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Capillary electrophoresis as a technique has many applications in a broad range of fields including forensics, environmental analysis, and biological analysis and as a separation method for samples such as oligonucleotides, peptides, proteins, and pharmaceuticals. The research conducted herein is for the antibiotic pharmaceutical ciprofloxacin HCl and three impurities. Sample stacking was used to overcome the poor limits of detection generally associated with CE, which successfully improved the limits of detection when compared to results from a previous feasibility study. This thesis encompasses three stages: method development, limit of detection determination, and proof of successful method development by analysis of tablets of ciprofloxacin. The method developed is reproducible, yields high resolution, and provides intense signals with respect to the detection generally achievable with capillary electrophoresis. The limits of detection of the impurities were determined to be below 0.05% (by concentration) of that of the active ingredient, and a brief tablet analysis showed that formulated tablets could be analyzed with this method.

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Employing Capillary Electrophoresis as a Separation Method for Pharmaceutical Analyses

by
Laura L. Lessard

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This thesis is dedicated to Keitha, David, Benjamin, Peggy and Daniel. I couldn't have done this without you.

EMPLOYING CAPILLARY ELECTROPHORESIS AS A SEPARATION METHOD FOR PHARMACEUTICAL ANALYSES

1 INTRODUCTION

1.1 HISTORY

Capillary electrophoresis is a technique that is relatively new to the landscape of analytical instrumentation and analysis. The concept of electrophoresis was established in the late 19th century by pioneers such as Helmholtz, Hittorf, and Kohlrauch [1]. Difficulties arose leading to band broadening and began the use of anti-convective supporting media such as paper or polyacrylamide. By using the supporting media in a buffer, the heat created by the electrical field applied became less of a problem. Paper was one of the first supporting media; paper electrophoresis was introduced in the mid 20th century. Placing analytes in aqueous solution on the wetted paper and applying a voltage across the paper induced movement of the molecules, and the rate of movement is dependant on the size of the molecule and the direction of movement is based on its charge. The molecules would stop at their neutral point, also called their isoelectric point.

Use of a gel such as polyacrylamide as the support medium has been common for approximately 30 years. With gel electrophoresis, molecules will be separated based on size. Protein analysis is commonly performed in this medium, and now CE is becoming a popular technique for protein analysis. With the gel as medium, the proteins must first be denatured. SDS (sodium dodecyl sulfate) is a detergent that is used for this purpose. It has both polar and non-polar ends and therefore can bind to

any protein. After the separation occurs under the influence of the applied electrical potential gradient, the slab is cut and stained and analyzed to determine approximate molecular weights. Although this method requires quite a bit of preparative work and yields no definitive quantitative data, this method is widely employed in the fields of biology and biochemistry.

Decreasing the separation size to narrow inner diameter conduits was introduced as early as the 1950s [2] by Haglund and Tiselius and picked up interest again in the late 1960 with Hjerten, to aid in the reduction of joule heating [1]. In the small diameter conduit separations, Teflon tubing as well as glass capillaries of 50-200 micrometer diameter were used and proved to be beneficial in reducing band broadening caused by poor dissipation of the heat produced [1].

The disadvantage that was realized early on with the capillary format was the decreased sensitivity due to the decreased path length. The advent of mass spectrometry and capillaries with increased pathlengths greatly improved the detection of the capillary format. A benefit that was recognized early was that with large surface area to volume ratios and therefore efficient heat dissipation, analysis time could be greatly decreased with use of higher applied voltages.

Capillary electrophoresis instrumentation became commercially available in the late 1980s, and the application of this technique has seen a dramatic rise since 1981 [1]. It is

now uncommon not to see capillary electrophoresis instruments in the pharmaceuticals industry, as a complement to liquid chromatography. Figure 1 shows the instrumental setup of the capillary electrophoresis instrument.

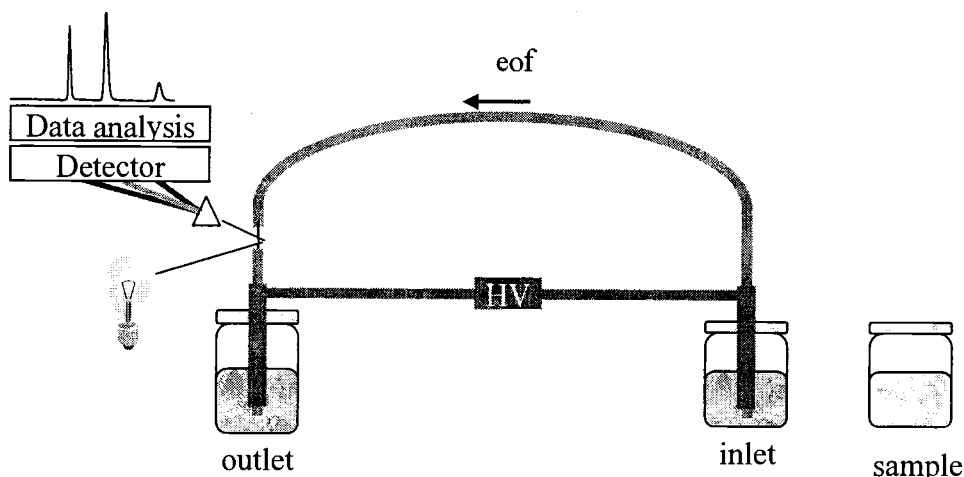


Figure 1.1 Instrument configuration for capillary electrophoresis

The sample vial contains the sample for analysis in the sample buffer. Prior to analysis, the capillary has to be conditioned and then filled with separation buffer, which may or may not be the same concentration as the sample buffer. The sample can be injected using various pressure modes, or electrokinetic injections by applying voltage. Once the sample is in the capillary, the inlet of the capillary is placed back into the run buffer and the separation ensues due to the applied high voltages. The detectors available and the electroosmotic flow (EOF) will be discussed later.

Use of capillary electrophoresis for pharmaceutical analysis is still relatively new and is becoming quite popular as a secondary method to high performance liquid chromatography [3-7]. A fully developed method using CE is a sensible secondary analysis to liquid chromatography. HPLC has a wide range of working methods for analyzing pharmaceuticals, and although capillary electrophoresis is becoming recognized for its utility in pharmaceutical analysis now, there are disadvantages to this technique.

1.2 ADVANCES IN ELECTROPHORESIS

Greatly decreasing the scale of the separation by performing it in a capillary resolved some of the complications involved with slab gel electrophoresis. Capillaries have a greater surface to volume ratio which aides in the dissipation of heat that may be produced from applying voltages through solution. Joule heating, as it is termed, leads to convection which broadens peaks[1,2,8]. Joule heating will be discussed further in the theory section. Also, by decreasing the size of the separation medium, the sample size required is decreased to the nanoliter range, and eluent volumes consumed are on the order of microliters[8]. Further advances have helped to overcome some of the less appealing features of this technique, such as poor limits of detection, which was addressed by the introduction of longer path lengths through extended detection windows, and solution manipulations can that also increase signal of a given analyte.

1.3 DETECTORS

In capillary electrophoresis there are many options for detectors available as well. UV/vis, fluorescence, laser-induced fluorescence, refractive index, conductivity, amperometry, and mass spectrometry are all available, and most of these are also common for HPLC systems[9].

Most common in the commercially available instruments is UV detection. With a UV/vis detector and photodiode array detector, the maximum wavelength of absorption can be chosen globally (for the entire sample) or for individual analytes in the sample. UV/vis like any detector requires that the sample has physical properties that allow it to be detected by the detector, in this case a UV chromophore.

Fluorescence detection requires that the molecule of interest be fluorescent. This detection method yields high sensitivities so long as the quantum efficiency of excited molecules is high. If the molecules are not fluorescent, the analyte would have to be derivatized into a fluorescing species for detection to be possible. LIF is similar to fluorescence, however a laser such as argon ion, helium-cadmium, or helium-neon is used to excite the electrons as opposed to a deuterium, tungsten, or xenon lamp[9].

Mass spectrometry can be coupled to CE with the proper interface. The only limitation for this detector in the samples that can be measured is the mass range of the instrument. There are no requirements such as a UV chromophore, or a fluorescent

species. In certain modes it can be highly selective to certain mass-to-charge ratios, or it can be used over wide mass-to-charge ranges.

1.4 ADVANTAGES TO USING CAPILLARY ELECTROPHORESIS (CE)

Capillary electrophoresis has its advantages in the range of samples that can be studied, the ability to analyze samples of small volumes, the robustness of a method within a set of samples, and broad range of pHs that can be used.

This technique has been used for oligonucleotides, carbohydrates, biomolecules, chiral compounds, environmental samples, isomer compounds, surfactant separations, dyes, amino acids, peptides, proteins, pharmaceuticals [3,7,10-11] and many other sample types. The volumes that are used in capillary electrophoresis are on the scale of nanoliters, where as the volumes required in HPLC are significantly larger in terms of both sample size and eluent needed. Another advantage to this technique is the potential for one working method to be applied to many different matricies. One method for basic pharmaceutical drugs may be applied to many other basic pharmaceuticals with little or no alteration to the method [8]. This emphasizes the robustness of this technique in particular to a set of pharmaceutical samples. Studies conducted with HPLC allow for less pH variation because of the stationary phase. The capillary wall in CE is capable of performing analyses from the low acidic to the high basic pHs[8]. Another important benefit to using CE over HPLC is the cost. CE capillaries can be bought relatively cheap and the life is dependant on the conditioning procedures followed. HPLC columns are very expensive, and due to the nature of the stationary phase,

dissolved pharmaceuticals depending on their formulation are likely to coat the stationary phase. Therefore the life of a HPLC column tends to be less than a CE capillary and they are more expensive.

1.5 DISADVANTAGES ASSOCIATED WITH CE

HPLC provides lower detection limits than CE in general because of the larger sample volumes and larger path length of detection [8, 12-16]. Employing a bubble cell capillary, or using a z-cell detection window with CE increases the path length approximately 3 to 10 fold respectively[17], and using a "sample stacking" methodology (discussed later) will enhance the signal by increasing analyte concentration in the capillary [12-16, 18-21]. Another disadvantage associated with capillary electrophoresis is irreproducibility. The proper conditioning steps must be taken in order to ensure that the capillary wall is equilibrated properly[8]. The inner surface of the capillary must be "etched" or cleaned with sodium hydroxide to remove any residual contaminants, preventing ghost peaks, as well as removing any cations that may have become bound to the silanols. Flushing with fresh separation buffer will then get the capillary equilibrated with the solution that will be used as a run buffer. A progressive, unidirectional shift in analyte elution time indicates that reproducibility is not sufficient.

1.6 APPLICATIONS

Capillary electrophoresis has many applications in analyses of oligonucleotides, biomolecules, proteins, pharmaceuticals, and many other areas [3-8, 10-11]. Utilizing CE

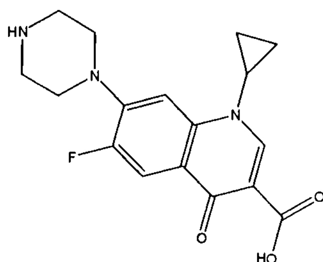
for the separation of ciprofloxacin from its impurities is similar to previous research that was conducted on other pharmaceuticals including an antibiotic with the same functionality as ciprofloxacin.

1.7 PREVIOUS WORK ON PHARMACEUTICALS USING CE

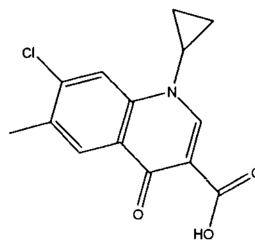
There is plenty of literature on the topic of pharmaceuticals being determined with capillary electrophoresis [3-7]. Basic pharmaceuticals should in general be dissolved in lower pH buffers, such as phosphate around pH 2.5, while acidic compounds should be dissolved in higher pH buffers such as borate at its natural pH of around 9.2 [8]. The compound that was studied here, ciprofloxacin HCl, is a quinolone which is negatively charged when dissolved in buffer. Altria et. al. studied a quinolone antiobiotic, similar to ciprofloxacin, using CE at a time previous to analysis using HPLC on this pharmaceutical. The CE method that was developed provided good limits of detection and quantitation, and yielded desirable linearity. HPLC has pH limitations due to the nature of the stationary phase, which meant that this technique could not offer the same results [5].

