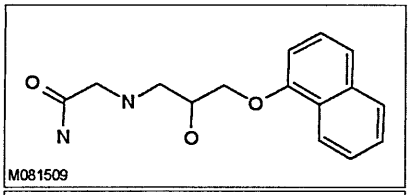
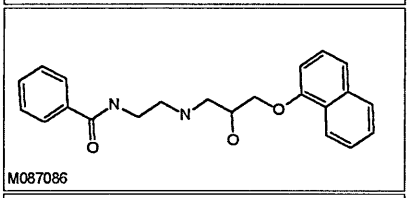
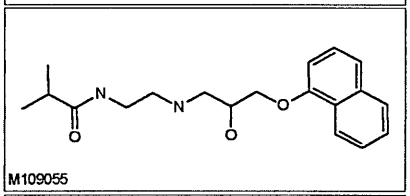
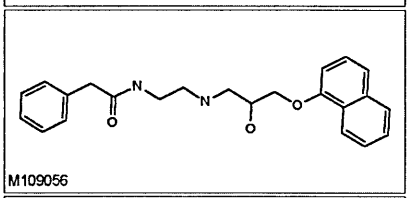
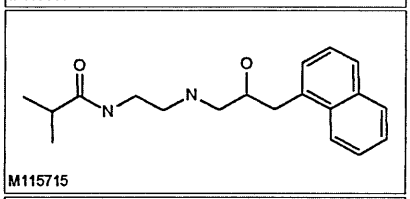
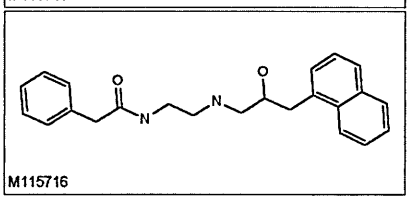
aminoalcohol with the smallest molecular weight was the quickest to elute from the capillary, closely followed by the remaining aminoalcohols. The amides have smaller charge to size ratios with correspondingly slower electrophoretic velocities. M081509 and M087086 were among the latest amides to migrate through the capillary, possibly due the their lower pKa values and hence a lesser degree of ionisation.

Table 8a: Propranolol Analogues (aminoalcohols) - Physicochemical Properties, HPLC and CE Results (3.4.4)

	Structure CHEMISTRY	Mol.Wt	pKa	Log D	Calc Log P	HPLC RT (mins)	CE MT "a" (mins)	CE MT "b" (mins)
M045520	 M045520	259	9.45	3.13	2.75	6.7	NR	NR
M045655	 M045655	273	9.45	3.31	3.5	9.3	1.583	3.328
M047070	 M047070	273	9.45	1.22	2.7	8.2	1.521	3.083
M049666	 M049666	289	9.45	3	2.84	7.6	1.574	3.269
M051932	 M051932	307	9.45	2.44	2.26	4.5	1.609	3.313
M052092	 M052092	289	9.45	3.28	2.8	6.9	1.583	3.284
M052487	 M052487	289	9.45	3.37	2.8	6.1	1.582	3.058
M065318	 M065318	243	10.6	3.52	3.61	8.6	1.492	2.93

NR = No Result

Table 8b: Propranolol Analogues (aminoalcohols+amides) - Physicochemical Properties, HPLC and CE Results (3.4.4).

	Structure CHEMISTRY	Mol.Wt	pKa	Log D	Calc Log P	HPLC RT (mins)	CE MT "a" (mins)	CE MT "b" (mins)
M081509	 M081509	274	7.0	1.22	0.82	5.9	1.798	3.935
M087086	 M087086	364	7.5	4.07	3.11	10.4	1.791	3.93
M109055	 M109055	330	7.9	4.04	2.25	8.1	1.793	3.922
M109056	 M109056	378	7.9	3.93	NC	12.2	1.796	3.937
M115715	 M115715	314	7.9	2.34	2.24	6.7	1.791	3.918
M115716	 M115716	362	7.9	3.16	3.12	10.2	1.793	3.918

4.4 A comparison of HPLC versus the Beckman and the HP^{3D} CE Instruments.

The 2 mg/ml propranolol solutions prepared previously in experiment 3.4.1 were also run, using the same conditions, on the Hewlett Packard HP^{3D} to provide a direct comparison of the two types of CE instrumentation.

The individual results, mean values and associated standard errors for the retention/migration times and peak areas of propranolol run by HPLC, Beckman P/ACE and HP^{3D} CE are given in Table 10 overleaf.

Both the HPLC and the two CE methods gave good, symmetrical, Gaussian peak shapes. The retention/migration times by HPLC and the HP^{3D} CE were comparable, with mean values of 4.2 minutes and 4.5 minutes respectively, however, the propranolol peak run by CE was much narrower and sharper than its HPLC equivalent which gave a much broader baseline peak width. This is characteristic of the greater efficiencies achievable when using CE analysis; even the later running propranolol peak on the Beckman P/ACE, with an average migration time of 6.3 minutes, had a narrower baseline time than the corresponding value for HPLC. The relative efficiencies of the three methods can be compared directly (Table 9), by calculating the number of theoretical plates (N) available for separation using the equation:

$N = 16(t/w)^2$ t = retention/migration time (minutes)

w = peak width at base (minutes)

Table 9 : Theoretical Plate Numbers for HPLC column and CE capillaries used.

2mg/ml Propranolol	HPLC	Beckman P/ACE	HP^{3D} CE
t (minutes)	4.2	6.3	4.5
w (minutes)	0.5	0.2	0.09
N (actual plate count)	18,000	254,000	640,000

Table 10 : Propranolol Analysis Results by HPLC and CE. (results from section 3.4.4)

HPLC		Beckman P/ACE		HP^{3D} CE	
Retention Time (min)	Peak Area (AU)	Migration Time (min)	Peak Area (AU)	Migration Time (min)	Peak Area (AU)
4.26	366	6.39	6.96	4.49	10.9
4.19	368	6.32	7.38	4.49	10.8
4.19	366	6.32	6.76	4.49	10.8
4.19	367	6.31	7.21	4.50	10.6
4.19	366	6.32	7.04	4.49	10.7
4.19	364	6.33	7.24	4.50	10.5
Mean RT	4.20	Mean MT	6.33	Mean MT	4.49
S.E.	0.547	S.E.	0.012	S.E.	0.002
Mean Area	366	Mean Area	7.10	Mean Area	10.7
S.E.	0.543	S.E.	0.092	S.E.	0.060

S.E. = Standard Error (standard deviation divided by the square route of [n = 6])

The migration times recorded for propranolol on the Beckman P/ACE were reproducible within runs, but between day variability was high with peaks observed at approximately 4, 6 or 9 minutes. The same length of the same specification of capillary was used, with the same run buffer and the same run parameters as the HP^{3D}. Problems were encountered with the turning cycle of the autosampler on the P/ACE which caused several capillary breakages. The variability in migration times could be due to inaccurate temperature control of the capillary or insufficient conditioning/equilibration of the new capillaries, although every effort was made to ensure

consistency both within and across instruments. Reference standards were run prior to each piece of work to assure monitoring of the correct component.

All three instruments were also assessed for precision, reproducibility and linearity to assess the robustness of each method, the results of which will be presented and discussed in section 4.7.

4.5 The Development of a CE Assay for Chlorogenic Acid

The development of a suitable CE assay for chlorogenic acid proved to be relatively straightforward. A set of standard, literature recommended conditions[4] based on those outlined in the method development section produced satisfactory results first time (for operation conditions refer to section 3.4.5).

