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Effects of Eluent pH and Different Types of Acidic Modifiers on the Retention and Electrospray Ionization Efficiency of Basic Analytes in LC-ESI-MS.

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Effects of Eluent pH and Different Types of Acidic Modifiers on the Retention and Electrospray Ionization Efficiency of Basic Analytes in LC-ESI-MS

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We certify that we have read this thesis and that, in our opinion, it is adequate in scope and quality as a dissertation for the degree of Doctor of Philosophy.

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Abstract

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Electrospray ionization (ESI), coupled to liquid chromatography (LC-MS) has become the instrument of choice in the research laboratory today. This soft ionization process which involves the efficient transfer of ions from the liquid phase to ions in the gas phase is well suited of the qualitative and quantitative analysis of proteins, peptides carbohydrates and other thermally labile compounds. Applications in pharmaceutical analysis bring a significant demand for investigations of the influence of typical chromatographic parameters on the efficiency of electrospray ionization. Reversed-phase HPLC retention of ionizable analytes is greatly dependent on their ionization and solvation states, which are influenced by the pH of the mobile phase, ionic strength, pK_a of the acidic modifier and solvent composition. HPLC mobile phase type and composition exhibit noticeable effects on the spray performance and formation of ionic species in the gas phase. In this study we investigate the effects of various mobile phase additives, solution pH, mobile phase composition and counter-anion concentration on the retention in HPLC and electrospray ionization efficiency of structurally related tricyclic antidepressants. We demonstrate how the use of different acidic modifiers affect HPLC retention of basic analytes and correlate these effects with the electrospray ionization efficiency in LC-MS. The development of efficient analysis of a complex mixture of ionizable components is a compromise between the HPLC separation selectivity and ESI detection sensitivity.

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DEDICATION

This work is dedicated to my son John Ebodi Peri-Okonny and in loving memory of my late father David Jonathan Mitchell

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Chapter 1.

Introduction

Retention Mechanism

High Performance Liquid Chromatography has been employed for the separation of basic polar analytes on a non-polar stationary phase such as octadecyl-silica with aqueous eluents [1]. Retention in RP-HPLC occurs when the distribution of the solutes favors the stationary phase. The extent at which the solutes interact with the stationary phase will determine their elution time and the order in which they will elute from the column. However, if the distribution of the solute is favored by the mobile phase the solute will elute very rapidly. The mechanism by which the solutes are held up in either phase is a result of their interactions between the solute molecules with the molecules of either the mobile or the stationary phase. Further selectivity (the rate at which these solutes interact with either phase) can be achieved by changing the nature of either the stationary phase or the mobile phase.

Retention can be described as either adsorption or partitioning process. Partitioning involves a stationary phase whose composition is different from that of the moving liquid phase. Sample molecules distribute themselves between the mobile and stationary liquid phases [2]. Therefore, the analyte retention occurs by a distribution of the analyte between the bulk mobile phase and the bulk stationary phase. Some models of reversed phase chromatography attribute the retention to the partitioning of the analyte between the hydro-organic mobile phase and the alkyl-silica surface of the stationary

phase [2,3]. However, this theory is disputed in favor of pure adsorption process or a composite of adsorption/partition mechanism [4].

The adsorption process occurs at the solid-liquid interface [2]. For HPLC, this type of retention process may exist for a liquid mobile phase and a porous, solid stationary phase that is impervious to the analyte [4]. In this case, retention occurs when the analyte in the mobile phase migrates to the solid phase and displaces adsorbed components of the mobile phase. Such a displacement or competitive adsorption process dominates the retention in the liquid chromatography utilizing polar stationary phases (i.e. NPLC) [4,5].

The Mobile Phase

General Effects

Both the stationary phase and the mobile phase contribute to the retention of the analyte in RP-HPLC. Retention generally varies much more with alterations in the mobile phase composition than with changes in the hydrocarbon chain length of the column [6]. Therefore the mobile phase plays a dominant role in controlling the retention process and variations in sample retention for optimum separation are achieved almost exclusively by changes in the mobile phase.

In most reversed-phase separations, a mixture of buffered aqueous eluents with varying compositions of organic solvents is employed as the mobile phase. These

buffered systems are particularly important for the use of separating basic polar or ionizable compounds on reversed phase columns. In RP-HPLC, the retention of these analytes is greatly dependent on their ionization and solvation states, which are influenced by the pH, ionic strength of the buffer and the eluent composition [7].