Ciprofloxacin HCl is an acidic, commercially-available drug that has been prescribed in the event of exposure to Anthrax. As stated before, it is a quinolone antibiotic, and its structure and the structures of the impurities associated with it are found in Figure 1.2.

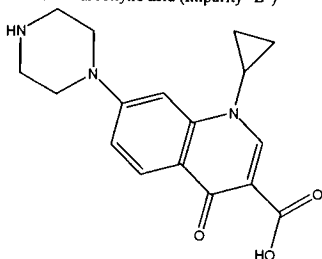
Ciprofloxacin HCl



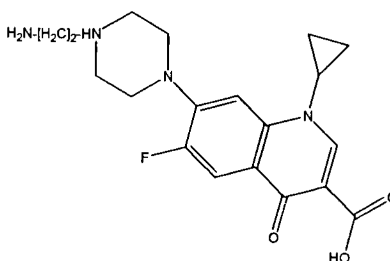
7-Chloro-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihyd roquinoline-3-carboxylic acid (Impurity "A")



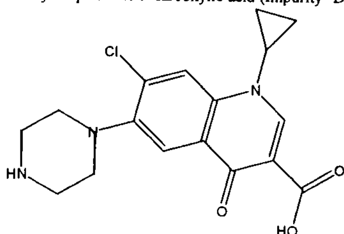
1-cyclopropyl-4-oxo-7-(piperazin-1-yl)-1,4-dihydroqui noline-3-carboxylic acid (Impurity "B")



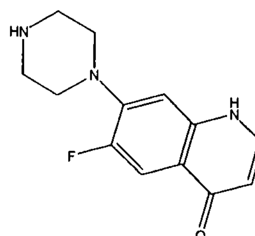
7-[(2-aminoethyl)amino]-1-cyclopropyl-6-fluoro-4-oxo-1 ,4-dihydroquinoline-3-carboxylic acid (Impurity "C")



7-Chloro-1-cyclopropyl-4-oxo-6-(piperazin-1-yl)-1,4 -dihydroquinoline-3-carboxylic acid (Impurity "D")



1-cyclopropyl-6-fluoro-7-(piperazin-1-yl) quinolin-4(1H)-one (Impurity "E")



1-cyclopropyl-6-hydroxy-4-oxo-7-(piperazin-1-yl)-1,4-di hydroquinoline-3-carboxylic acid (Impurity "F")

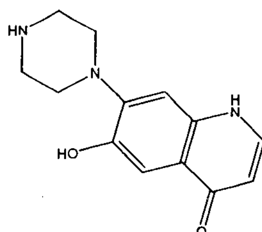


Figure 1.2 Structure of Ciprofloxacin HCl and impurities "A", "B", "C", "D", "E", and "F". The functional groups on all of these compounds except Impurity "E" will be acidic upon dissolution as they can lose a proton at a high pH. Impurity "E" is a neutral species.

2 THEORY

2.1 THE ELECTROCHEMICAL DOUBLE LAYER AND ELECTROOSMOTIC FLOW (EOF)

Inside the capillary coating, which is polyimide, there is bare fused silica. Figure 2.1 below shows the wall of the capillary with the negatively charged surface.

The purpose of the polyimide coating is to enhance flexibility and protect the surface of the capillary. The purpose of using a silicate inner wall is to make use of the silanol groups on the surface. This charged surface serves to attract the cations from the buffer solution. This attraction forms a layer that is essentially static, or immobile against the capillary wall. A more loosely held layer of charge forms next to that layer, or closer to the center of the capillary, which contains more charged species are attracted weakly to the static layer in an attempt to balance the charge at the surface of the all. These layers are coined the static (Stern) layer and the mobile (Gouy) layer respectively[1,2,8,9]. Figure 2.1 shows the charge effects that create the electroosmotic flow.

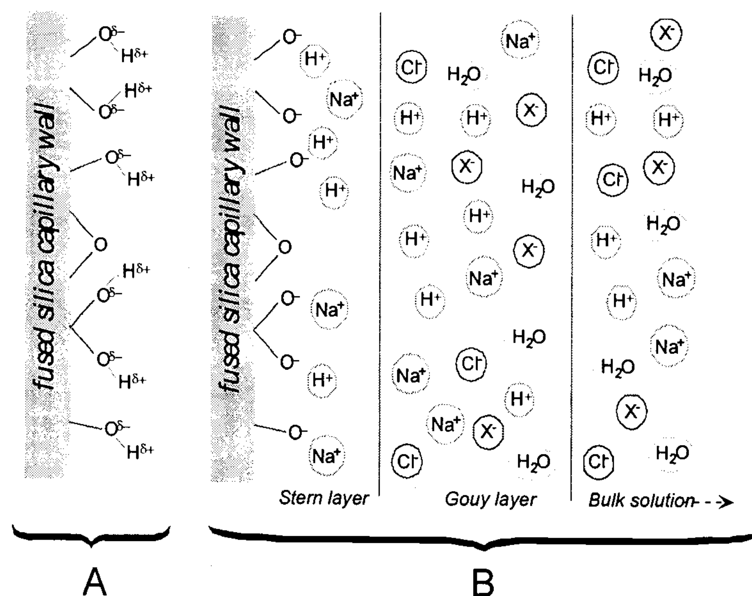


Figure 2.1 The electrochemical double-layer (adapted from Remcho, V. T., *Chem. Educator*, **1997**, 2.

When a voltage is applied, the bulk of the solution will migrate toward the cathode, and thereby the electroosmotic flow (EOF) is established. The EOF rate is proportional to the voltage applied and therefore it will migrate in a direction depending on the polarity of the applied voltage, and at a rate that is dependent on the magnitude of the voltage. The EOF velocity can be determined by injecting a neutral marker into the capillary and applying the desired voltage with the proper polarity. The time that it takes for the neutral marker to reach the detector is dependent only on the electroosmotic flow of the solution, and therefore the EOF can be determined experimentally.

A benefit to using the solution pump system (EOF) as opposed to the mechanical pump used in HPLC is the profile created. With the proper experimental conditions and a properly equilibrated capillary inner surface, a flat profile is generated. This is an advantage especially with electrophoresis on the capillary format because the small volumes of sample that are being used. As the sample bands are moving through the system, a laminar profile will yield broad peaks due to the dispersive nature of the broad range in flow velocities. With a flat profile, there is significantly less dispersion since the origin of flow is at the wall of the capillary [9]. Figure 2.2 shows the difference between laminar profile and flat plug profile.

Figure 2.2 Laminar profile vs. flat plug profile.

The profile of the electroosmotic flow that is generated under the proper solution, surface and voltage conditions is illustrated on the left in figure 2.2. This flat profile is desirable mainly for the tight focusing of component zone that will reach the detection window over the course of a small but finite amount of time. This is important not only because the sample volumes are so low, but also because the path length of the detection window is very small. The narrow peaks produced using flat plug versus laminar will also decrease errors in peak area measured.

The pump-driven profile on the right in figure 2.2 is laminar. The laminar profile is undesirable because of the broad peaks that are generated due to the long periods of time that the analyte is passing the detection window. The pump driven mechanism is therefore not used as a driving force in the separation using CE for the grand errors that would be introduced.

2.2 ELECTROPHORETIC MOBILITY

Separation of analytes in CE is due to differences in their electrophoretic mobilities, μ, which was first demonstrated by Tiselius' work on proteins[1]. The pH of the buffer

will essentially determine the charge of the molecule and will therefore determine the direction that the molecule will migrate under an applied field.

(1) $$\mu = q/6\pi\eta r$$

Here q is the charge of the molecule; η is the viscosity of the buffer system, which can easily be determined using a viscometer (cP); r is the Stokes radius of the particle. Without knowing the magnitude of the electrophoretic mobility, the direction of migration can be easily determined using this equation. A more straightforward equation to determine the direction of the electrophoretic mobility is the electrostatic force.

(2) $$F_e = qE$$

Where F_e is the electrostatic force on the molecule; q is the charge of the molecule; E is the force applied in both magnitude and direction (V).

Knowing the electrophoretic mobility can therefore indicate the polarity of the system that should be employed. In terms of the effect of molecule size on migration, the smaller the particle (smaller r) the faster the molecule will migrate to the detector, and vice versa [1,2,8,9]. Therefore, as figure 2.3 shows the relationship between the EOF and the electrophoretic mobility is dependant on the molecules mass to charge ratio.

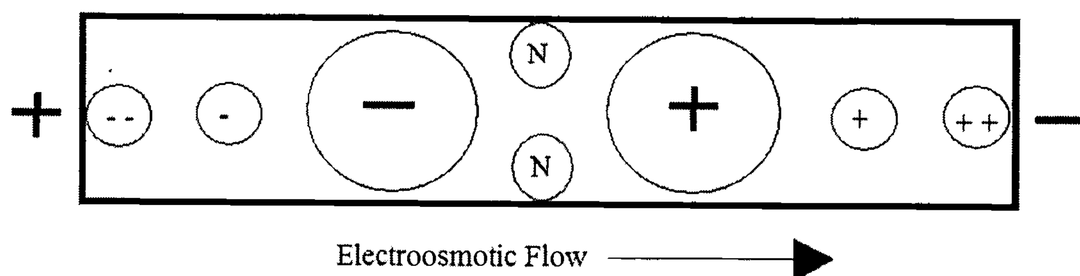


Figure 2.3 Process of separation in the capillary demonstrating EOF and electrophoretic mobility (Figure adapted from Baker, D. R., *Capillary Electrophoresis*, Wiley-Interscience, New York, 1995)

Figure 2.3 shows that as the mass to charge increases for a cation, the faster it moves. The cations in the system are going to travel the fastest through the system than the electroosmotic flow where as the anions will move slower. Because the neutral species have no charge or electrophoretic mobility, they will move at the same rate as the electroosmotic flow. Since the negative species have no secondary separation mechanism of separation, they will be separated from the charged species, but not from other neutral species without modifications being made to the molecule; a topic that is beyond the scope of this thesis. Separating anions from other anions is based on their mass-to-charge ratios. The smallest mass-to-charged anion will be most attracted to the positive electrode and therefore will be retained the longest. The large molecules will little charge will have a high mass-to-charge ratio and will therefore have less force opposing the electroosmotic flow and will come out soon after the neutral species. The separation works the same for the cations of different sizes and charged; as drawn in figure 2.3 above, the smaller molecule with the highest charge

will be most attracted to the negative electrode so it will be traveling much faster than the electroosmotic flow with respect to all the other analytes in the sample.

With the combined use of the electrophoretic mobility and the electroosmotic flow, it is possible to analyze both anions and cations in one run where as, without the electroosmotic flow acting to "push" everything through the system, the anions would never reach the detector in a positively-charged system and the same with cations using a negative separation voltage. The one criterion is that the electrophoretic mobility of the anions must be smaller than the EOF in the schematic drawn above showing positive polarity. Also, the electrophoretic mobility must be in the opposite direction of the electroosmotic flow in order for a separation to occur. If the electrophoretic mobility were in the same direction as the EOF, the analytes would not have time to separate.

2.3 FIELD STRENGTH

The length of the capillary will effect the analysis time, the resolution of the analytes, as well as field strength that can be achieved. The field strength is determined by dividing the voltage applied by the total length of the capillary.

$$\mathbf{E(v/cm) = V_{app}/L_{total}} \quad \textbf{(3)}$$

Here V_{app} is the voltage applied (V); L_{total} is the total length of the capillary. Theoretically using this equation, field strengths as high as 1500 V/cm can be

achieved using a capillary 20 cm in length and applying a voltage of 30 kV (which is the practical limit of the instrument used for this study).

2.4 JOULE HEATING

When a voltage is applied to the capillary, the buffer will conduct current of a certain magnitude depending on conductivity of the buffer used, K_{buffer}. In this process heat is created. If insufficient dissipation of the heat occurs, the resultant electropherogram will have broad peaks due to convection which will have detrimental effects on the efficiency of the analysis.

(4) $$\Delta H = K_{buffer} V_{app}^{2} / L_{total}^{2}$$

Here ΔH is the heat produced; K_{buffer} is the conductivity of the buffer; V is the voltage applied (V); L is the length of the total capillary (cm). The last portion of the equation, $V_{app}^{2}/L_{total}^{2}$ is proportional to the square of the field strength from equation 3 above, and therefore, as the field strength increases the likelihood of joule heating also increases. Utilizing small inner diameter capillaries at longer lengths increases the surface to volume ratio of the capillary and decreases the probability of joule heating while being able to use higher voltages.