This assay gave a migration time for chlorogenic acid of 4.3 minutes (Figure 18). These conditions were modified in a later experiment to investigate the optimum conditions, but were used as stated in section 3.4.5 for all other work.

The relative ease and speed with which the CE method development for chlorogenic acid was accomplished may have been fortuitous, but in this instance the assay responded well to small changes in the operating conditions as can be seen from the results in the following section in which the method development will be discussed in more detail.

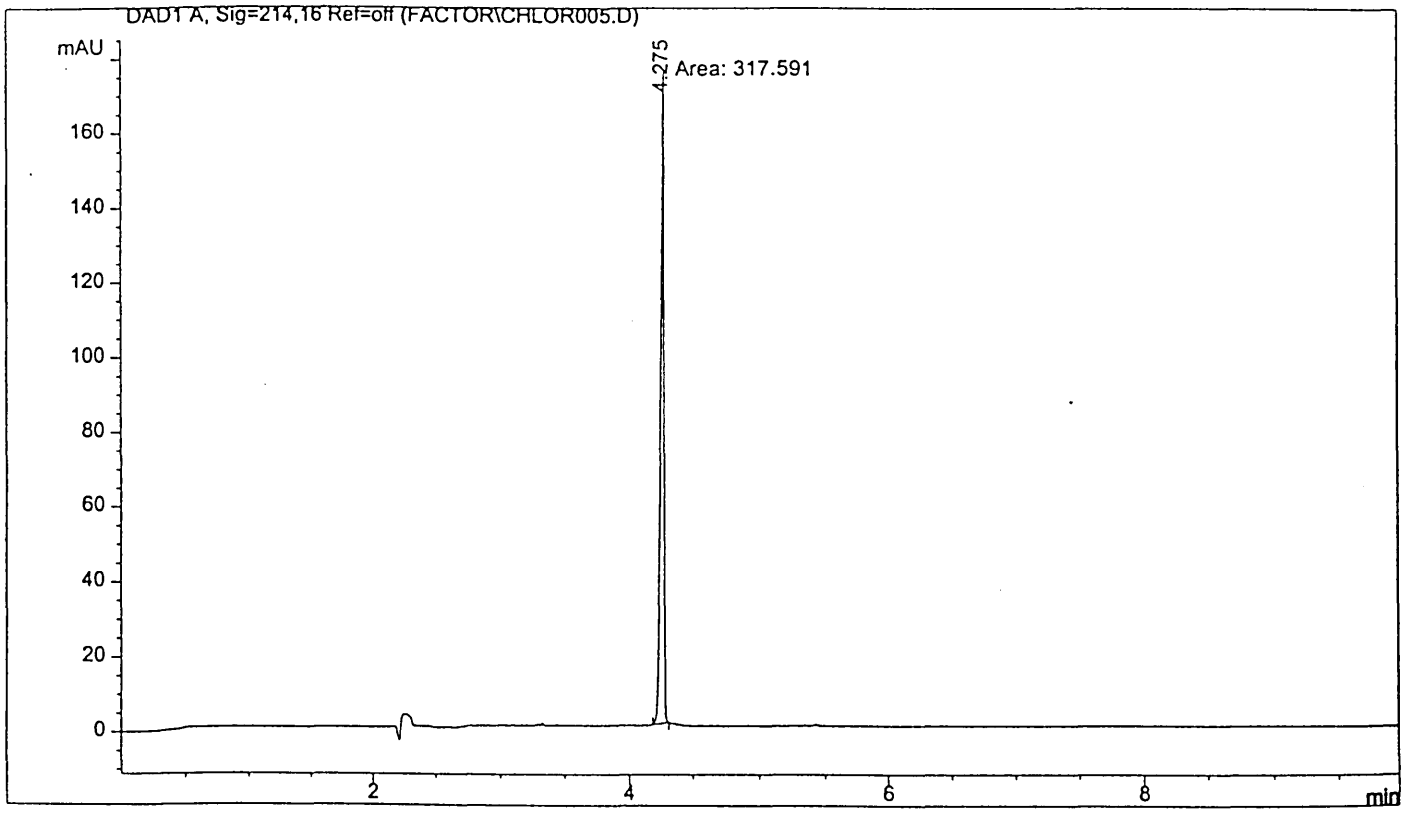


Figure 18 : Chlorogenic Acid Electropherogram by HP^{3D} CE

(for operating conditions refer to section 3.4.5)

4.6 Statistical Analysis for the Method Development of Chlorogenic Acid.

A 0.1 mg/ml methanolic chlorogenic acid solution was prepared and a single injection made for each run with different sets of conditions. A full 2^4 factorial experimental design (Table 11) was used to examine the effects of buffer pH, the ionic strength of the buffer and the applied voltage (which dictates the electric field strength) whilst operating at a constant temperature of 25°C. The additional effect of temperature was investigated by performing a duplicate set of experiments at 30°C. The migration times for chlorogenic acid recorded under these various conditions are presented in the Table 11 below.

Table 11 : Migration Times for Chlorogenic Acid.

Buffer pH	Buffer Conc.	Applied voltage.	Migration Time (min)	
	(mM)	(kV)	T = 25°C	T = 30°C
9.2	50	30	4.6	4.3
9.2	50	20	9.1	8.4
9.2	20	30	3.0	2.8
9.2	20	20	4.6	4.2
8	50	30	4.3	3.9
8	50	20	7.2	NR
8	20	30	5.7	6.6
8	20	20	4.5	9.5

NR = No Result due to instrument malfunction.

These data were analysed using SAS version 6.11[54], a statistical computer program capable, amongst other things, of performing ANalysis Of VAriance (ANOVA)[55]. Loosely, the analysis of variance attempts to partition the total variability of a number of samples into individual components. If the samples are drawn from normally distributed populations with equal means and variances, the *within* variance is the same as the *between* variance. If a statistical test shows that this is not the case, then the samples have been drawn from populations with different means and/or variances. If it is assumed that the variances are equal (and this is an underlying assumption in ANOVA) then it is concluded that the discrepancy is due to differences between *means*. Thus :-

H_0= samples are drawn from normally distributed populations with equal means and variances.

H_1= populations' variances assumed to be equal and therefore samples are drawn from populations with different means.

When partitioning the total variability, it is simpler to work with sums of squares because adding and subtracting variances is complicated (usually) by varying degrees of freedom. However, in the final stages of the analysis the sums of squares are converted to variances by dividing by the degrees of freedom in order to apply the F-test to compare them[56].

Variance describes the spread of the data by its deviation from the mean calculated from the sum of squares of each data point according to the equation:-

$$\text{variance} = \sum \frac{(x - \text{mean})^2}{n-1} \quad \text{or} \quad \frac{\text{total } \textit{sum of squares}}{n-1}$$

where n = number of data points.

The ANOVA data tables were generated within SAS and can be found appended at the end of this section (Tables 12,13 and 14).