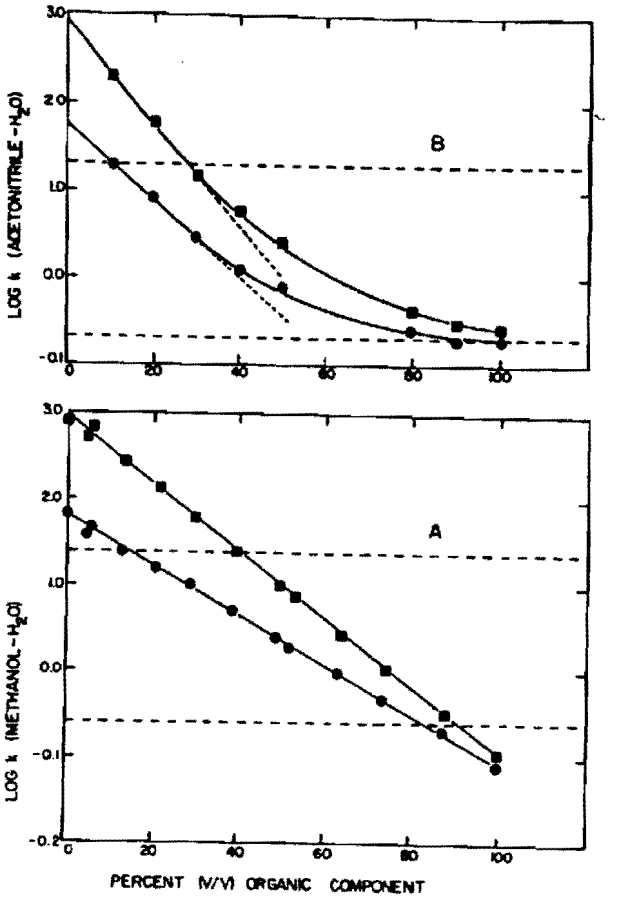
A well-chosen buffer should have a buffering capacity between pH range of 2 –8, be optically transparent, compatible with organic eluents, should have a capacity for enhancing equilibration rates and the potential for masking any residual silanol groups on the surface of the adsorbent. The most commonly used organic solvents in RP-HPLC are acetonitrile and methanol. The physical properties of these solvents such as viscosity, surface tension, and dielectric constant are all important in determining the selectivity of the analysis. These solvent properties will change as a function of their concentration in aqueous-organic mixtures.

The Linear relationship between the logarithm of the retention factor and the volume fraction of the organic co-solvent is the rule in RPLC. However, exceptions to the linear relationship of the log retention factor with the volume fraction of methanol can occur at high methanol concentrations. Special effects can occur and cause this rule to be violated as reported by [8]. These effects were observed with antibiotic amines and other basic drugs. These analytes showed pronounced minima in the corresponding retention factor versus the solvent composition. These were attributed to the interaction of the basic analyte with the accessible silanol groups of the stationary phase.

The composition and type of the organic modifier will affect the retention of the solute in reversed-phase chromatography. A number of researchers have shown the affects of the composition of organic modifiers on the retention factor [9-11]. A plot of the logarithm of the retention factor versus the volume fraction of the concentration resulted in a linear decrease in the capacity factor. The logarithm of the capacity factor of several aromatic compounds also showed a linear relationship and was reported by [10]. **Figure 1-1** shows data obtained by Karger et al for the retention of *n*-hexanol and *n*-octanol in methanol-water mixtures on the octyldecyl silica stationary phase.