2.5 OVERCOMING POOR DETECTION LIMITS

The poor limits of detection associated with CE using absorbance detection are due to the small sample volume and small detection window as stated before. There are ways to alleviate this problem and lower the LOD, such as altering the buffer system in

some way or increasing the path length. Sample stacking, large volume sample stacking, bubble cell capillaries, z-cell interfaces, and multi-reflection capillaries are methods of sample concentration and path length magnification that are used to improve the limits of detection.

2.5.1 INSTRUMENT ALTERATIONS

The instrumental modifications that have historically been employed to overcome poor LODs are using a capillary with an extended detection window. The diameter of the capillaries that are used for the separation are on the order of 50 μm to 75 μm generally. Figure 2.4 can be referred to for the types of cells for detection enhancement in capillary electrophoresis.

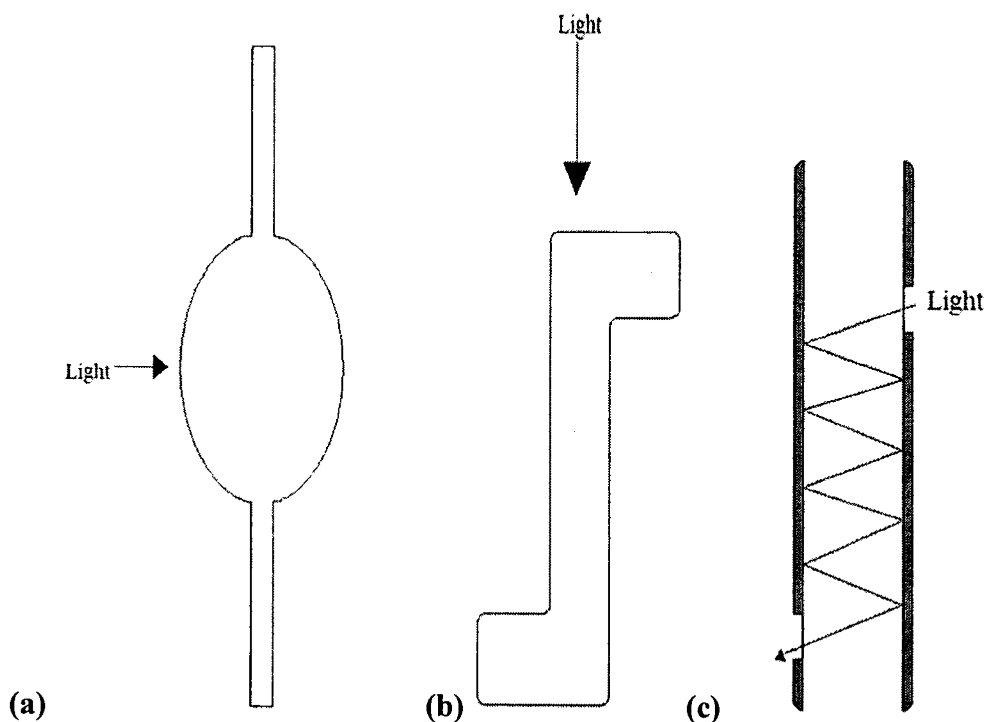


Figure 2.4 (a) Bubble cell capillary; (b) z-cell capillary; (c) multi-reflection capillary

Using a commercially available bubble cell capillary (a), the path length can increase up to 5 fold, which greatly enhances the signal without sacrificing all the appealing properties associated with small inner diameters. Of the extended pathlength capillaries shown above, this is the best choice without loss of resolution due to closely eluting analytes.

The z-cell interface (b) increases the path length to the millimeter range, which enhances the signal even more over the bubble cell capillary, as well as increasing the linear dynamic range up to 4 orders of magnitude [17]. Using the z-cell capillary is an expensive way of increasing the pathlength, and may decrease resolution of closely eluting analytes. Without acceptable resolution, the increase in sensitivity is useless.

A less common type of detection cell is the multi-reflection cell (c) which provides an effectively longer pathlength of the cell by allowing light to reflect off the walls of the capillary multiple times. A coating of silver is on the outside of the capillary wall which acts like a mirror which reflects light through the capillary multiple times. The number of reflections before detection is proportional to the amount of magnification of signal read at the detector.

2.5.2 BUFFER ALTERATION

Sample stacking and large volume sample stacking (LVSS) are methods that can be used to enhance the signal of a low concentration analyte thereby decreasing the LOD.

In both stacking methods, a magnification of the signal occurs from buffer concentrations being different in the sample matrix and the run buffer. A ten-fold increase in concentration from the sample buffer to the separation buffer has shown to improve the signal up to 2,000 fold [12-16, 19-21]. For clarification purposes, the terms run buffer and separation buffer are synonymous, and are used interchangeably throughout the text.

2.5.2.1 SAMPLE STACKING

Sample stacking takes advantage of different conductivities of buffers present in the sample and in the eluent. Equation 5 relates the concentration of a solution to the its conductivity.

$$K_{buffer} = F\Sigma_i (|z_i| u_i C_i) \quad (5)$$

Here F is Faraday constant; z_i is the magnitude of the charge of the ion; u_i is the mobility of the ion which is a function of viscosity; C_i is the concentration of the buffer. Holding z_i, F, and u_i constant, increasing the concentration will increase the conductivity of the buffer.

The sample buffer in which the sample is prepared is made at a concentration 10 times less than that of the separation buffer. This 10 fold increase in concentration from the sample buffer to the run buffer gives a 10 fold decrease in the conductivities from the sample buffer to the run buffer. In the lower concentration sample buffer, the analytes move at higher velocities, accelerating all of the analytes toward a convergence zone

at the sample buffer-run buffer interface. This rapid process also sorts the analytes into bands based on their charge and size. The EOF also acts to move the bulk of solution from the inlet to the outlet, so the sample is moving at a largely accelerated rate compared to moving under just the force of the EOF. When the analytes exit the sample buffer, or "hit" the wall where the separation buffer zone begins, the samples are forced to slow down due to the decrease in conductivity in the run buffer. This decrease in the velocity causes the individual analytes to slow down thereby focusing the bands that have been created in the first separation step further based on their electrophoretic mobility. [8, 12-16, 19-21]. Figure 2.5 below shows the process of separation using sample stacking.

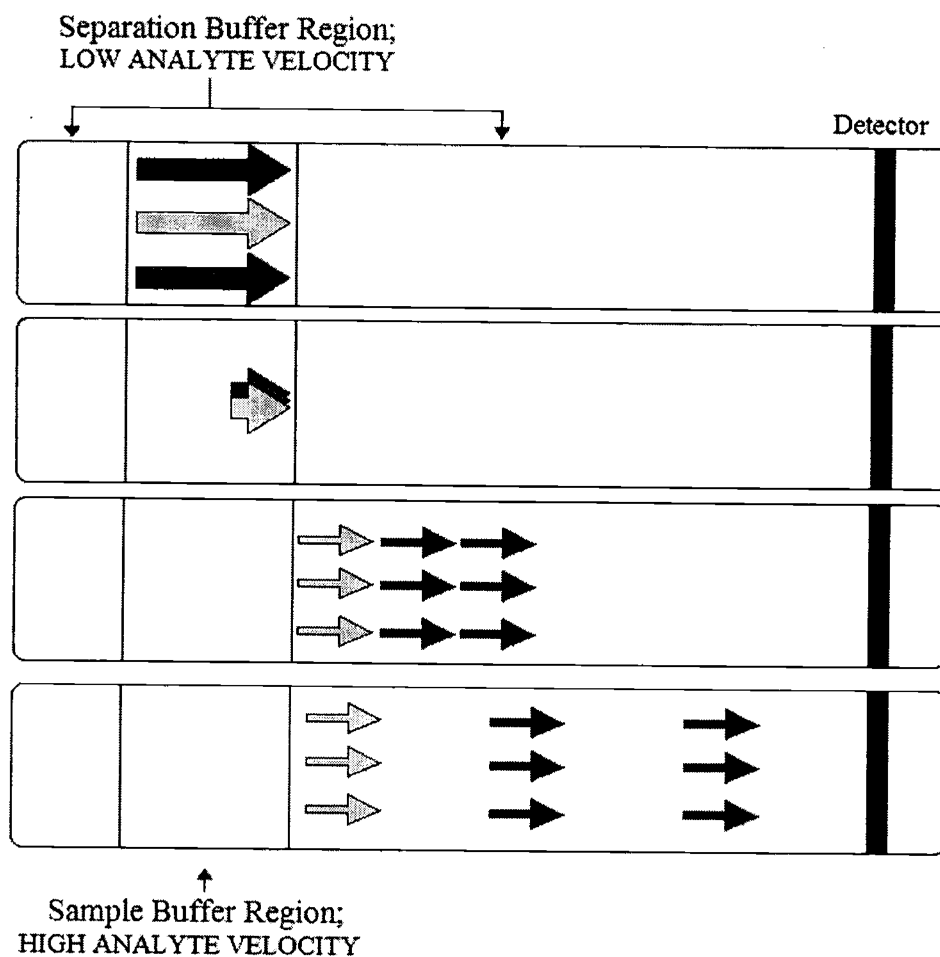


Figure 2.5 Sample stacking with separation buffer at 10 times the concentration of the sample buffer.

2.5.2.2 LARGE VOLUME SAMPLE STACKING (LVSS)

LVSS is similar to sample stacking in the resulting effects and use of different conductivities to enhance the signal. However, with LVSS larger volumes are injected and the sample buffer must be removed to compensate for the overly large volumes injected in LVSS.

The viscosity of the sample matrix must be determined in order to control the length of capillary that will be filled with sample. The volume of sample injected can be determined by using Poiseuille's Law [15].

(6) $$Pt = 3200 L_{inj} \eta L / d^2$$

Where P is the pressure applied (dynes/cm^2); t is the length of time that the sample is injected (variable seconds); L_{inj} is the length of capillary that will contain the sample (cm); η is the viscosity of the sample matrix (dynes/cm^2); L is the total length of the capillary (cm); d is the diameter of the capillary (cm).

At a given pressure, the longer the injection, the larger the volume of sample injected in the capillary. Increasing the volume injected can have negative effects on resolution; however, it will undoubtedly produce larger signals for the same concentration of analyte. Resolution can suffer using this method because in the capillary, if an appreciable volume of sample buffer is left in the capillary for the duration of the separation, mixing can occur and the stacking effects will be decreased and a laminar profile can result [9, 12, 16, 20].

To reduce the chance of decreasing the resolution by increasing band broadening, the sample buffer must be removed after injection without removing the analytes. This is sometimes coined the "sample buffer back-out" method. Figure 2.6 below illustrate the general method of the sample buffer back-out method used for on-capillary sample concentration with large volumes.

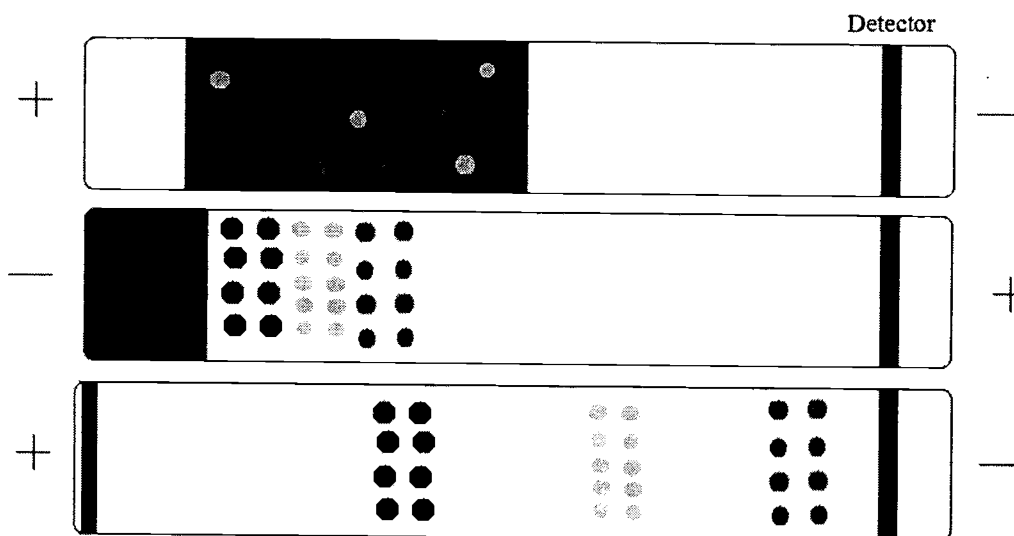


Figure 2.6 LVSS using sample buffer back-out.