The first analysis (procedure 1, Table12) included all available combinations of factors. Note that a four-way interaction term cannot be obtained because of the NR result. The total sum of squares value calculated for this data set was 64.8 with 14 degrees of freedom. As stated previously, ANOVA partitions this total sum of squares into components due to main effects (e.g. pH, temperature, electric field strength and buffer ionic strength) and their 2 and 3-way interactions. The contribution of each of the sum of squares values for these factors, or combinations thereof, towards the total (64.8) is directly proportional to the effect on migration time, the higher the value the greater the difference in migration time seen by varying the parameter(s) in question. The sum of squares values for the four 3-way interactions contributed very little to the total sum of squares and therefore these 3-way interactions were pooled to construct an error term. In this re-analysis (procedure 2, Table 13), the model sum of squares value was 60.3 (10 degrees of freedom) and the error term was 4.512 (4 degrees of freedom) giving an unbiased estimate of *within* variability of 1.128. The significance, or importance, of the remaining individual and 2-way interactions on the overall effect on migration time can be evaluated statistically using F-tests. The F value is the ratio of the mean square value (estimate of variability for a particular factor(s)) to the unbiased estimate of variability. If this ratio is close to 1 then the factor being assessed is not statistically important and has little or no effect on migration time. Whereas if the ratio is very much greater than 1 the term in question is statistically important and causes a significant change in migration time.

The parameter which showed the highest variability was electric field strength alone (by altering the applied voltage) with a mean square value in excess of 20 and an F value greater than 18. The probability[57] of seeing such an extreme change occurring by chance (Pr>F) is given as 0.0126, (1.26%), or about one in a hundred. The assumption tested is that there is no change in migration

time, with a 95% confidence limit, so of the remaining factors, any with a probability value less than 0.05 (5%) disproves the theory. The only other parameter to fall into this category was the combination of altering pH and buffer ionic strength, with a probability of 4.28% - although these two factors alone also each had a fairly low Pr value (7.5-8%). The combination of electric field strength and buffer ionic strength also had a low probability of 6.2%.

In procedure 3, Table 14, all terms involving temperature were dropped from the model. This had little effect on the partition of the total sum of squares among the other terms in the model. Temperature, being the least important of the four factors in this analysis (contributed only 1.09 to the model sum of squares) can therefore be removed and 3D cubes can be used to describe the effect of moving from the low level to the high level of the other three factors. The remaining three factors, pH, electric field strength and buffer ionic strength, have their high and low values placed at the corners of a cube along with their corresponding migration times as shown below (Figure 19) for each temperature setting.

From the data within the mean temperature cube it is possible to calculate the effect each parameter has on migration time. This calculation can be simplified by first replacing the individual migration values at each corner by their mean, then examining the change in migration time from one corner of the cube to the other. For example, the effect of increasing the electric field strength from 20 kV to 30 kV is:

7.0 - 6.15 = - 1.15 (a reduction in migration time of 1.15 minutes)
4.4 - 2.9 = -1.5 (a reduction in migration time of 1.50 minutes)
7.2 - 4.1 = -3.1 (a reduction in migration time of 3.10 minutes)
8.75 - 4.45 = -4.3 (a reduction in migration time of 4.30 minutes)

giving a combined effect of:-

i) Increasing the applied voltage from 20 kV to 30 kV, produced an overall decrease in migration time.

1/4 [-1.15 + -3.1 + - 4.3 + - 1.5] = <u>-2.51</u>

In the same way:

ii) Increasing buffer ionic strength from 20 mM to 50 mM produced an overall increase in migration time.

1/4 [-2.05 + - 0.3 + 4.35 + 1.55] = 0.888

iii) Changing the buffer pH from 8 to 9.2 produced an overall decrease in migration time.

1/4 [- 3.1 + - 3.25 + 1.55 + 0.35] = -1.11

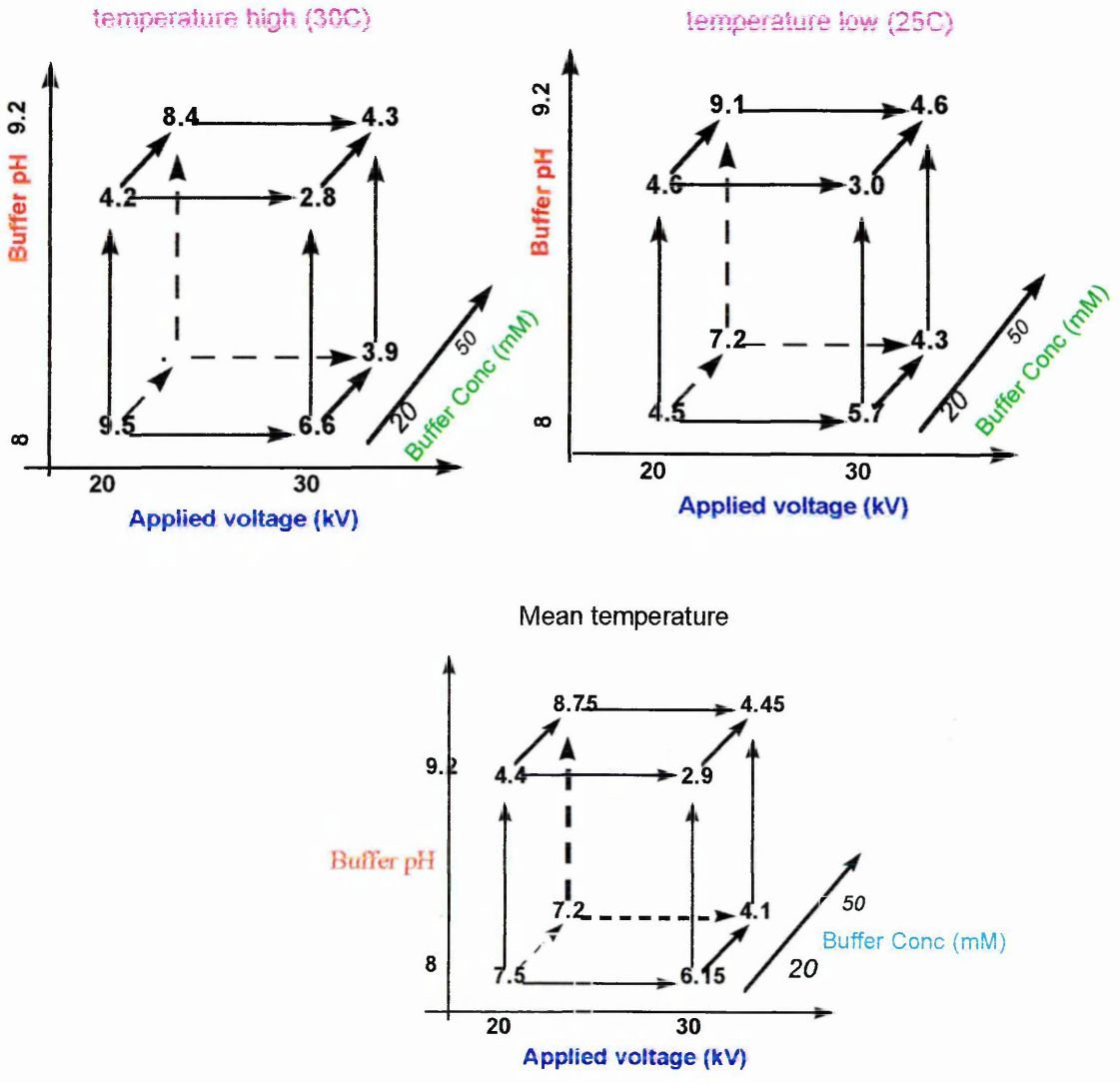


Figure 19 : Chlorogenic Acid Method Development - FED Cubes for Migration Times.