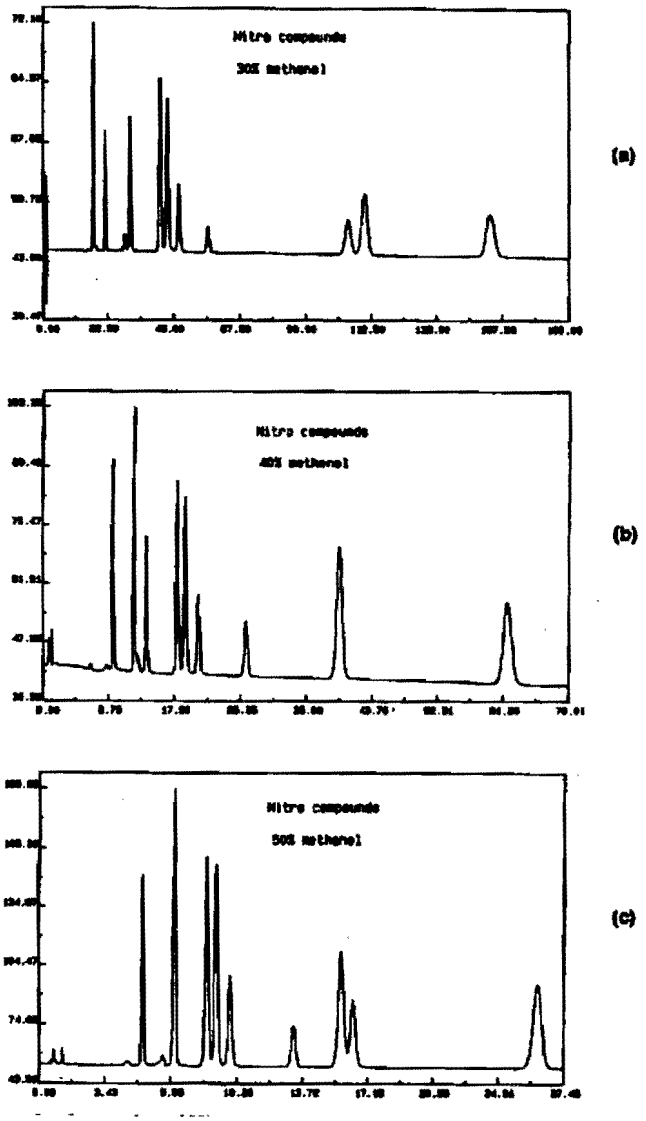
The eluent composition of the organic modifier will also affect the selectivity or the separation of solute mixtures. In RPLC the solvent strength increases with the percent of organic in the aqueous-organic mobile phase. Typically, an increase in the percent organic by 10 % will decrease the analyte retention by a factor of 2 to 3. An example is shown in **Figure 1-2** for nitro-aromatic mixtures with the methanol composition varied from 30 to 70 %. In the 30 % methanol (panel A), separation has a run time of three hours, but all ten compounds are well resolved. The 70 % methanol (panel E) has a run time of only six minutes, but only eight of the ten peaks can be seen and the resolution is minimal.

Figure 1-1: Logarithm of the Retention Factor of n-hexanol (•) and n-octanol (▪) on C-18 Column as a Function of Mobile Phase Compositions. [from reference 10]



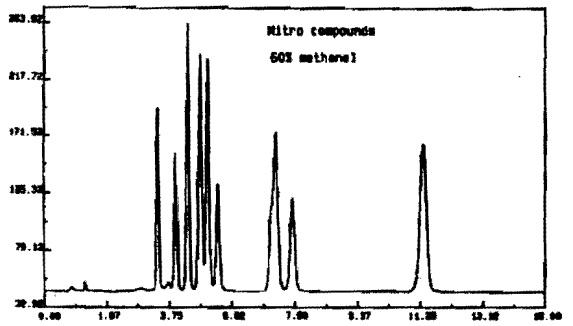
In (A), the eluents were methanol-water mixtures of various volume fractions. In (B), the eluents were acetonitrile-water mixtures of various volume fractions.

Figure 1-2: The Effects of Percent Methanol on the Separation of Nitro Compounds. [from reference 12]

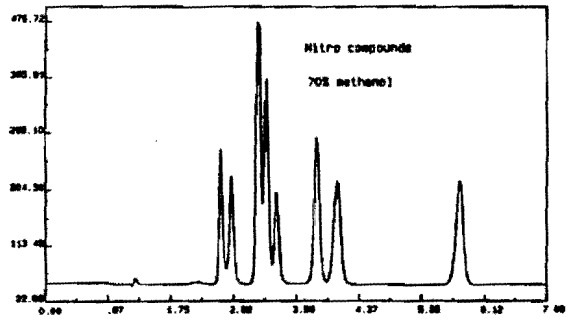


Chromatographic conditions: Column, 25 x 0.46 cm Zorbax C-8; mobile phase, Methanol/water; composition of methanol varied as shown [12].