The purple colored region with the molecules is the sample matrix, or sample buffer region while the white regions are the run or separation buffer. In sample stacking, the concentration of the run buffer is 10 times that of the sample buffer. First the sample is injected. For large volume sample stacking the period of injection is longer than with traditional sample stacking. As shown above, the voltage being applied initially is positive, therefore driving the EOF towards the outlet. Since the sample volume injected with LVSS is larger than the sample volume with traditional sample injection, the sample buffer must be removed to avoid mixing the sample and run buffers which would lead to band broadening from inconsistencies in flow velocity. One way to achieve this is by applying a voltage that is opposite in polarity but equal in magnitude to the run voltage used for the separation, thus removing the sample buffer. To determine if the sample matrix has been removed, the current should be monitored.

Doing so will determine the necessary duration of reverse polarity [15, 20-21]. An associated pitfall to the "sample buffer back-out" method for LVSS is the resultant inconsistent EOF if the current is not properly monitored [12, 16, 20-21]. Another problem associated with the sample buffer back-out method used with LVSS is that sometimes partial removal of analytes is experienced if the polarity is switched for too long. For this reason, recovery of the current back to 100% the current in run buffer alone should not be attempted [15].

3 MATERIALS

All standards of the impurities used (7-chloro-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid "A", 7-[(2-aminoethyl)amino]-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid "C", and 1-cyclopropyl-6-fluoro-7-(piperazin-1-yl)-1,4-dihydroquinoline-3-carboxylic acid "E") as well as the ciprofloxacin HCl standard were available from the US Pharmacopeia, Rockville, MD., USA. Milli-Q water was used throughout. All the buffers and the dissolved tablet were filtered through a 0.45 μm PVDF filter manufactured by Lida Manufacturing Co., Kenosha, WI., USA. Acetonitrile was purchased from J.T. Baker, Phillipsburgh, NJ., USA, sodium tetraborate decahydrate was purchased from Sigma, St. Louis, MO., USA, sodium hydroxide from Mallinkrodt, Paris, KT., USA, sodium chloride and concentrated hydrochloric acid (for preparation of modified simulated gastric fluid (mSGF) in the tablet dissolution) were purchased from Fisher, Pittsburgh, PA., USA. 75 μm ID bare fused silica capillary was purchased from Polymicro Technologies, Phoenix, AZ., USA. An Agilent 3D/CE, Palo Alto, CA., USA, equipped with a deuterium lamp for UV detection at 254 nm, purchased from Sonntek, Upper Saddle River, NJ., USA was used throughout the experiment. A Cannon-Ubbeholde viscometer No. N293 with a 75 μm capillary was borrowed from Dr. Skip Rochefort's lab for the viscosity measurements.

4 METHOD

Resolution in capillary electrophoresis depends on factors such as the field strength, buffer composition, pH of the buffer system, and degree of dissipation of heat created in the capillary. In this study, the buffer conditions, run voltages and sample volume injected were studied in an effort to develop a robust CE method for acidic pharmaceutical analyses.

There were three steps involved for the analysis of Ciprofloxacin HCl and its available impurities. The first step was the method development, in which the resolution and detectivity of all the available analytes are optimized. Examining pH effects as well as using sample stacking and LVSS, and testing the effect of organic solvents on the stacking procedure took place in the method development portion of the study. Samples were either individually analyzed or analyzed in a sample mixture spiked to determine the order of elution. The second step that is critical from the pharmaceutical industry standpoint is determining the limit of detection for each of the impurities. This step involves quantitating the signals due to each of the impurities at a certain percent concentration with respect to the concentration of the active ingredient. This was performed using blank measurements to account for noise, and the LOD's determined were 3 times the signal due to noise. Finally, a real sample of the pharmaceutical tablet was analyzed using the developed method to determine if the impurities are above the limit of detection that was determined in the previous step, and to ascertain if the presence of excipients (inactive ingredients in the tablet

formulation) has an effect on the separation. This portion of the study is the reinforcing step ensuring that the method can be used for analysis of pharmaceutically relevant samples.

4.1 BUFFERS

Ciprofloxacin HCl is an acidic pharmaceutical. When dissolved in a high pH buffer, the proton on the R-group is lost and the compound is negatively charged. To determine the buffer that should be used, the literature was consulted for analyses of acidic drugs [3, 5, 7, 8]. Borate had been used for several CE analyses on pharmaceutical agents of similar structure. This buffer has appealing aspects to it, such as its naturally high pKa, and number of moles of borate atoms relative to the molecular species [3, 7, 8, 18]; $Na_2B_4O_7 \cdot 10H_2O$ has four moles of borate to the molecule. Other high pKa systems that could have been prepared include a phosphate buffer prepared with the monohydrate and dihydrate species. Another appealing aspect of borate buffers is the life time of the buffer compared to that of phosphate and acetate buffers. After preparation, the buffer proved to be stable for over 3 weeks when kept refrigerated without growing microbes which give noisy baselines using absorbance like phosphate and acetate buffers do.

Sample stacking and LVSS were to be studied to enhance the detection limits of the analytes. Sample stacking works best when the concentration of the separation buffer to the sample buffer is 10:1. Lower concentration buffers are desirable to minimize joule heating and so a 5 mM borate buffer was prepared as the sample buffer. In a previous feasibility study, it was determined that the impurity standards would not dissolve in a purely aqueous medium but would with some added acetonitrile (ACN). For this reason, 33% acetonitrile (by volume) was added to the 5 Mm borate buffer. All stock solutions and further dilutions were performed using this 5 mM borate buffer with 33% ACN. For the sample buffer, an extensive pH study was not conducted due to the small amounts of standard samples on hand and the inability to obtain more. The pH that was ultimately selected for the sample buffer was ~9.25.

Per the sample stacking procedure to use 10:1 (run buffer: sample buffer) ratio of concentrations a 50 mM borate buffer was prepared. In a previous study, using a run buffer without acetonitrile, a signal due to ACN was present from the sample. This is undesirable especially if ACN is eluting at or near one of the analytes. To determine which concentration to use with that in mind, parallel studies were conducted with a 50 mM borate buffer with 33% acetonitrile (by volume) and with 3.3% (by volume). Both the solutions had very similar pH's measured prior to addition of the ACN.

A pH study was conducted using three different pH values with all other solution characteristics held constant. The first studied was at the natural pKa of borate, 9.25,

secondly at a pH of 8.22, and finally at a pH of about 10.05. The two pH values that were adjusted from the natural pH were adjusted to pH 8.22 and 10.05 with concentrated HCl and 1 M NaOH respectively. All pH measurements were performed before the addition of the organic.

Prior to using the buffers, they were all filtered through a 0.45 micron PVDF filters. Filtering the buffers helps to prevent the capillary from getting clogged.

In order to use LVSS, the viscosity of the sample solution had to be measured. To conserve the standards available, the viscosity measurement was performed using just the sample buffer (5 mM borate buffer pH 9.25 with 33% acetonitrile (by volume)) without the analytes present on a 75 μm Cannon-Ubbeholde viscometer. For the tablet analysis, the viscosity of the sample being injected will change dramatically relative to the standards due to the nature of the tablet in solution. Excipients and other ingredients present in the tablet formulation yield a more viscous solution which will decrease the volume injected under the same pressure and time conditions. The measurements were performed against deionized water. The viscometer capillary was rinsed and filled with fresh sample buffer. The solution was drawn up into the capillary and then allowed to drain from it by force of gravity. This experiment was performed in triplicate. Next, water was measured, again in triplicate. Performing the measurements in this order ensured that the sample buffer solution was not diluted by the water. The times (s) were used to set up a proportion comparing the viscosity of

water at 21^{0}C and the time it took to pass through the capillary to the time it took for the sample buffer to pass through the capillary. The viscosity of the sample buffer was determined to be 1.0764 cP compared to 0.98 cP for water at 21^{0}C. Knowing the viscosity allowed for calculation of the volume of sample that was injected when a certain pressure was applied for a certain length of time using Poiseuille's Law in equation 5 above; ($Pt = 3200\ L_{inj}\ \eta\ (L/d^2)$).

4.2 CAPILLARY

4.2.1 PREPARATION OF THE CAPILLARY

A bare fused silica capillary with an inner diameter of 75 μm was cut to 50 cm, and a detection window burned at 41 cm. This was performed on a fiber optic fusion splicer, which removes the polyimide coating by burning it off leaving behind only the fused silica for the detection window.

4.2.2 CONDITIONING THE SURFACE OF THE CAPILLARY

Borate buffer quickly equilibrates at the surface of a capillary, however it was allowed to sit for at least 2 hours in the capillary before flushing prior to analysis. In the previous study, phosphate buffer was used, which requires a much longer time to equilibrate and should be allowed to sit against the capillary walls overnight to ensure proper surface conditioning[8]. In the event that the capillary interior wall was exposed to air or the capillary had sat for a long period of time draining some of the liquid from the inlet and outlet, the capillary was again conditioned with fresh run buffer to

regenerate the surface. This step should also be performed if the buffer itself is changed, and then copious amounts of buffer should be flushed through. A poorly equilibrated capillary is distinguished by an irreproducible electroosmotic flow (EOF). In this study to condition the capillary from dry, the capillary was etched with 1 M NaOH, flushed with water and then filled with run buffer and left to sit for 2-4 hours to allow the surface to become equilibrated in the buffer. This buffer is then flushed with fresh run buffer and the capillary can then be used for analysis. This method was repeated if the run buffer was changed in any way.

4.3 SAMPLE PREPARATION

Stock solutions of impurities "A", "C" and "E" and ciprofloxacin HCl were prepared in volumetric flasks. Each solid was weighed directly into a volumetric flask and the exact concentration was calculated in ppm (w/v). Stock concentrations did not exceed 120 ppm (w/v) and were above 80 ppm (w/v). Higher concentration stock solutions were not needed; for sample stacking, concentrations in excess of 5 ppm (w/v) were unnecessary. A mixture of all the analytes in equal concentration was prepared and run under each of the conditions studied. Most of the runs were performed at 5 ppm (w/v) in the method development portion of the study. To determine the order of elution, the stock solutions of each analyte was run individually. This was only done when the method being studied proved to be promising in both signal intensity and resolution.

For the limit of detection (LOD) portion of the study, concentrations of 10, 5, 2.5, 1, and 0.5 ppm (w/v) were prepared. A goal of the LOD study was to be able to detect impurities at concentrations 0.05% (by concentration) of that of the active ingredient. The lowest concentration studied (0.5 ppm (w/v)) is 0.05% of that of ciprofloxacin HCl when it is prepared at 1000 ppm (w/v).

The tablet preparation involved dissolving the tablet which contained the API, ciprofloxacin HCl, and all the impurities shown in figure 1.2 in a solution that resembled gastric fluid. The solution was diluted to 1000 ppm (w/v) with the sample buffer and then analyzing using the developed method. Crushing the tablet was decided against to avoid contamination, loss of sample, and also to simulate a more realistic scenario. The stock solutions for the tablets were prepared at 5000 ppm (w/v). Each ciprofloxacin HCl tablet is 500 mg API. By dissolving the tablet in a 100 mL volumetric flask, this yields 5000 ppm (w/v) ciprofloxacin HCl. A 1:5 dilution was then made using the sample buffer as the diluant. Prior to dilution, the 5000 ppm (w/v) tablet was filtered through a 0.45 μm PVDF filter. This helped remove residual excipients that make the analyte into a solid tablet form. It should be noted here that the viscosity of the dissolved tablet solution is noticeably higher than that of the sample buffer. A viscosity measurement of the tablet was not performed since LVSS was not chosen as a means of sample preconcentration. Two tablets were analyzed for this study, being analyzed only once due to the duration of the run with proper conditioning procedures.