(results from section 3.4.6)

These values show the overall effects on migration on time from varying the individual settings for each parameter.

The extent of the effect of these parameters (electric field strength, buffer ionic strength and pH) can be demonstrated by plotting their interactions graphically as shown below in Figure 20.

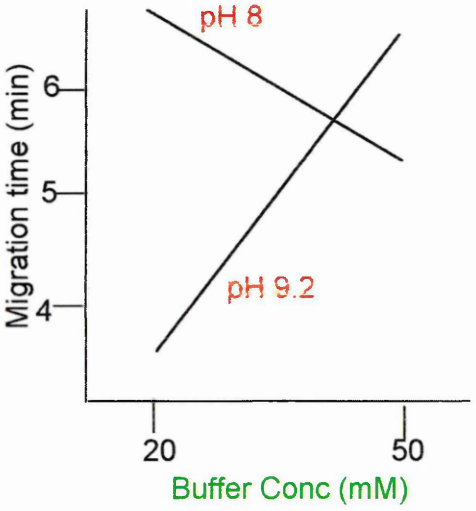


Figure 20a

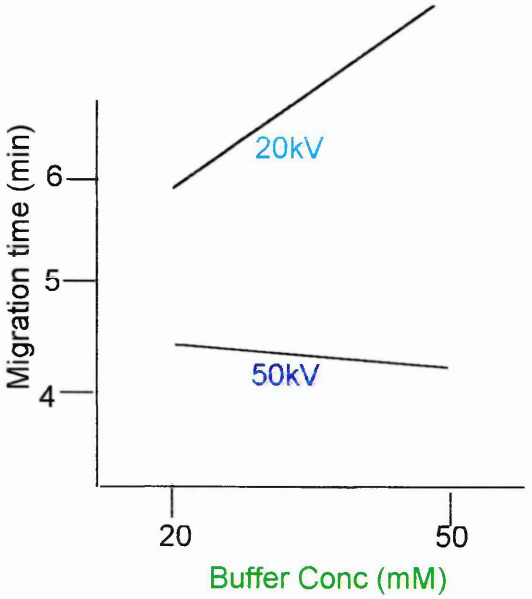


Figure 20b

Figures 20a and b : Chlorogenic Acid Method Development - FED Interactions.

This confirms the ANOVA results: that the most effective and straightforward way to vary migration time is to alter the electric field strength by changing the voltage applied to the capillary, especially when working with a more concentrated run buffer, as the lines on figure 20b can be seen to be diverging. This can be achieved even with a small increase in voltage which also keeps any increase in Joule heating to a minimum. These graphs also indicate which parameter(s)

is/are fairly stable with respect to their effect on migration time to avoid non-reproducible results between samples or between runs. In this case, if a poor buffer preparation lead to a variation in buffer concentration, whilst operating at the higher applied voltage this would not result in a detrimental variation in migration time (a relatively flat lower line in figure 20b). However, the steeper sloped and crossing lines in figure 20a dictate that great care should be taken to ensure that the run buffer is made to the correct pH value or migration times could be observed to vary in either an increasing or decreasing manner, especially if also combined with a slight error in buffer concentration.

The factorial experimental design approach to method development was successful in highlighting the most influential parameter in capillary zone electrophoresis. Although the more obvious factor may appear to be temperature, on closer inspection of the tabulated data, an increase in temperature produced a much less marked effect than increasing the electric field strength. This style of method development also has several advantages. Each variable can be assessed in one run on the same day using the same equipment, run buffer and compound solutions, thereby reducing variability introduced by performing separate experiments on different days using different solutions and/or buffers. This therefore reduces the number of experimental runs, with resulting savings in time and solvent/buffer usage and disposal. Further, if the initial conditions chosen did not prove to give the desired results, graphical representation of the collective data may indicate which parameter(s) to alter in order to optimise migration time.

Table 12 : General Linear Models **Procedure (1)**

Dependent Variable: SEP_TIME

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	14	64.8	4.63	.	.
Error	0	.	.		
Corrected Total	14	64.8			

R-Square	C.V.	Root MSE	SEP_TIME Mean
1.000000	0	0	5.51

Source	DF	Type II SS	Mean Square	F Value	Pr > F
PH	1	5.92	5.92	.	.
IONIC_ST	1	7.73	7.73	.	.
PH*IONIC_ST	1	6.75	6.75	.	.
ELECT	1	18.7	18.7	.	.
PH*ELECT	1	0.701	0.701	.	.
IONIC_ST*ELECT	1	7.84	7.84	.	.
PH*IONIC_ST*ELECT	1	0.180	0.180	.	.
TEMP	1	1.09	1.09	.	.
PH*TEMP	1	3.41	3.41	.	.
IONIC_ST*TEMP	1	0.241	0.241	.	.
PH*IONIC_ST*TEMP	1	0.180	0.180	.	.
ELECT*TEMP	1	1.021	1.021	.	.
PH*ELECT*TEMP	1	2.31	2.31	.	.
IONIC_ST*ELECT*TEMP	1	0.005	0.005	.	.

Table 13 : General Linear Models **Procedure (2)**

Dependent Variable: SEP_TIME

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	60.3	6.023	5.34	0.060
Error	4	4.51	1.128		
Corrected Total	14	64.8			

R-Square	C.V.	Root MSE	SEP_TIME Mean
0.930368	19.3	1.06	5.51

Source	DF	Type II SS	Mean Square	F Value	Pr > F
PH	1	6.21	6.21	5.51	0.079
IONIC_ST	1	6.45	6.45	5.72	0.075
PH*IONIC_ST	1	9.69	9.69	8.59	0.043
ELECT	1	20.9	20.9	18.5	0.013
PH*ELECT	1	0.176	0.176	0.16	0.713
IONIC_ST*ELECT	1	7.45	7.45	6.60	0.062
TEMP	1	1.14	1.14	1.01	0.372
PH*TEMP	1	4.18	4.18	3.71	0.127
IONIC_ST*TEMP	1	1.22	1.22	1.08	0.357
ELECT*TEMP	1	1.73	1.73	1.53	0.284

Table 14 : General Linear Models **Procedure (3)**

Dependent Variable: SEP_TIME

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	51.4	7.35	3.84	0.048
Error	7	13.4	1.91		
Corrected Total	14	64.8			

R-Square	C.V.	Root MSE	SEP_TIME Mean
0.794	25.1	1.38	5.51

Source	DF	Type II SS	Mean Square	F Value	Pr > F
PH	1	5.44	5.441	2.85	0.135
IONIC_ST	1	4.33	4.33	2.26	0.176
PH*IONIC_ST	1	13.3	13.3	6.96	0.034
ELECT	1	20.7	20.7	10.9	0.013
PH*ELECT	1	0.720	0.720	0.38	0.559
IONIC_ST*ELECT	1	5.88	5.88	3.08	0.123
PH*IONIC_ST*ELECT	1	0.067	0.067	0.04	0.857

Table 14 : General Linear Models **Procedure (3 continued)**

Parameter	Estimate		T for H0: Parameter=0	Pr > |T|	Std Error of Estimate
INTERCEPT		4.45	4.55	0.003	0.977
PH	8	-0.35	-0.25	0.807	1.38
	9.2	0.000	.	.	.
IONIC_ST	20	-1.55	-1.12	0.299	1.38
	50	0.	.	.	.
PH*IONIC_ST	8*20	3.60	1.84	0.108	1.95
	8*50	0.000	.	.	.
	9.2*20	0.000	.	.	.
	9.2*50	0.00	.	.	.
ELECT	20	4.30	3.11	0.017	1.38
	30	0.000	.	.	.
PH*ELECT	8*20	-1.20	-0.55	0.600	2.19
	8*30	0.000	.	.	.
	9.2*20	0.000	.	.	.
	9.2*30	0.000	.	.	.
IONIC_ST*ELECT	20*20	-2.80	-1.43	0.195	1.95
	20*30	0.000	.	.	.
	50820	0.000	.	.	.
	50*30	0.000	.	.	.
PH*IONIC_ST*ELECT	8*20*20	0.550	0.19	0.857	2.93
	8*20*30	0.000	.	.	.
	8*50*20	0.000	.	.	.
	8*50*30	0.000	.	.	.
	9.2*20*20	0.000	.	.	.
	9.2*20830	0.000	.	.	.