Figure 1-2 (continued)



(d)



(e)

The Effects of Mobile phase on the Separation of Ionizable Compounds

For the analysis of most pharmaceutically active compounds, reversed-phase HPLC using modified silica surfaces with C-8 or C-18 is the preferred method. This method is preferred, due to the resulting efficient separation and symmetrical peaks that are often obtained, which enable the quantification of analytes at low concentration levels. However the HPLC analysis of basic compounds can be problematic [13].

The main problem encountered during the analysis of these basic analytes is due to the two retention mechanisms occurring simultaneously. These two mechanisms are interactions of the basic analyte with residual silanols on the silica surface of the column and ion exchange retention mechanism [13]. This mixed retention mechanism will depend on the character of the stationary phase, and will result in varying peak shapes. Work done by [6] has shown that the mobile phase contribution to the retention varied much more with changes in the mobile phase than did the stationary phase contribution with changes in hydrocarbon chain length. As a result they concluded that the mobile phase composition plays the dominant role in controlling the retention in RPLC.

Most reversed phase separation of basic analytes employs the use of buffered aqueous components as initial eluent in the mobile phase. These buffered components are primarily important in the separation of polar ionizable compounds on reversed-phase columns. Variations in the aqueous portion of the mobile phase such as the pH, the type

and concentration of the salts used to prepare the buffers and the type and concentration of the solvent composition can drastically affect the retention of these ionogenic solutes [14]. Therefore, the retention and separation of these ionogenic solutes changes significantly with variations in mobile phase pH.

Effects of pH

The pH of the mobile phase plays a crucial role in optimizing the separation of ionogenic compounds and for controlling the reproducibility of the analyte retention and the ruggedness of a HPLC method [15]. A properly chosen pH can significantly alter the retention of polar or ionizable compounds through secondary equilibria. The degree of ionization of solute, stationary phase and the mobile phase additives may be affected by the pH of the mobile phase [16].

Weak basic analytes can dissociate in the mobile phase. This makes their retention and eventual separation more challenging. According to [17], the dissociation of these weak bases can be defined in terms of the secondary equilibria that follows the main adsorption/desorption process in the column. The ionization of a weak base, B, in the mobile phase is governed by the following equilibrium:

$$BH^+ \Leftrightarrow B + H^+ \qquad [1\text{-}1]$$

Where, BH^+ is the protonated base. The equilibrium is characterized by the dissociation constant of the protonated base in the mobile phase, K_{am}, which is given by:

$$K_{am} = \frac{[H^+]_m[B]_m}{[BH^+]_m} \qquad [1\text{-}2]$$

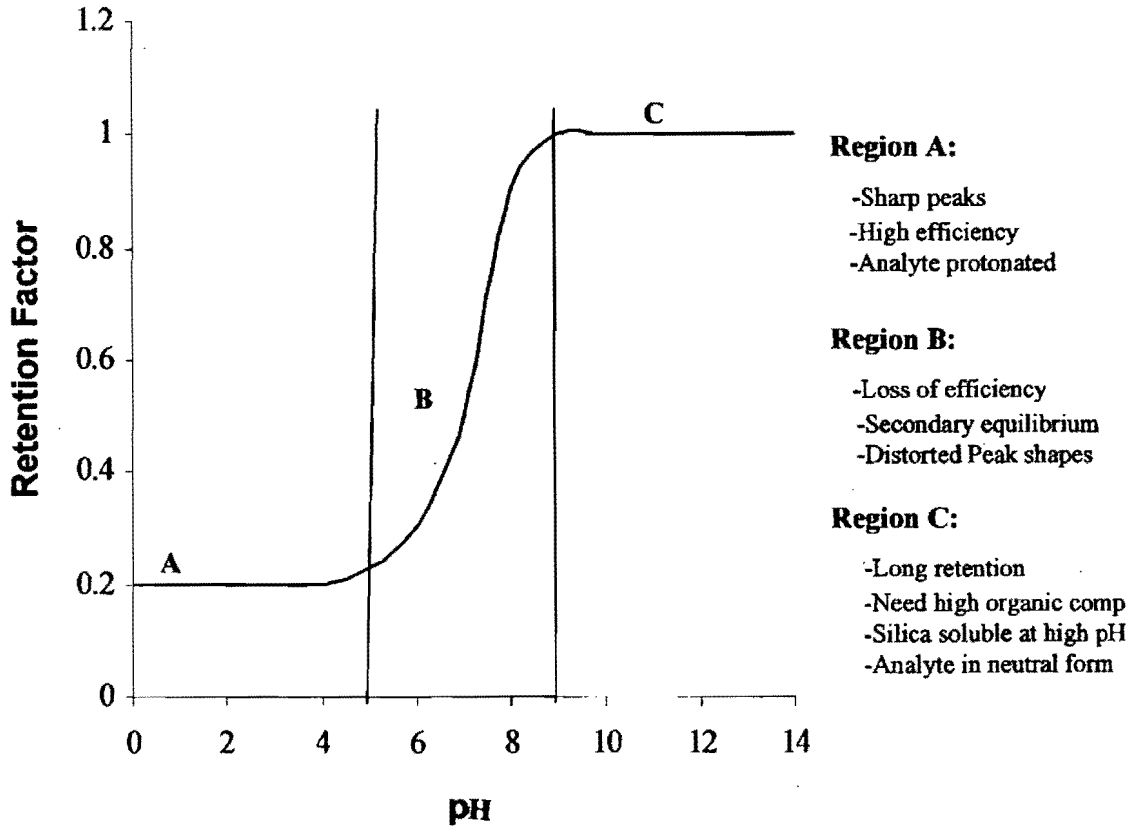
The most widely used model [18-20] for the description of HPLC retention of ionizable compounds considers that the observed capacity factor is a weighted average of the capacity factors of the ionic and neutral forms of the analyte.