4.4 METHOD OF INJECTION

Injecting by pressure, where pressure is applied to the inlet and forces the sample up into the capillary, and is less discriminatory to the sample than electrokinetic injection. Other pressure injection methods are by applying a vacuum at the outlet of the capillary, or holding the inlet at a higher level than the outlet creating a siphon effect. These are all non-discriminating modes of injection; however, injecting by applying voltage would introduce a bias on the sample introduced leaving lower mobility analytes behind. For this study, pressure was applied to the inlet vial to inject the sample. The length of time varied in the study depending on the length of capillary that was desired to be filled. A pressure of 25 mBar that was instrumentally controlled was used as a constant throughout the duration of the study.

4.5 INJECTION: LENGTH OF INJECTION (SAMPLE STACKING AND LVSS)

The 5 ppm (w/v) mixture of "A", "C", "E" and Ciprofloxacin HCl was injected at 25 mBar for 5 s, 12.23 s, and 61 s. These times correspond to injecting 0.41 cm, 1 cm, and 5 cm respectively. These lengths were determined using Poiseuille's Law which was discussed earlier. The latter two times correspond to the LVSS mode. The large volumes that were injected demanded that the sample buffer matrix be removed fully or a laminar flow profile could result [12, 16, 20].

4.6 LOD DETERMINATION

4.6.1 ANALYSIS

For the limit of detection portion of the study, mixtures of the impurities (only) were prepared at 10, 5, 2.5, 1.0 and 0.5 ppm (w/v). Prior to the LOD study, the mixtures were run in at least triplicate to ensure reproducibility, and during the LOD study, each standard concentration was run in duplicate.

4.6.2 DATA ACQUISITION AND ANALYSIS

The electropherograms that were produced from each run are plots of absorbance intensity (mAU) versus the time. The wavelength of detection for the entire study including the LOD and tablet analysis was 254 nm since it yielded the highest absorbance signals for all the analytes. The migration time is the critical factor in qualitatively determining the species that is being seen. A reproducible EOF is therefore critical in the data acquisition portion of any capillary electrophoresis study. To determine the limits of detection, a paper by G.L. Long et. al [22]was referred to which described the IUPAC method of determination of LOD. Calibration curves for impurities "A" and "C" were prepared.

Calibration curves consisted of plots of the areas for each impurity at the corresponding concentrations. The areas of the peaks were determined manually by using an integration tool on the ChemStation (Agilent, Palo Alto, California, USA) software. The area of the peaks were measured from the baseline. For the blank

signal, the average elution time and its standard deviation around the average elution time for all the runs of "A" and "C" were considered. The blank was run in duplicate, and the areas of average elution, +/- the standard deviation was manually integrated and used for the LOD calculations. The concentrations that were determined to be the limit of detection yield a peak area that was three times that of the noise in the baseline. The noise was determined by measuring the sample buffer without analytes present (blank).

The following equations were used to determine the limits of detection for impurities "A" and "C".

(7) $$\mathbf{x_L = x_B + K(s_B)}$$

Where x_L is the smallest signal that can be detected for a given analyte; x_B is the average area integrated from the blank; s_B is the standard deviation of the blank measurements made; K is defined to yield a certain confidence level. K values are generally between 2 and 4, with 2 giving the least confidence for the concentration limits that are detectable.

(8) $$\mathbf{C_L = (x_L - x_B)/m}$$

Where C_L is the lowest concentration that an analyte is detectable at; m is the slope of the calibration curve that is obtained when a series of concentrations of standards are run and areas integrated for at least 4 concentrations. The two equations can be combined to yield equation 8.

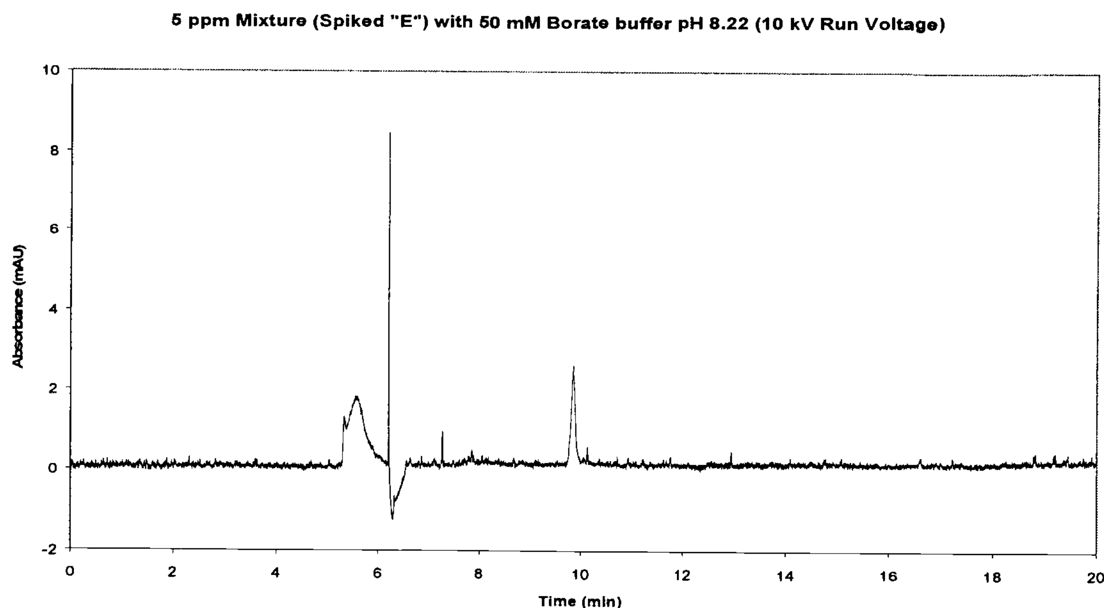
(9) $$\mathbf{C_L = K(s_B)/m}$$

Equation 9 shows that as the standard deviation of the areas resulting from the blank measurements decreases the confidence level increases. Two blank measurements were used for the background correction to determine the LOD for both impurities studied.

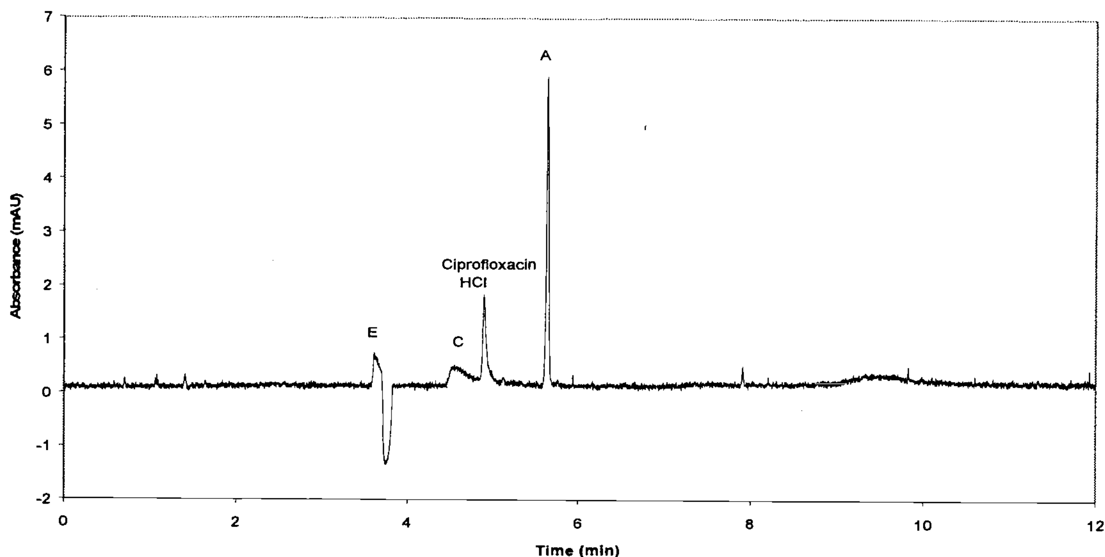
5 RESULTS AND DISCUSSION

5.1 pH: EFFECTS ON STACKING

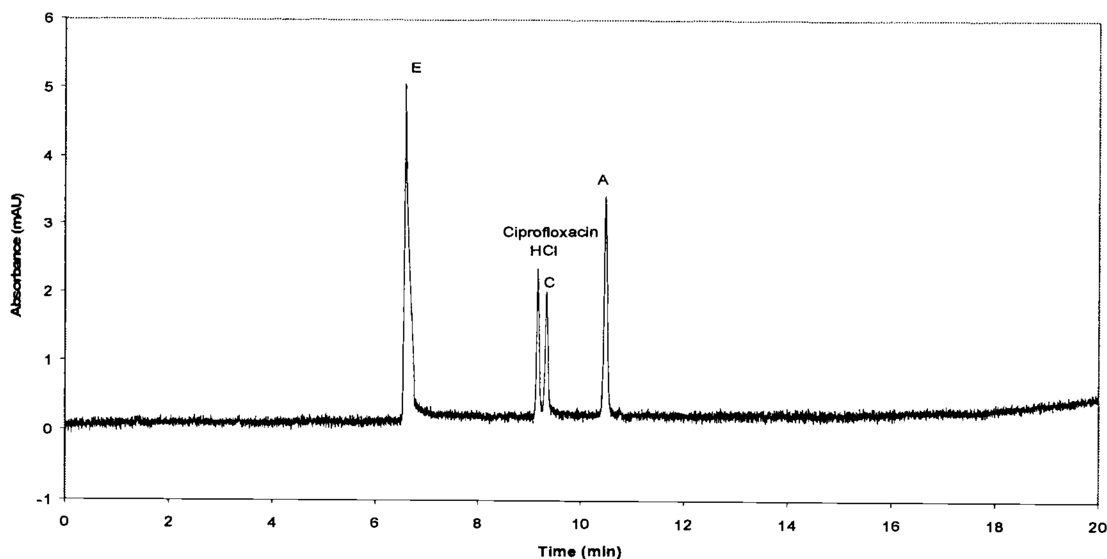
The effects of pH were also studied using 50 mM borate buffer with 3.3% acetonitrile. With 1 M NaOH or 37% HCl the pH was adjusted in the range of plus or minus 1 pH unit from the pKa of borate, 9.25. pHs studied include 8.22, 9.25, 10.05, and 10.19. Decreasing the pH by making the run buffer more acidic proved to be detrimental to the resolution and general shape of the signals in the electropherograms, while increasing the pH to 10.05 actually improved the shape and increased the signal due to impurity "C". The following figures show the same samples that were run using the pH's as listed. Figure 5.1 illustrates the effects that the pH had on signal shape (stacking effect) as well as elution order or analytes.



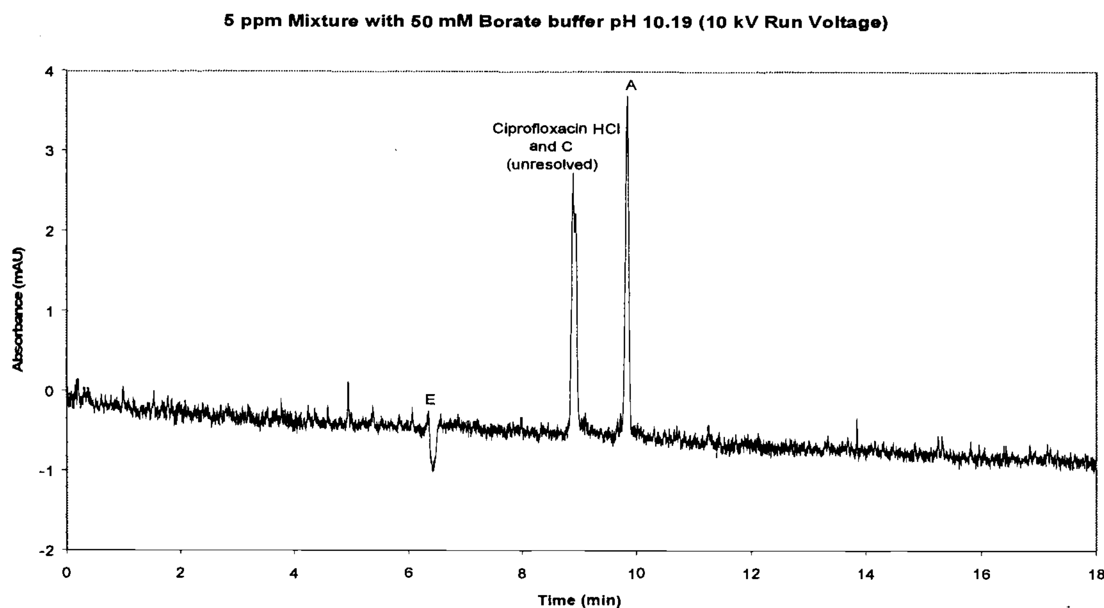
5.1 (a) 5 ppm (w/v) mixture spiked with "E" with 50 mM borate buffer with pH 8.22, 3.3% ACN (10 kV separation voltage). The peaks are unidentifiable and therefore are not labeled.