4.7 A Comparison of HPLC versus CE for Precision, Linearity and Reproducibility.

Six replicate injections were performed for each of a range of concentrations, (0.005 mg/ml to 1 mg/ml) noted in the Tables 15 and 16 below, for both propranolol and chlorogenic acid using the HPLC and CE conditions stated in the previous sections. These results were used to produce calibration plots from which the precision, linearity and reproducibility of the systems could be calculated. The relative ease of use, robustness and accuracy of the two techniques were also assessed.

The individual data for each of these experiments are presented in the appended excel spreadsheets (Tables 17 and 18), whilst the summarised version for propranolol with mean and standard error (SE) values are given in the following table (Table 15). For ease of handling and to allow a clear comparison of the three calibration curves on one graph with a single scale the results have been normalised towards the Beckman P/ACE data which is unmodified. The HPLC values have been divided by one hundred and the HP^{3D} results have been multiplied by a factor of ten.

Having become familiar with each instrument over the previous experiments and as they were all fully programmable the setting up and operation of this investigation was equally straightforward for HPLC and both CE instruments. Similarly, each analytical technique proved to be robust and reliable, completing the run without any problems.

The precision and reproducibility of CE versus HPLC can be measured by looking at the repeated injections at each concentration, their mean values and associated standard errors. Perhaps the most obvious point which becomes immediately apparent in the mean results is the difference in response of the various instruments, hence the normalisation. The most sensitive technique by far was HPLC, which was more than ten times more sensitive than the HP^{3D} and two orders of magnitude more sensitive than the Beckman P/ACE. The response capability of the Beckman seemed to become saturated in this experiment above a concentration of about 0.1 mg/ml propranolol, however, this was later rectified in the *in vitro* incubation experiment by raising the attenuation on the UV detector to give a linear response across the entire concentration range. Apart from these skewed results from the Beckman at the top end of the curve, the reproducibility of the three techniques was very good. This can be more effectively compared by observing the

standard errors stated as a percentage of their mean values, given in brackets in the Table 15 below, to enable a proportional comparison to be made and to give a measure of precision.

Table 15 : Propranolol Calibration Data (results from section 3.4.7)

Concentration (mg/ml)	Peak Area Response (AU) : Mean Value ± SE (SE % Mean)		
	HPLC (÷100)	CE-HP^{3D} (x10)	CE-Beckman P/ACE
0.005	0.188 ± 0.005 (2.72)	0.40 ± 0.002 (0.05)	1.58 ± 0.015 (0.95)
0.01	0.885 ± 0.011 (1.20)	0.73 ± 0.010 (1.39)	3.62 ± 0.079 (2.18)
0.025	2.55 ± 0.049 (1.91)	1.78 ± 0.016 (0.89)	5.68 ± 0.012 (0.21)
0.05	6.22 ± 0.013 (0.21)	3.29 ± 0.016 (0.49)	14.1 ± 0.087 (0.62)
0.1	14.8 ± 0.113 (0.76)	6.51 ± NC # (NC)	29.7 ± 0.123 (0.41)
0.25	38.6 ± 0.090 (0.23)	16.8 ± 0.130 (0.78)	36.2 ± 1.25 (3.45)
0.5	80.9 ± 0.039 (0.05)	32.8 ± 0.090 (0.27)	38.8 ± 0.749 (1.93)
1	155 ± 0.142 (0.09)	65.9 ± 0.260 (0.39)	46.8 ± 1.70 (3.64)
r	0.9997	0.9999	0.827

r = correlation coefficient

NC # = Not Calculated, n = 2

A general trend, as would be expected, is for the standard error to be higher at the lower concentrations where accuracy and precision can be affected by very slight changes in injection volume and response. This is true for each of the instruments. When expressed as a percentage of the mean, the standard error values indicate that precision is greatest for the HP^{3D} CE, followed by the Beckman CE, then HPLC. It was perhaps less surprising that the precision of the latest, state of the art Hewlett Packard CE equipment was greater than the older, less technically

advanced Beckman CE instrument; but quite interesting to discover that both CE systems (P/ACE up to 0.1 mg/ml only) gave greater precision than HPLC. This may be because the electrokinetic introduction of the sample slug onto the capillary in CE is more reliable than the on-line loop and valve injection used in HPLC. Even though the HP^{3D} CE was close to its limit of detection at the lower end of the calibration, its precision remained impressive giving a tight data set. The HPLC, however, although less precise, could have been operated at lower levels of quantification than CE due to its greater sensitivity.

To further investigate the precision of these instruments, the total data set was analysed by linear regression to give a correlation coefficient, r, for the calibration curves run by CE and HPLC. The mean values at each concentration have been plotted in the graph below to depict the calibration lines (Figure 21), whilst the correlation coefficients have been quoted in Table 15 above.

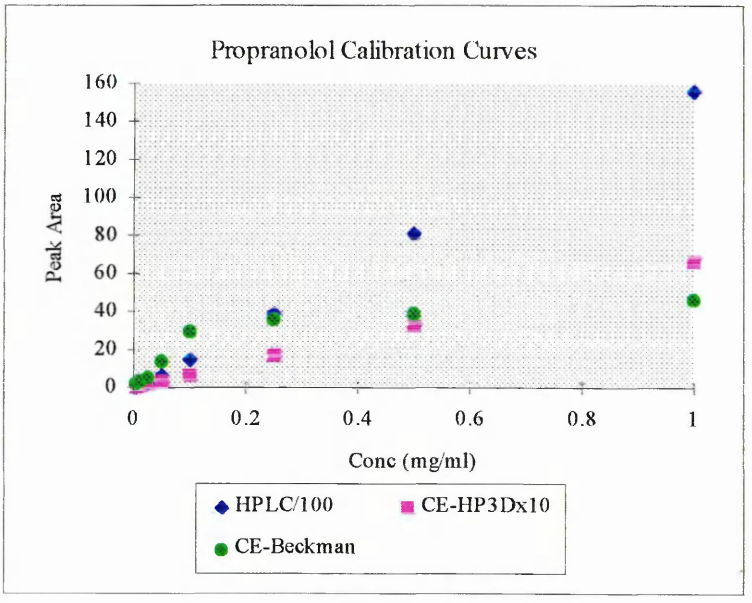


Figure 21 : A Graph of the Propranolol Calibration Curves.