On the basis of the above equilibrium, Hovarth [17] derived an equation for the dependence of the analyte capacity factor on the mobile phase pH as:

$$k = \frac{k_o + k_1 \frac{[H^+]_m}{k_{am}}}{1 + \frac{[H^+]_m}{k_{am}}} \qquad [1\text{-}3]$$

Where k_0 and k_1 are the capacity factors of the neutral and fully ionized fractions of the analyte respectively. The pH dependence of the capacity factor for a monoprotic base is expressed by equation 1-3. A plot of the analyte retention dependence versus the pH of the mobile phase (**Figure 1-3**), shows a typical sigmoidal curve, where the inflection point represents the apparent pK_a of the analyte.

Figure 1-3: Theoretical Variations of Capacity Factor of Basic Analyte as a Function of Mobile Phase pH

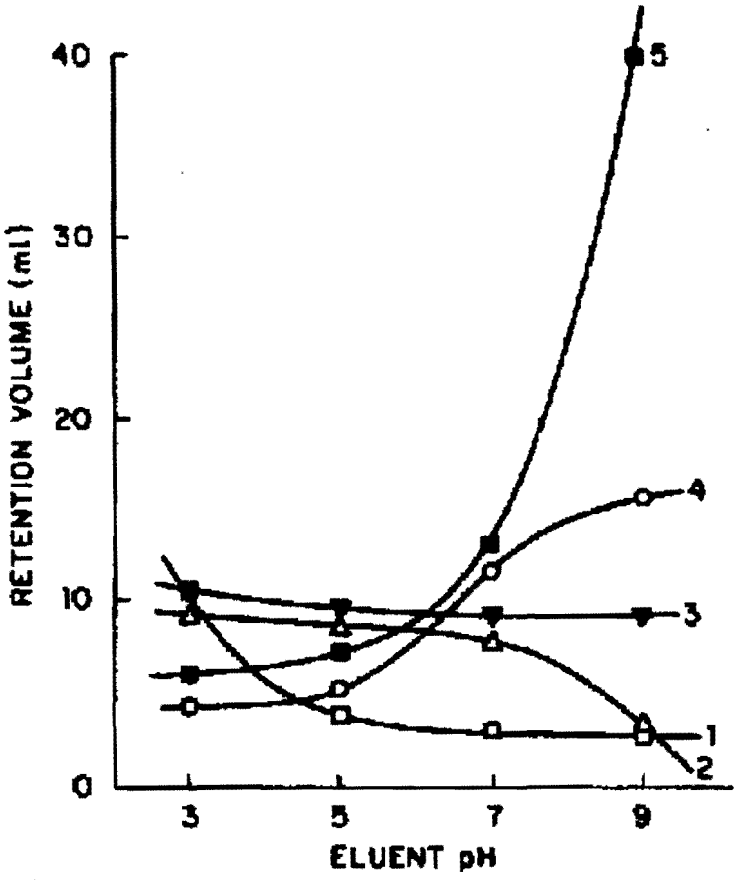


The major variations of *k* with respect to the pH of the mobile phase occur within ± 2 pH units around the pK_a value of the analyte. The greatest retention of the basic analyte occurs when the pH of the mobile phase is above the pK_a (neutral form) and the lowest retention is obtained when the pH of the mobile phase is below the pK_a (protonated form). Once the analyte is fully protonated, further lowering of the pH, by addition of more acidic modifier should not have an effect on the analytes retention.