5.1 (b) 5 ppm (w/v) mixture with 50 mM borate buffer pH 9.25, 3.3% ACN (15 kV separation voltage).



5.1 (c) 5 ppm (w/v) mixture spiked "E" with 50 mM borate buffer with pH 10.05, 3.3% ACN (10 kV separation voltage).



5.1 (d) 5 ppm (w/v) mixture with 50 mM borate buffer with pH 10.19, 3.3% ACN (10 kV separation voltage).

Figure 5.1 pH effects on resolution, analysis time and elution order.

In figure 5.1 (b) and (d), a refractive index for the ACN can be seen, where as in 5.1 (a) and (c), there is no peak. Because of impurity "E's" elution time just before the refractive index of ACN, higher concentrations of impurity "E" will mask the ACN peak. The peak is still present, however it cannot be seen and therefore impurity "E" cannot be quantitatively determined.

With the 50 mM borate buffer with 3.3% ACN at a pH of 8.22 the peak shape was poor for three of the analytes. The resolution is essential for both the quantitative and qualitative identification. Borate at its natural pKa provided good peak shape for all analytes, except impurity "C" for which the peak was broad which would be difficult

to quantitate at low concentrations. The analysis time with this pKa was shorter; however the separation voltage for this pH was 15 kV as opposed to 10 kV which all the other runs were. The pH 10.05 provided the best resolution, peak shape, and signal intensities for all the analytes. It is clear from above that the signals for all the analytes were best in the pH 10.05 borate buffer with 3.3% ACN (c) above. With the pH 10.19 buffer that was adjusted using 1M NaOH, the impurity "C" and ciprofloxacin were not resolved. The analysis time appeared to be unaffected by the pH of the separation buffer, only the run voltage.

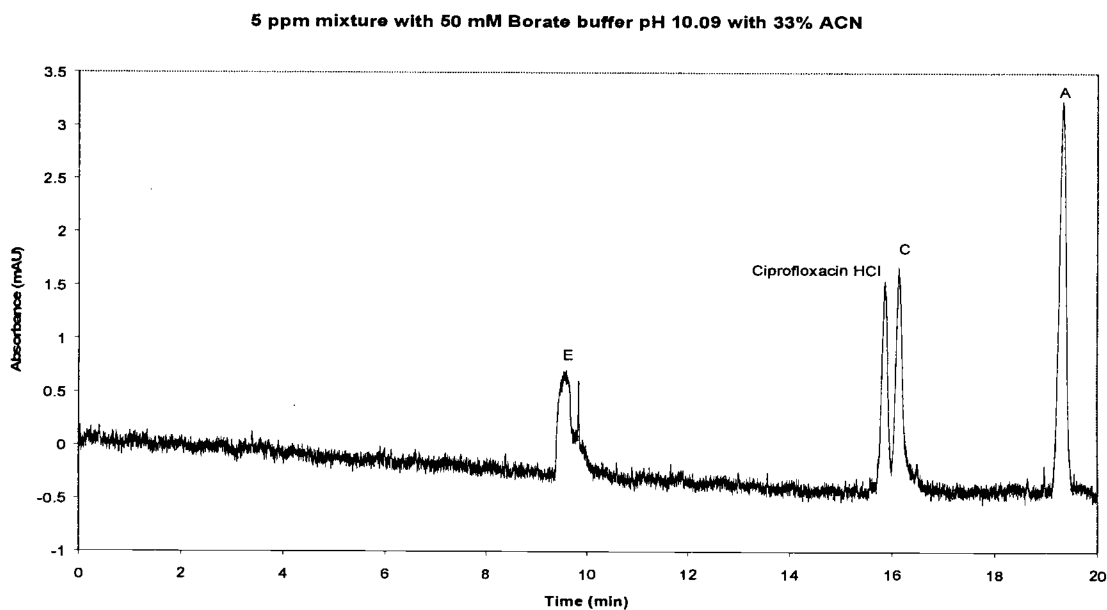
5.2 ELUTION ORDER: BUFFER EFFECTS

The elution order of ciprofloxacin HCl and impurity C changed when the pH of the buffer changed from pH 9.25 to the other pH values tested with the exception of pH 8.22 which the peaks were nearly indistinguishable. This can be seen in figure 5.5. With the pH 10.05 borate run buffer with 3.3% ACN, the peak shape is better for impurity "C". With pH 9.25, the analysis time was shorter, however it should be noted that this is because the run voltage for this pH was 15 kV.

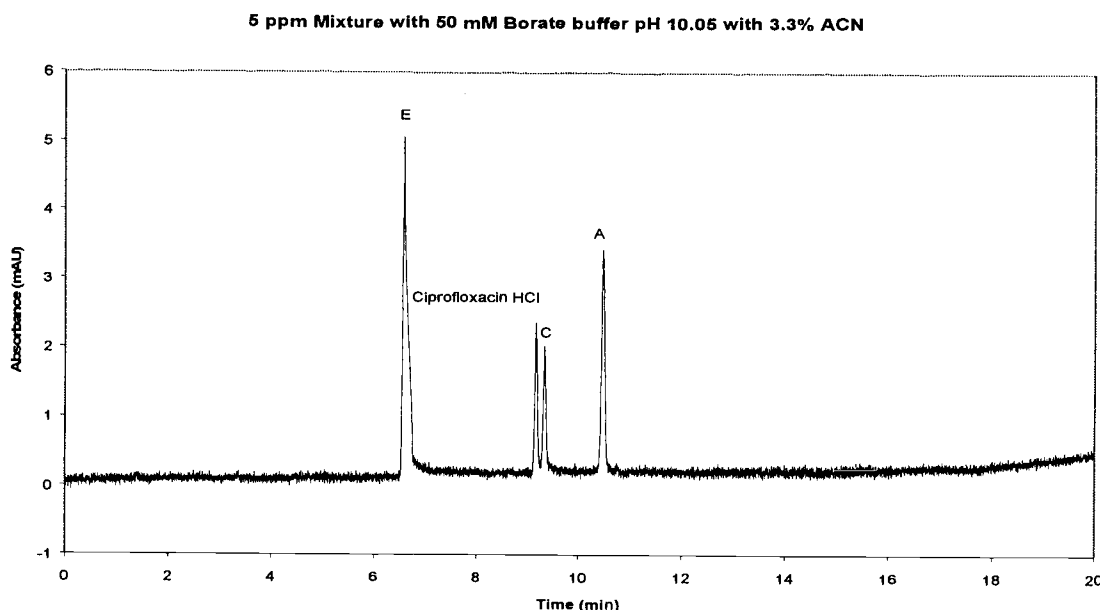
5.3 BUFFER SYSTEM- ORGANIC CONCENTRATION

The amount of acetonitrile in the run buffer was studied to ascertain its effect on stacking. With the run buffer having an organic composition of 33% ACN, the refractive index peak for ACN is no longer present and the stacking effects are also less extreme. Another run buffer with essentially the same pH was studied with 3.3%

acetonitrile by volume and an enhanced stacking effect and therefore improved resolution and detectivity for of impurity "C" and ciprofloxacin HCl. Figure 5.2 shows the effect of acetonitrile concentration on the stacking effects and analysis time.



5.2 (a) 5 ppm (w/v) mixture spiked with impurity "E" with 50 mM borate buffer pH 10.09with 3.3% ACN, injected for 5 seconds (0.409 cm of capillary; 72.24 nL)



5.2 (b) 5 ppm (w/v) mixture injected for 5 seconds (0.409 cm of capillary; 72.24 nL) Sample is spiked with impurity "E" for larger signal.

Figures 5.2 Electropherograms illustrating the effects of the concentration of organic solvent in the run buffer on the separation. (a) and (b) differ only in concentration of ACN and only an insignificant amount in pH. This illustrates the detrimental effects that high percentages of ACN cause in both peak shape and elution time. Both analyses utilized 10 kV run voltages.

The sample was a 5 ppm (w/v) mixture of impurities "A", "C", and "E" with ciprofloxacin HCl. Impurity "E" was spiked due to low signals in this buffer system. In comparing the two run buffers that had similar pHs but different amounts of acetonitrile, it was found that the buffer with 3.3% acetonitrile yielded the best signal peak shape and resolution. It was found that the peak shape of impurity "E" and the resolution between impurity "C" and ciprofloxacin were poor using the 33% ACN buffer. It appeared that having considerable amounts of ACN present in the separation buffer actually decreased the stacking performance. It is apparent that the peak shape

and elution time suffered from the increased amount of organic present in the run buffer.

It was conclusive comparing these run buffer conditions that the higher percentage by volume organic was detrimental to the shape, run time, and stacking ability for this sample.

A previous study conducted in our research lab using the same set of samples at the same concentrations using phosphate buffer proved unsuccessful in achieving good separation and low detection limits. However, it is not easy to compare the past method with the current method because of the significant differences between the two. This current method using the borate buffer however did show improved limits of detection with respect to using the phosphate buffer system of approximately 2 fold.

5.4 SAMPLE STACKING VS. LVSS

The injection time was studied at constant pressure. 25 mBar of pressure was in all cases applied to inject the sample, and volumes ranging from normal sample volumes to LVSS volumes were studied. With the LVSS experiments, 1 and 5 cm of the capillary were filled with the sample. The sample buffer was removed by reversing the polarity for a sufficient amount of time while monitoring the current level but more importantly the refractive index peak of acetonitrile was monitored.

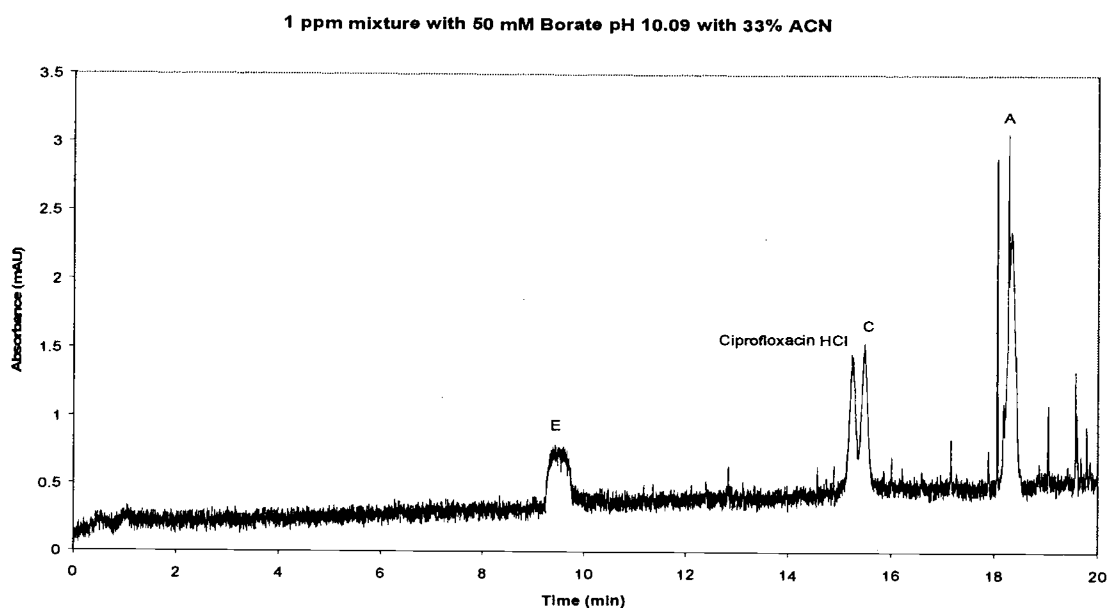
To monitor the current level, the capillary is filled with run buffer only and a voltage is applied that is the same magnitude as the run voltage. When any amount of sample in introduced into the capillary, the current will decrease due to the voltage drop across the sample buffer plug. When the polarity is switched, the sample buffer is removed from the capillary to the inlet vial. As the sample buffer is removed, the current will restore to its original value. When the current is restored to 1% of the initial level, the polarity can be switched to begin the separation as this restoration in current indicates that enough of the sample buffer has been removed to ensure that a plug-flow profile remains.