(data plotted are the mean results from section 3.4.7, detailed in Table 15, individual values are quoted in section 4.7, Table 17, pages 89, 90)

The linearities of these data sets were extremely impressive. There was little to choose from between the HP^{3D} CE and the HPLC with r values of 0.9999 and 0.9997 respectively. The Beckman was only linear over the concentration range 0.005 mg/ml - 0.1 mg/ml in this experiment, which had a correlation coefficient of 0.9967, due to the UV detector off-scaling. An r value of 0.992 was achieved over the complete concentration range from the attenuated data collected from the *in vitro* calibration results presented in the following section.

Table 16 : Chlorogenic Acid Calibration Data (results from section 3.4.7)

Concentration (mg/ml)	Peak Area Response (AU) - CE-HP^{3D}		
	Mean	SE	SE % Mean
0.005	1.66	0.087	5.24
0.01	4.09	0.053	1.30
0.025	10.9	0.210	1.93
0.05	22.9	0.561	2.46
0.1	43.5	0.438	1.01
0.25	104	0.253	0.240
0.5	186	6.00	3.23
1	376	3.381	0.900
r	0.9996		

In addition to propranolol, this experiment was also performed for chlorogenic acid, on the HP^{3D} CE instrument only, to assess the suitability of CE for the analysis of acidic molecules as well as bases. The method performed robustly and reliably, giving reproducible results at each

concentration. A closer inspection of the standard errors revealed a reversal in the trend for precision, with higher concentrations having more variable results, although these were less obvious when normalised as a percentage of the mean (Table 16). In spite of this, the precision of CE for an acidic component was still high with a correlation coefficient of 0.9996 (Figure 22), thus demonstrating that CE can be equally applicable to the analysis of both acidic and basic compounds.

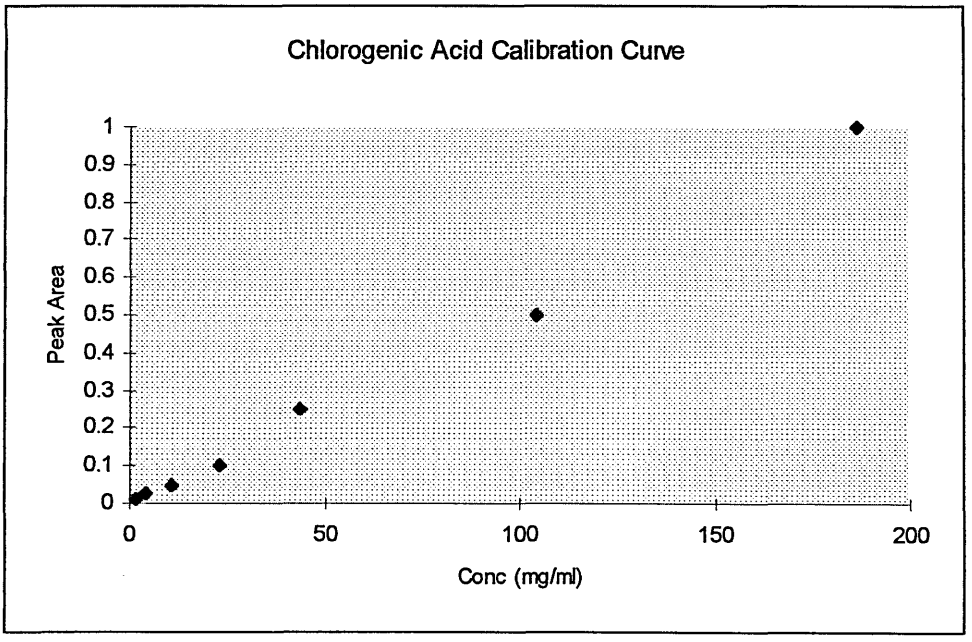


Figure 22 : A Graph of the Chlorogenic Acid Calibration Curve.

(graphical presentation of results from section 3.4.7, detailed in Table 16, individual values are quoted in section 4.7, Table 18, page 91)

To conclude, the reproducibility and precision of the three analytical techniques were more than adequate for the purposes of this experiment. Once familiar with the equipment, each analytical technique performed robustly and reliably, for both the acid and the base, with controllable, programmable capabilities.

The final challenge was to apply this knowledge, having fully tested the methods involved, to investigate the use of these analytical techniques in the field of pharmaceutical science with some actual, matrix derived samples rather than pure standard solutions.

Table 17 : Experiment 4.7 Propranolol Results (data from section 3.4.7)

Reproducibility/Linearity/Calibration n = 6

CE - HP3D				HPLC			
Conc. (mg/ml)	Area (AU)	Area mean	Area SE	Conc. (mg/ml)	Area (AU)	Area mean	Area SE
0.005	0.04019			0.005	17.872		
0.005	0.03985			0.005	17.727		
0.005	0.04001			0.005	18.052		
0.005	0.0402			0.005	18.934		
0.005	0.04058			0.005	19.391		
0.005	0.03914	0.039995	0.000198	0.01	21.03	18.83433	0.513
0.01	0.0768			0.01	84.78		
0.01	0.0748			0.01	89.28		
0.01	0.0751			0.01	85.766		
0.01	0.07264			0.01	89.884		
0.01	0.0709			0.01	91.334		
0.01	0.07054	0.073463	0.00102	0.01	89.994	88.50633	1.066
0.025	0.1824			0.025	243.531		
0.025	0.1817			0.025	244.348		
0.025	0.1766			0.025	247.739		
0.025	0.1783			0.025	254.31		
0.025	0.1774			0.025	268.779		
0.025	0.1717	0.178017	0.00158	0.025	269.858	254.7608	4.86
0.05	0.3347			0.05	623.54		
0.05	0.3247			0.05	617.989		
0.05	0.3322			0.05	620.186		
0.05	0.3273			0.05	626.877		
0.05	0.3264			0.05	619.293		
0.05	0.3265	0.328633	0.0016	0.05	622.28	621.6942	1.321
0.1	0.6495			0.1	1497		
0.1	0.6524			0.1	1514		
0.1	NR			0.1	1496		
0.1	NR			0.1	1476		
0.1	NR			0.1	1462		
0.1	NR	0.6506	NC	0.1	1438	1480.5	11.323
0.25	1.711			0.25	3892		
0.25	1.681			0.25	3871		
0.25	1.699			0.25	3860		
0.25	1.675			0.25	3852		
0.25	1.657			0.25	3840		
0.25	1.625	1.674667	0.013	0.25	3830	3857.5	8.954
0.5	3.301			0.5	8084		
0.5	3.298			0.5	8098		
0.5	3.283			0.5	8102		
0.5	3.264			0.5	8100		
0.5	3.248			0.5	8093		
0.5	3.256	3.275000	0.009	0.5	8079	8092.667	3.865
1.0	6.599			1	15512		
1.0	6.634			1	15538		
1.0	6.649			1	15521		
1.0	6.567			1	15521		
1.0	6.594			1	15548		
1.0	6.471	6.585667	0.026	1	15606	15541	14.191

r = 0.9999 (excluding 0,0)
r = 0.9999 (including 0,0)

r = 0.9997 (excluding 0,0)
r = 0.9997 (including 0,0)

Table 17 (continued) : Experiment 4.7 Propranolol Results (data from section 3.4.7)