Others [20,21] have also shown the effect of mobile phase pH on the retention of acidic, neutral and basic analytes and is shown in **Figure 1-4**. From **Figure 1-4**, the retention of a weak acid, phenobarbitone (compound 2), shows little change until the mobile phase has a pH greater then 7. At this point, the molecule begins to ionize and its retention drops precipitously. The retention of a neutral compound, such as phenacetin (compound 3), shows little dependence on pH. A strong base, such as methylamphetamine (compound 5, $pK_a = 8$), shows increasing retention above pH 8, as the molecule becomes less ionized. As a result, the retention decreases steeply at a lower pH, but approaches a constant retention for $pH < 6$.

This figure clearly shows the pH of the mobile phase will have a profound effect on the retention behavior and the separation of these ionogenic analytes in RPLC. In this particular example, it can be seen that all five analytes are well separated at a pH of either 5 or 8; pH 5 will be preferred because of shortened analysis time and will increase column life.

Figure 1-4: Effects of Mobile Phase pH on Separation in Reversed-Phase HPLC. [from reference 21]



The data suggests that if the pK_a values are known for a group of compounds to be separated by RPLC, and if their values are different, then it is likely that the pH near (± 2 units) the average pK_a value of the mixture should provide good separation. The reason is that pronounced changes in separation are expected as pH is varied in the region $pH = pK_a$.

pH and pK_a Shift

It has been shown earlier that the composition of organic modifier will affect the retention or the elution time of solutes in RPLC. The operational pH in mixed aqueous-organic solvents is usually measures assuming that the pH of the mobile phase is the same as that of the aqueous fraction. However, the pK_a values of the acids used to prepare the buffers are dependent on the solvent composition [22,23], as is the pH of the buffer [24,25]. Therefore, the pK_a of these acidic modifiers used in HPLC cannot be directly correlated to those obtained potentiometrically.

The analyte ionization may be affected by the composition of organic modifier in the mobile phase at a constant pH. Additionally, this will affect the chromatographic profile of the ionogenic species. In some instances the pH of the mobile phase is measured after the addition of the organic modifier [26]. However, even in this case, the potentiometric system is usually calibrated using aqueous standards and the pH recorded is not the true pH of the aqueous-organic mixture.

The organic portion of the mobile phase will change the pH of the aqueous phase to different degrees [27-28]. Barbosa *et al.* show that the pH of the aqueous/acetonitrile buffer will shift linearly by approximately 0.3 pH units for every 10% acetonitrile used. This will have a consequential effect on the retention of the basic analyte since the pH of the aqueous phase is not the same as the mobile phase eluent.

Barbosa *et al* [27], have studied the effect of ionic equilibria on the retention behavior of a series of quinolones and evaluate their ionization constants using chromatographic data in acetonitrile-water mixtures. These quinolones are acidic in nature due to the terminal carboxylic acid group on the analyte structure. The authors have shown that the pK_a values of these compounds increase with increasing concentration of acetonitrile in the mobile phase. This decrease in acidity was attributed to the intramolecular H-bond formation with the neighboring keto function, resulting in a stabilization of the proton species.

Other researchers [7,28] have shown that the pK_a of basic analytes is shifted to lower values and acidic compounds shifted to higher values when an increase in the composition of methanol is added to the mobile phase. Barcella's group measured the pK_a of several carboxylic acids and three anilinium compounds in methanol-water solvents containing up to 95% methanol. The results are shown in **Table 1-1** together with available pK_a values in 100% methanol.