Another method of monitoring the removal of the sample buffer that was used was monitoring the RI peak for the ACN. If the sample buffer was completely removed, there would be no refractive index peak for ACN because all of the ACN from the sample buffer would have been removed. Reversing the polarity for durations that led to 100% recovery of the current yielded no refractive index peak for ACN. Under 100% recovery of current levels, (incomplete removal of sample buffer) there was still a refractive index peak due to ACN. This means that if there is sample buffer remaining, there will be a refractive index and if it is removed there will no longer be an RI peak for ACN.

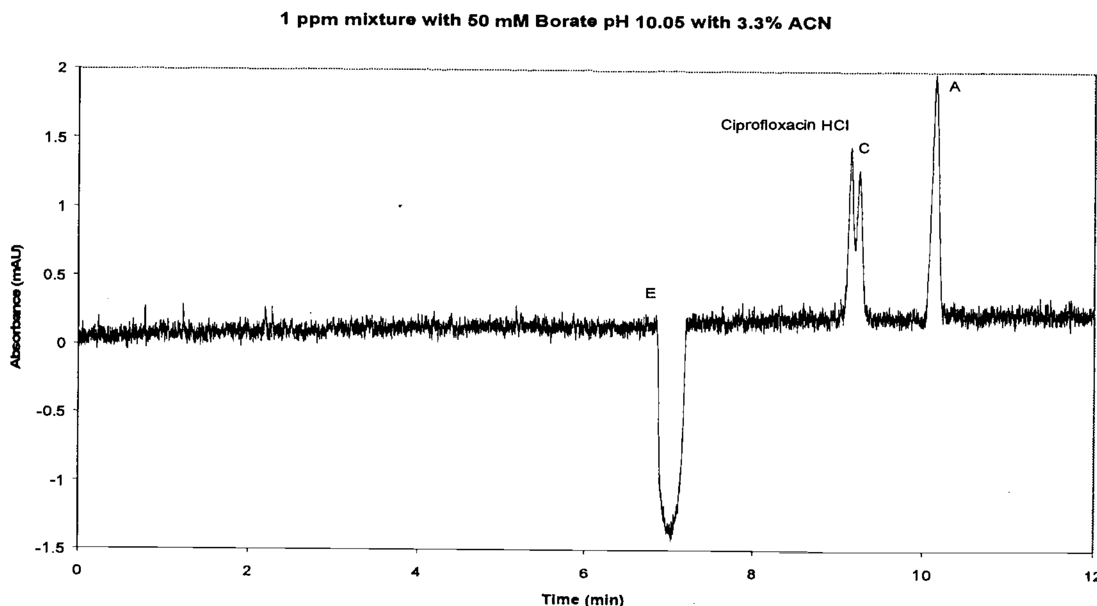
Impurity "E" is neutral in this buffer, and therefore could not be adjusted to elute at times other than just before the acetonitrile refractive index peak. Therefore, complete

removal of the run buffer was impossible and LVSS was deemed inappropriate in this situation. The insufficient removal of the sample matrix could have yielded a distorted, laminar-like flow profile as discussed in section 2.1, which leads to band broadening.

Figure 5.3 shows two resultant electropherograms obtained using LVSS with sample buffer back-out.



5.3 (a) LVSS of a 1 ppm (w/v) mixture with 50 mM borate pH 10.09 with 33% ACN concentration in run buffer.



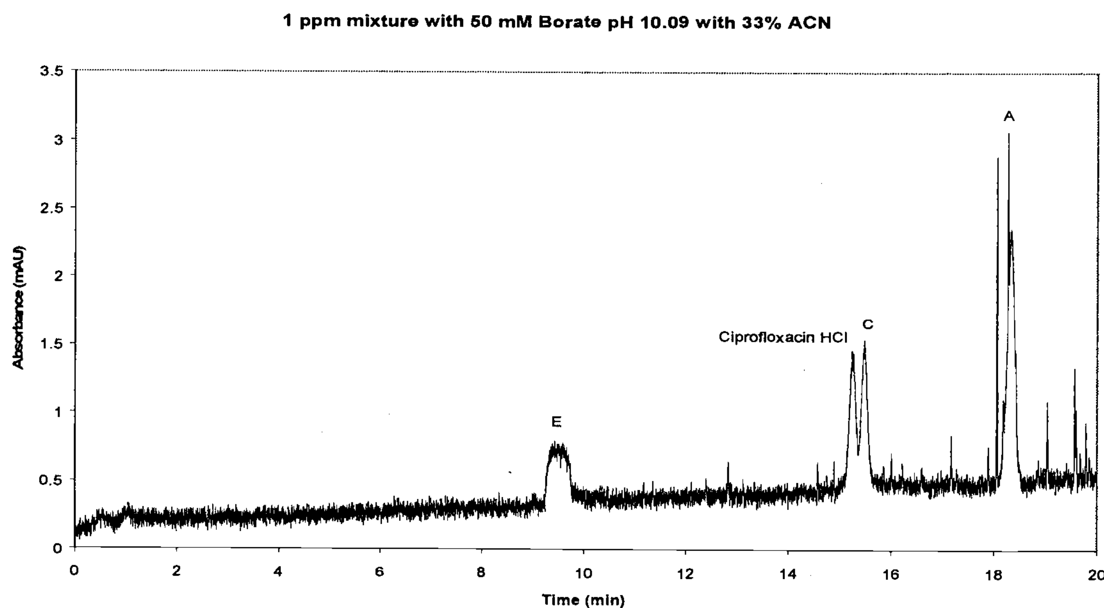
5.3 (b) LVSS of a 1 ppm (w/v) mixture with 50 mM borate pH 10.05 with 3.3% ACN concentration in run buffer.

Figure 5.3 Electropherograms representing the effects of LVSS. (a) and (b) above show the effects of acetonitrile concentration using large volume sample stacking. The 1 ppm (w/v) mixture was injected for 12.23 s which filled 1 cm of the capillary with the sample solution. The polarity was reversed for a total of 0.5 min.

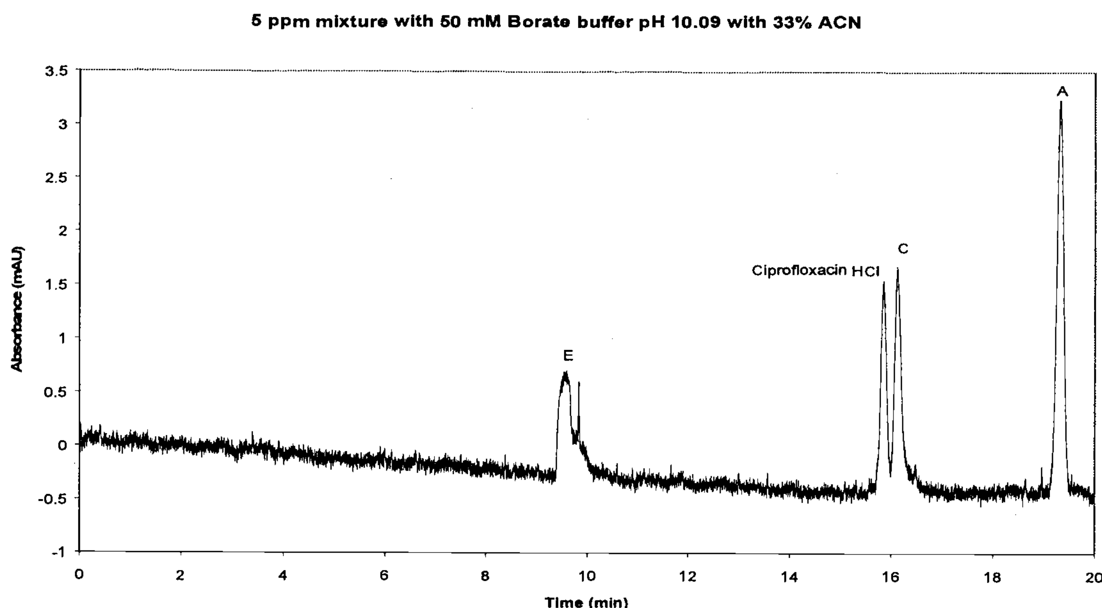
It can be seen that the RI peak for acetonitrile is still present even after applying the negative voltage for half of a minute. It is desirable in this situation to actually retain some of the sample matrix because of the elution of impurity "E". Removing more of the sample matrix proved to remove analytes as well. Since impurity "E" elutes just before the RI peak for acetonitrile, LVSS was not effective.

Sample stacking in normal mode was only studied for one injection duration; about half of a centimeter (0.409 cm) of the capillary was filled with sample. With this small volume of sample buffer present, removal is not needed and the stacking effects

were observed. With this method decreased peak broadening and increased sensitivity and resolution of the analytes when compared to using LVSS on the same sample. Figure 5.4 shows the stacking effects when conventional sample stacking was used; injecting for shorter periods of time which eliminates the need to remove the sample matrix for a 5 ppm (w/v) mixture with 50 mM borate buffer pH 10.09 with 33% ACN. The following figures are repeated for comparison illustrating the lack of improvement in sample shape and analysis time. Figure 5.4 (a) has areas approximately the same as 5.4 (b). Since 5.4 (a) is the analysis of a 1 ppm (w/v) mixture and 5.4 (b) is a 5 ppm (w/v) mixture, approximately 5 fold increase in signal was achieved using LVSS with sample buffer back-out.



5.4 (a) LVSS of 1 ppm (w/v) mixture with 50 mM borate pH 10.09 with 33% ACN using sample buffer back-out



5.4 (b) 5 ppm (w/v) mixture with 50 mM borate pH 10.09 with 33% ACN using Conventional sample stacking

Figures 5.4 Electropherograms illustrating sample stacking vs. large volume sample stacking. (a) and (b) show that when comparing the conventional sample stacking method (b) to the LVSS method (a) there is a signal enhancement of approximately 5 fold using LVSS over conventional sample stacking, however no improvement in signal shape or analysis time.

It is obvious that LVSS produced more intense signals for all the analytes of interest. LVSS could not be used effectively or efficiently in this study, however, unless some derivative of impurity "E" were made to change its migration behavior.

5.5 REPRODUCIBILITY ASSOCIATED WITH THE CAPILLARY CONDITIONING STEP

One of the difficulties associated with capillary electrophoresis is achieving a reproducible EOF. Conditioning the capillary properly and frequently enhances the reproducibility of elution times.

In this method, the capillary was conditioned at the beginning of each run. The capillary conditioning involved flushing with water (2.5 min), 1 M NaOH (2.5 min), water (2.5 min) and finally run buffer (5.0 min). Insufficient conditioning of the capillary can result in a continuous increase or decrease in the EOF. After every third analysis, or before switching sample composition or concentration, a more rigorous capillary conditioning, procedure was used to better clean the surface of the capillary. This involved etching with 1 M NaOH for 5.0 minutes instead of 2.5 minutes. This method of conditioning showed a reproducible EOF. The following figure illustrates the reproducibility of the EOF and therefore sufficient capillary conditioning procedures.

Figure 5.5 below shows the reproducibility produced by using an efficient conditioning procedure. Electropherograms of 10 and 5 ppm (w/v) are shown. Each concentration was run in duplicate. The overlay of the electrophreograms illustrates the reproducibility of the method.