Reproducibility/Linearity/Calibration n = 5

CE - Beckman

Conc. (mg/ml)	Area (AU)	Area mean	Area SE
0.005	1.601		
0.005	1.56		
0.005	1.545		
0.005	1.627		
0.005	1.566	1.5798	0.015
0.01	NR		
0.01	3.405		
0.01	3.499		
0.01	3.606		
0.01	3.75		
0.01	3.834	3.6188	0.079
0.025	5.71		
0.025	5.703		
0.025	5.686		
0.025	5.656		
0.025	5.648	5.6806	0.012
0.05	13.982		
0.05	14.401		
0.05	13.987		
0.05	14.186		
0.05	13.937	14.0986	0.087
0.1	29.276		
0.1	29.817		
0.1	29.592		
0.1	30.017		
0.1	29.644	29.6692	0.123
0.25	31.373		
0.25	38.407		
0.25	37.766		
0.25	36.448		
0.25	36.794	36.1576	1.246
0.5	37.413		
0.5	36.66		
0.5	39.208		
0.5	40.347		
0.5	40.269	38.7794	0.749
1.0	43.041		
1.0	43.138		
1.0	46.377		
1.0	49.865		
1.0	51.37	46.7582	1.703

r = 0.8257 (excluding 0,0)
r = 0.8266 (including 0,0)

Table 18 : Experiment 4.7 Chlorogenic Acid Results (data from section 3.4.7)

Reproducibility/Linearity/Calibration n = 6

HPLC

Conc. (mg/ml)	Area (AU)	Area mean	Area SE
0.005	1.49		
0.005	1.77		
0.005	1.73		
0.005	NR		
0.005	NR		
0.005	NR	1.66	0.087
0.01	NR		
0.01	4.19		
0.01	4.21		
0.01	3.98		
0.01	4.11		
0.01	3.95	4.088	0.053
0.025	11.29		
0.025	11.17		
0.025	10.83		
0.025	11.49		
0.025	10.34		
0.025	10.29	10.902	0.21
0.05	NR		
0.05	21.05		
0.05	22.53		
0.05	22.73		
0.05	23.51		
0.05	24.43	22.85	0.561
0.1	42.48		
0.1	44.46		
0.1	43.05		
0.1	45.11		
0.1	43.19		
0.1	42.55	43.473	0.438
0.25	103.7		
0.25	104.4		
0.25	104.1		
0.25	104.2		
0.25	103.5		
0.25	102.7	103.767	0.253
0.5	185		
0.5	185.7		
0.5	162.3		
0.5	179.6		
0.5	204.4		
0.5	197.8	185.8	6
1.0	372		
1.0	380.9		
1.0	380.6		
1.0	363		
1.0	372.4		
1.0	386	375.82	3.381

r = 0.99957 (excluding 0,0)
r = 0.99957 (including 0,0)

4.8 The Application of CE to *In vitro* Metabolism - HPLC versus CE.

Propranolol is known to undergo oxidative phase I metabolism to a 4-hydroxy propranolol metabolite. A metabolism profile was produced over 2 hours by incubating propranolol at a final concentration of 25 μM (7.4 μg/ml) with rat liver microsomes as a shaking suspension at 37°C.

Control incubations demonstrated that there was no inherent breakdown of propranolol under the

The 7-Ec samples, analysed using an established HPLC method, showed a 75% turnover to the hydroxy metabolite, which demonstrated the metabolic viability of the microsomes. The 7-Hc response is disproportionately larger than that of the 7-Ec as it has a greater fluorescence.

Having demonstrated that the incubation was successful, the propranolol samples were then analysed using the methods set up earlier for HPLC and both the Beckman P/ACE and HP^{3D} CE instruments.

The HPLC results were slightly disappointing. The chromatograms had a vastly elongated solvent front which extended out beyond the retention time for propranolol masking some of the parent peak and making a precise measurement of the metabolite almost impossible. However, itwas evident that there was a time related decrease in the parent propranolol peak, with an associated appearance of a metabolite. The explanation for the poor chromatography became apparent on analysis of the control samples. The large solvent front was seen in all chromatograms derived from the flasks which contained NADPH; those without NADPH were clean. This was further confirmed by running a UV check on the NADPH solution which showed significant absorption at 254 nm, the wavelength used to monitor propranolol. Whilst this meant that the HPLC data were compromised, the chromatograms were still integrated to obtain an approximate quantitative result, which depicted qualitative evidence of metabolism.

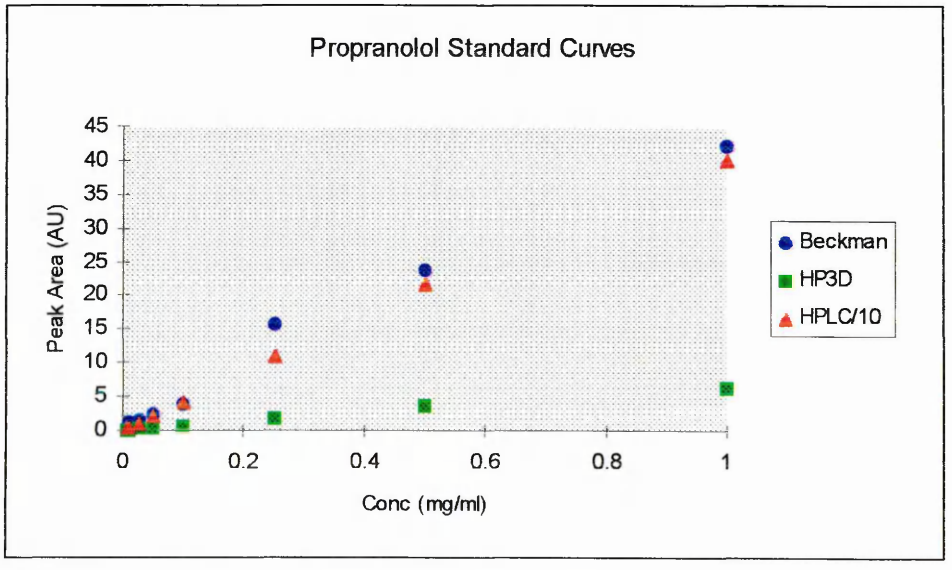


Figure 23 : Graph of Propranolol *In vitro* Calibration Curves.

(graphical presentation of data from section 3.8, detailed in Table 20 below)

Table 20 : *In vitro* Calibration Data for Propranolol. (results from section 3.8)

Concentration (μg/ml)	Peak Area (AU)		
	HPLC	Beckman P/ACE	HP^{3D} CE
5	1.66	0.348	0.041
10	3.66	1.054	0.097
25	9.38	1.515	0.192
50	19.8	2.338	0.368
100	41.9	3.833	0.704
250	110	15.667*	1.78
500	217	23.761*	3.43
1000	400	41.921	6.33
r	0.999	0.992 (0.999)*	0.999

r = correlation coefficient

* = these points were omitted from the calibration curve to enable more accurate quantitation

The linearity of the calibration curves was very good in each case, (r = 0.999), and was essentially unaffected by extraction from the rat microsomal protein. Two points were omitted from the standard curve on the Beckman as they biased the line below zero.