Table 1-1: pK_a Values of Analytes in Methanol-Water Systems at 25 ^{0}C. [adapted from reference 28]

Acid	Methanol, % by volume						
	0	20	40	60	80	95	100
Formic	3.745	3.919	4.159	4.597	5.282[a]	6.542	-
Acetic	4.756	5.011	5.334	5.808	6.500	7.858	9.720[b]
Propionic	4.869	5.150	5.572	6.053	6.813	-	-
Butyric	4.809	5.123	5.597	6.081	6.816	-	-
Benzoic	4.201	4.514	4.967	5.536	6.286	7.473	9.380[c]
Anilinium	4.620	4.463	4.322	4.168	4.068	4.613	5.804[c]
N-Methylanilinium	4.848	4.698	4.476	4.177	3.900	4.149	5.27[c]
N-Dimethylanilinium	5.150	4.964	4.726	4.306	3.821	3.953	5.02[c]

Thirty-three years later, Roses [7] group also showed similar pK_a values obtained from methanol-water mobile phase compositions. The authors have extended the study to accounting for the variation in retention parameters for the neutral and anionic forms of the solute ($t'_{R(HA)}$ and $t'_{R(A)}$). A plot of the adjusted retention time versus changes in the pH shows the expected sigmoidal curve. Values for the different anionic forms along with the pK_a and the standard deviations are shown in **Table 1-2**.

By assuming the retention factor is a weighted average of the neutral and fully ionized forms of the basic analyte, Goga *et al* [29], have extended equation 1-3 to account for the retention factor of both species in the mobile phase. The new equation is given as:

$$K_A \frac{1}{1 + K_a x 10^{pH}} + K_B \frac{K_a x 10^{pH}}{1 + K_a x 10^{pH}} \qquad [1\text{-}4]$$

Where, k_A and k_B are the retention factors for the acidic form (AH for the acid or BH^+ for a base) and the basic form (A^- for the acid and B for a base), respectively.

The authors have used equation 1-4 to calculate the pK_a of several basic analytes using different compositions of acetonitrile in the mobile phase and are shown in **Table 1-3.** These pK_a values reflect the degree of ionization of the solute in the aqueous-organic mixtures, resulting from changes in the dissociation constant of both solute and

Table 1-2: Adjusted Retention Times of Benzoic Acids in Methanol-Water Mobile Phase. [adapted from reference 7]

Acid	40 % MeOH				60 % MeOH				80 % MeOH			
	$t_{R(HA)}$	$t_{R(A-)}$	pK_a	SD	$t_{R(HA)}$	$t_{R(A-)}$	pK_a	SD	$t_{R(HA)}$	$t_{R(A-)}$	pK_a	SD
Benzoic	12.30	0.64	4.99	0.26	3.55	0.14	5.48	0.05	1.56	0.02	6.22	0.05
2-nitrobenzoic	6.23	0.28	3.02	0.14	2.13	0.06	3.67	0.03	1.30	0.00	4.32	0.04
3-nitrobenzoic	13.68	1.03	4.11	0.32	3.94	0.20	4.58	0.06	1.82	0.04	4.97	0.02
4-nitrobenzoic	15.65	1.07	4.04	0.35	4.37	0.22	4.47	0.11	1.94	0.05	4.94	0.03

Table 1-3: Effect of Acetonitrile Composition of the pK_a of Basic Analytes [adapted from reference 29]

Analyte Name	Percent Acetonitrile			
	0[a]	15	25	35
Aniline	4.63	4.24	3.87	3.20
Methoxy-2-aniline	4.52	4.11	3.75	2.79
Methoxy-4-aniline	5.34	4.94	4.63	4.28
Methyl-2-aniline	4.44	4.07	3.69	2.98
Methyl-3-aniline	4.73	4.37	4.01	3.12
Methyl-4-aniline	5.08	4.71	4.39	3.99
N,N dimethyl aniline	5.15	-	4.36	3.78
Pyridine	5.25	4.73	4.36	3.88

buffer when organic modifier is added to the mobile phase. They have compared these pK_a values in aqueous-organic mixtures to those obtained under pure aqueous condition (literature values), and found that there is a significant decrease in the pK_a values of the basic analytes with increasing concentration of acetonitrile concentration. The decrease in pK_a was attributed to the apparent pH change in the aqueous-organic mixtures.

Effect of Counter-anion Concentration

RPLC of ionogenic compounds is quite challenging. These compounds may undergo ionization in aqueous eluents and as a result, severe peak broadening may occur. This effect is reduced or eliminated by exploiting certain parameters of the mobile phase solution. Parameters such as the eluent type and composition [30, 31], the pH and the type and concentration of the buffer employed [32,33] can be varied to achieve the desired separation of these analytes. In practice a buffer concentration between 10 and 100 mM are commonly used.