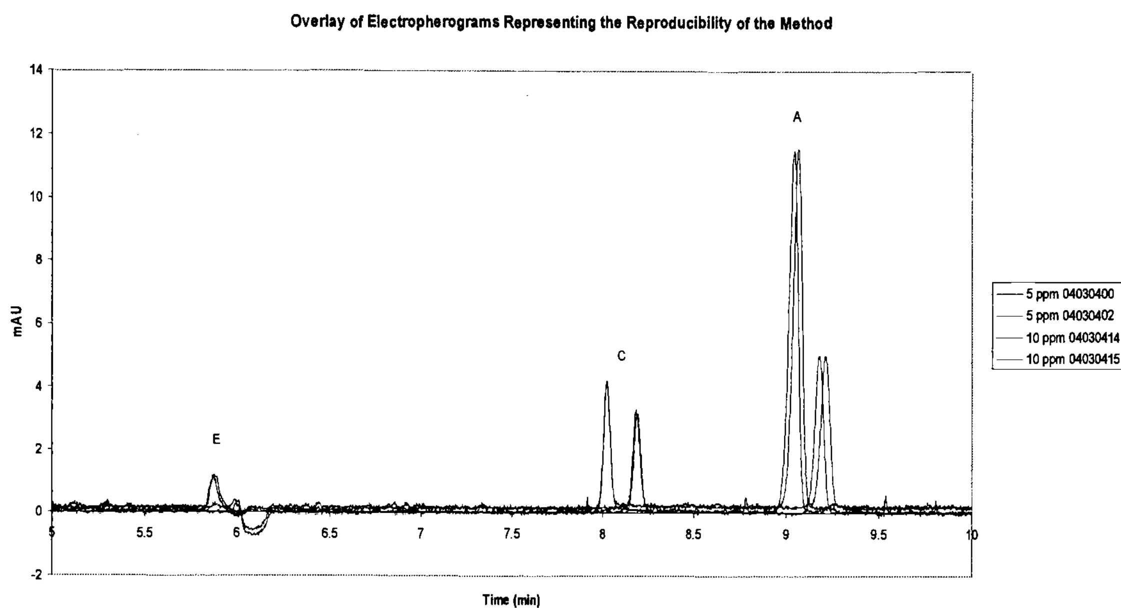


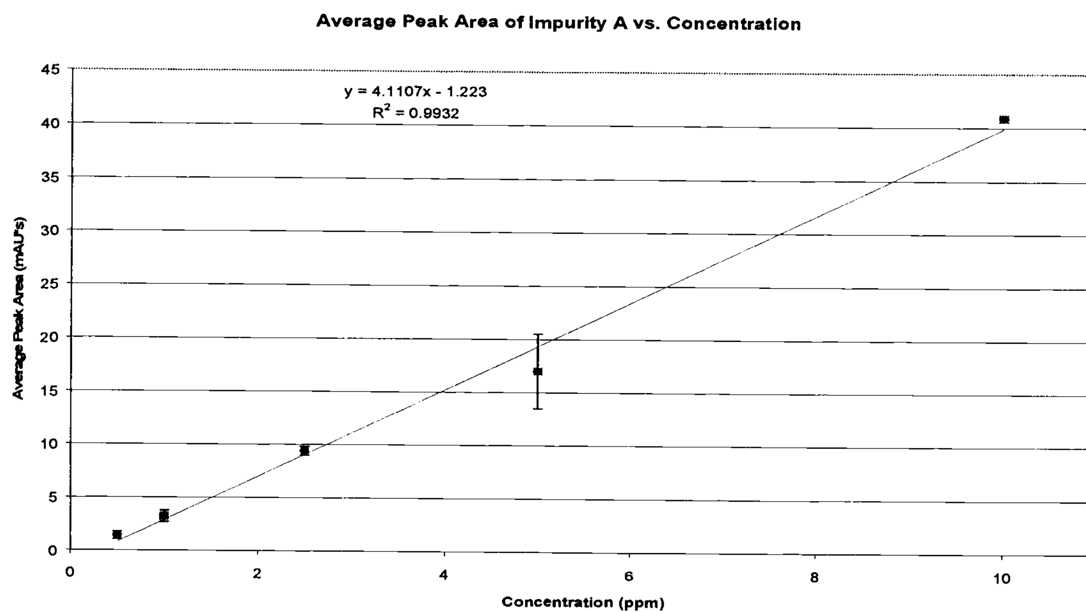
Figure 5.5 Overlay of electropherograms run at two concentrations, each in duplicate demonstrating reproducibility

It can be seen that shifts in the direction of migration of peaks is not continuously in one direction or the other. Had the peaks continuously taken longer to elute for the same analyte or consistently shorter periods of time, the capillary would be considered not properly conditioned. Shifts in elution times can be attributed to variables such as buffer depletion (loss of buffering ions in the capillary) and temperature changes throughout the day as well as incorrect capillary conditioning.

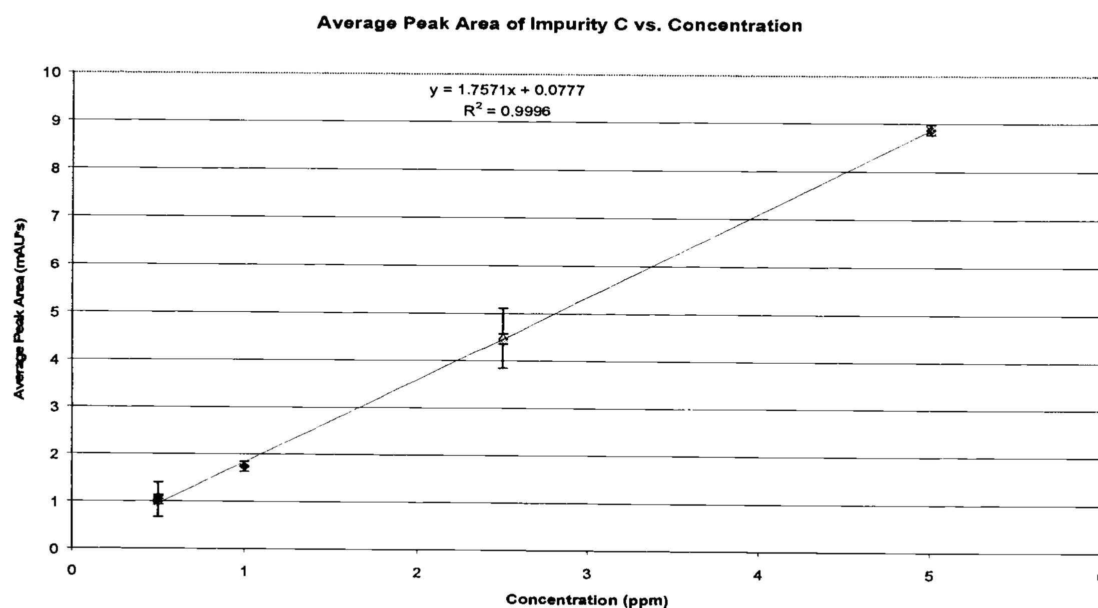
5.6 LIMIT OF DETECTION

To determine the limits of detection for impurities "A" and "C", the IUPAC method was used as described in G.L. Long et al. [22]. Each concentration was run in duplicate, and 5 concentrations were measured for the calibration curve. The LOD for impurity "E" could not be determined as a greater signal enhancement (such as is possible using an extended path length capillary) is necessary. Impurity "E" has a much less intense signal than impurities "A" and "C" at all the wavelengths studied when the sample was not spiked with impurity "E".

The following graphs in figure 5.6 show the calibration curves for impurities "A" and "C" that resulted from analyzing 0.5, 1.0, 2.5, 5.0 and 10.0 ppm (w/v) standard mixtures.



5.6 (a) Impurity "A" calibration curve



5.6 (b) Impurity "C" calibration curve

Figure 5.6 Calibration curve data for ciprofloxacin HCl LOD study.

From the calibration curve, the slopes were used to determine the LOD, and it was found that impurity “A” can be detected at 0.2 ppm (w/v) and impurity “C” at a concentration of 0.2 ppm (w/v). This means that each of the impurities can be detected at signal intensities three times that of the noise at concentrations 0.05% of Ciprofloxacin when a tablet is prepared at 1000 ppm (w/v).

5.7 TABLET ANALYSIS

From the LOD study, it was determined that by preparing the tablet at a concentration of 1000 ppm (w/v), impurities could be detected if present above 0.2 ppm (w/v) for impurity “A” and 0.2 ppm (w/v) for impurity “C” at 254 nm. Figure 5.7 is a representative electropherogram of a tablet analyzed using the method developed. The tablet was dissolved in the simulated gastric fluid and then diluted to 1000 ppm (w/v) with the sample buffer. In this instance, none of the impurities were detected at or above 0.05% by concentration of the active pharmaceutical ingredient (API).

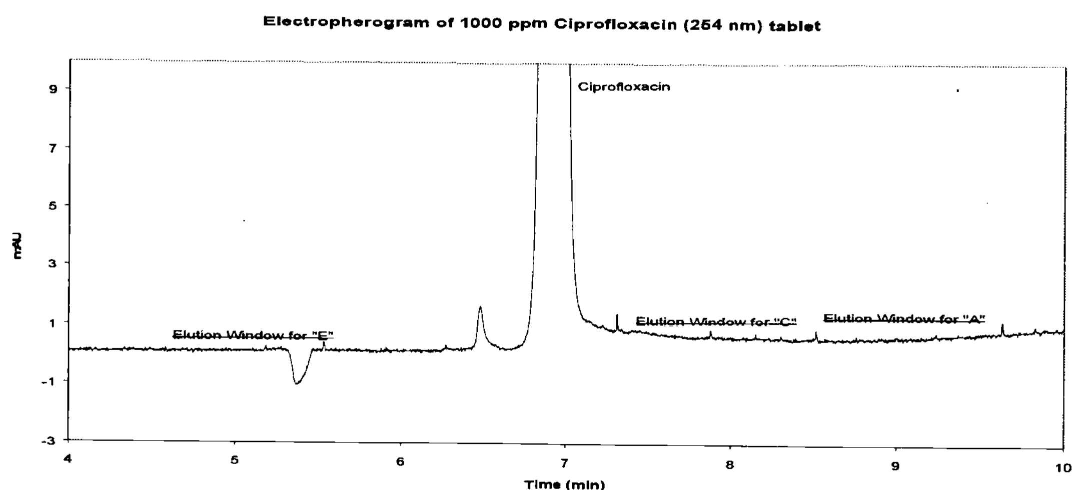


Figure 5.7 1000 ppm (w/v) Ciprofloxacin HCl with 50 mM borate buffer pH 10.05 with 3.3% ACN run buffer.

In figure 5.7, impurities "A" and "C" could not be detected at or below 0.05% (by concentration that of the API. The elution shift in ciprofloxacin HCl from approximately 9 min to 7 min illustrates the effect that the sample has on the analysis using CE. This pharmaceutical has other ingredients and excipients that make the solution more viscous and therefore less sample is actually introduced into the capillary, some of the sample may be sticking to the walls or other as shown here, the sample was retained in the capillary for less time. This shows the problems that can be associated with pharmaceutical analysis without proper sample preparation such as SPE or some other technique that would isolate the impurities from the API.

6 CONCLUSIONS

Employing capillary electrophoresis as a separation method proved to be successful in the analysis of ciprofloxacin HCl and three impurities. Detecting impurities "A" and "C" at concentrations 0.05% (by concentration) that of ciprofloxacin HCl was achieved using sample stacking with acetonitrile concentrations of 33% to 3.3% in the separation buffer and run buffer, respectively. Large volume sample stacking could not be fully utilized with this buffer system due to the order of elution of impurity "E". Instrumental alterations could be implemented to further improve the limits of detection of the impurities studied. Increasing the sensitivity of the instrument so as to be able to detect lower concentrations will greatly improve the limits of detection of the impurities, especially impurity "E" of the ones determined.

The HPLC method that has been developed from a pharmaceutical company whose name cannot be disclosed did resolve impurity "C" from ciprofloxacin HCl. A study that I conducted after this was an extensive tablet analysis, and the concentrations of impurity "E" are much greater in the tablet formulation than 10 ppm (w/v) based on the peaks observed during the LOD study and the extended tablet analysis. Due to the high concentrations of impurity "E" in the tablet, sample stacking proved to be a good technique even without modification. The HPLC method that has been developed also showed intense absorbance due to impurity "E". The results of the two methods are comparable, however the CE method that was developed provides a much cheaper route of ciprofloxacin HCl analysis.

Future work for the analysis of this pharmaceutical and its impurities would be to use an extended path length capillary, preferably a bubble cell capillary due to the price and ease of preparation and use. It may also be beneficial to derivatize impurity "E" to see if the electrophoretic mobility of the analyte could be changed so that LVSS could be used also to further enhance sensitivity.

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