Table 21 : Propranolol Incubation Results. (data from section 3.8)

Time	Peak Area (AU)											
	HPLC				Beckman P/ACE				HP^{3D} CE			
(min)	P	h-P	%	Q	P	h-P	%	Q	P	h-P	%	Q
0	13.3	/	100	27.5	0.76	/	100	7.68	0.064	/	100	8.90
15	6.88	NR	52	11.7	0.35	0.03	46	2.52	0.037	0.002	58	4.66
30	6.36	NR	48	10.4	0.31	0.16	41	1.55	0.031	0.017	48	3.71
60	5.72	NR	43	8.82	0.27	0.26	36	0.59	0.024	0.020	38	2.61
120	4.69	NR	35	6.28	0.24	0.67	32	ND	0.019	0.029	30	1.83
N(0)	18.1	/	100	/	0.74	/	100	/	0.113	/	100	/
N(120)	17.8	/	98	/	0.79	/	107	/	0.109	/	96	/

P = propranolol

h-P = hydroxypropranolol

% = percentage of parent at time zero

Q = quantified results by calibration (μg/ml propranolol)

NR = no result

ND = non detectable i.e. negative value given from the calibration line

N = no NADPH control

The integrated results demonstrated that each technique coped very well with the matrix-derived samples from the rat microsomal incubation. Unfortunately the precise measurement of the HPLC peaks was compromised by the background absorbance from the NADPH which gave falsely high concentrations, but a similar trend in percentage degradation of parent compound was seen on each instrument. The data produced by CE were very accurate, giving initial

measured concentrations of propranolol between 7.7 μg/ml and 8.9 μg/ml against the nominal spiking concentration of 7.4 μg/ml. Approximately 70% of the propranolol was metabolised by the rat liver microsomes over the 2 hour incubation, with a time-related increase in production of the 4-hydroxy metabolite. This profile was common to each analysis technique.

The UV interference problem could be avoided in future by altering the wavelength used to monitor propranolol by HPLC or switching to an acetonitrile based mobile phase. There was no issue with NADPH co-absorbance by CE as the wavelength used was lower (214 nm) in the absence of a solvent-based mobile phase.

In conclusion, both techniques, HPLC and CE, proved suitable for the analysis of *in vitro* metabolism samples, although a few points should be born in mind in each case. The disadvantage with the HPLC method was that a wavelength of 254 nm was chosen to avoid background absorbance from the methanol in the mobile phase, but this wavelength suffered from interference by NADPH. However, HPLC was ten-fold more sensitive than the HP^{3D} CE method. The advantage of the CE methods was that a much lower wavelength could be chosen to monitor propranolol which had no co-absorption from the NADPH or methanol as they used an aqueous-based run buffer. A potential problem with CE for the analysis of the *in vitro* incubates could be the presence of remaining buffer salts from the samples which can cause disruption to the EOF and affect migration times, selectivity and peak shape. Care should be taken to ensure that the run buffer is approximately 100 times more concentrated than the injected solutes or the symmetry of the peak could be adversely affected. Also, if the solute ions have a faster/slower mobility than the buffer ions this can result in peak fronting/tailing.

CE is a relatively recent and still evolving technique compared to HPLC which has a more sound and long-standing niche in pharmaceutical analysis. The familiarity of HPLC along with a solid foundation of knowledge makes method development and trouble shooting less of a challenge than in the comparatively novel arena of CE.

This thesis concentrated on the development and performance of CZE methods for both acidic and basic compounds, the suitability of this technique for use in *in vitro* drug metabolism and its performance compared to HPLC. A better understanding of instrument operation and the mechanisms involved in column chromatography was a definite advantage over a comparable naiveté towards electrophoresis. The HPLC equipment used was from Perkin Elmer with BBN, PeakPro data capture. Two different CZE instruments were used for the various experiments: a Beckman P/ACE 2050 and a Hewlett Packard HP^{3D}. The HP equipment was the most recent and top of the range available with multifunctional, programmable features run on a windows computer package; whereas the Beckman, with a DOS-based program, was older with fewer capabilities and generally less reliable. However, despite the lack of familiarity with electrophoretic separation, method development using the HP CE instrument proved relatively straightforward, given the precise, electronically controlled parameter settings, and leant itself well to FED; although a certain amount of serendipity cannot be denied. A wider range of compounds would need to be investigated to provide a true challenge to the technology.

In a direct comparison, the HP^{3D} CE readily out-performed the Beckman P/ACE in terms of reliability, robustness and reproducibility, although this result will be greatly affected by the age bias of the instruments. When using pure standard solutions both CE and HPLC gave equivalent results, with robust, reliable methods and reproducible peaks both in height/area and migration/retention times. It should be noted that HPLC analysis was more sensitive, giving greater UV responses, due to the longer pathlength possible when using an off-column detector versus the narrower, *in situ* detection window of a capillary in CZE. However, this reduction in sensitivity did not appear to have an adverse effect on the accuracy, reproducibility or linearity of the CZE results, even at lower concentrations. This phenomenon could be due to more accurate and reproducible hydrodynamic pressure injections by CZE compared to the on-line loop and valve injections used with HPLC. Statistical analysis of repeated injections across a 200-fold concentration range actually indicated that CZE performed slightly better than HPLC, having slightly lower standard errors (on the HP CE and the Beckman P/ACE) and a marginally improved linearity on the HP CE than by HPLC, although this was not statistically significant.

CZE also gave higher efficiencies, with sharper, narrower peaks than those obtained by HPLC; although selectivity and resolution of the propranolol analogues were better by reversed-phase chromatography than by electrophoresis.

Several problems were encountered when applying these techniques to the analysis of *in vitro* metabolism samples, although it was possible to see evidence of metabolism using all three instruments. The electropherograms produced by CZE contained a number of spikes; these were assumed to be either electrical spikes or a noisy baseline. HPLC chromatograms suffered from background absorbance from the presence of NADPH, causing an enlarged solvent front which did not reach baseline before the elution of the hydroxy-propranolol peak. Despite these disturbances it was possible to detect a decrease in parent peak height as well as the appearance of the metabolite, which could be measured qualitatively, against a retention standard, if not quantitatively in all cases. HPLC is also able to cope better with "dirty" samples as it is affected less by endogenous matrix components which may be filtered out by the inclusion of a guard column which protects the packing material of the main column from becoming clogged. The open capillary of CZE means that everything injected into the capillary passes through to the detector which can cause interference in peak detection as well as complications associated with additional, endogenous salts which may disrupt the electrical current and the EOF and therefore impair the reproducibility of migration times.

The advantages of using CZE include the flat, plug-flow profile of the EOF which elutes peaks in sharp bands with high efficiences and short run times. The use of narrow capillaries and low volume buffer vials also reduces solvent/buffer usage and disposal costs. However, the disadvantage of such a low capacity system is that it also restricts the volume of analyte which can be introduced into the capillary, hence limiting sensitivity unless very small (1 to 50 nl) volumes of highly concentrated solute are used. HPLC is better suited to larger injection volumes (up to hundreds of microlitres), although this technique can also be sensitivity-limited by weak sample concentrations of sub nanogram levels. Reversed-phase column chromatography experiences lower efficiencies and longer run times than CZE with greater organic solvent usage, but benefits currently from a greater depth and breadth of experience and understanding.

HPLC would still be the method of choice for bulk analysis, a field in which CZE with its small injection volumes cannot compete. However, the many different modes of CE enable a wide

variety of applications for numerous analytes and can certainly offer a viable alternative to HPLC in many areas, including *in vitro* metabolism in the pharmaceutical industry. Future developments in the area of capillary electrochromatography (CEC), an amalgamation of the two techniques, may well provide the analyst with the combined advantages of both technologies.

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With the exception of any statements to the contrary, all data presented in this report are the result of my own efforts. In addition, no parts of this thesis have been copied from other sources.