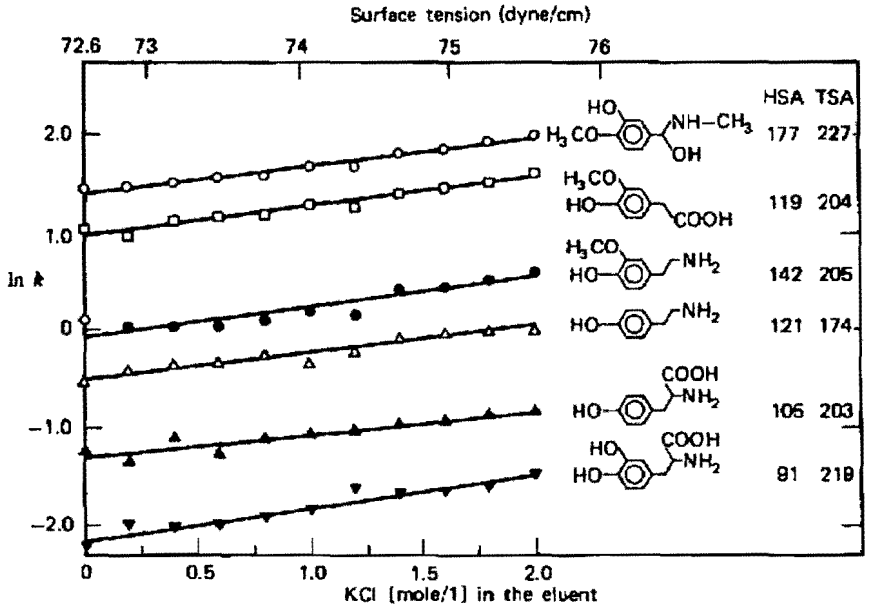
Counter-anions are generated from the dissociation of acid modifiers or from the salt used to prepare the buffers used in the mobile phase. Hovarth et al [34] have shown that increase in salt concentration in the eluent will augment the capacity factor of neutral solutes in hydrophobic chromatography. The authors have ascribe this effect to the increase in surface tension of the eluent and a concomitant increase in the energy required for the cavity formation in the stationary phase.

The addition of neutral inorganic salts to the mobile phase will cause a linear increase in surface tension of aqueous solutions, according to the following equation.

$$\gamma = \gamma_0 + \sigma_m \qquad [1\text{-}5]$$

Where γ_0 is the surface tension of pure water, m is the molarity of the solution, and σ is the molal surface increment of the salt. The logarithm of the capacity factor of the neutral solute increases linearly with the salt concentration, following an increase in the eluent surface tension. However, for ionized compounds the effect of added salt is more complexed. Hovarth et al [11] have shown the effect of increase salt on the logarithm of the capacity factor on ionogenic compounds (**Figure 1-5).**

Figure 1-5: Plot Illustrating the Effects of Counter-anion Concentration on the Retention Factor



Chromatographic conditions: column, Partisil 1025 ODS; mobile phase; KCl in 0.05 M KH_2PO_4 [11]

Negatively and positively charged species are produced from the dissociation of both the basic analyte and the acidic modifiers in solutions. These oppositely charged ions in the mobile phase have a tendency to be attracted to each other. The extent to which these ions are attracted to each other is dependent upon the dielectric constant of the mobile phase and the solvation of the individual ions.

It has been shown that the capacity factor of the protonated nitrogen compounds were altered when different acidic modifiers are used to prepare the mobile phase. The concentration [35,36] and the hydrophobic nature of the counter-anion [37-39] both play an important role in affecting the capacity factor of these protonated bases. The authors attribute this to this effect to the formation of ion-pairs between the analyte and the hydrophobic counter anion.

In addition to the analyte solvation, the structure of the analyte [39,40] may also affect the retention of these analytes. The retention of aminoindanol resulted in an increase when the pH of the mobile phase was changed from 3 to 1 using perchloric, trifloroacetic, nitric and phosphoric acids on a crown ether column [41]. The authors attribute the effect to the type and concentration of the counter-anion, which affected the solvation of the analyte.

Ishikawa et al [43], have shown the retention of primary, secondary and tertiary amines will increase with increase in the counter-anion concentration. The authors have also shown that the retention and separation of trimipramine (a basic racemate) will

increase on a CHIRALCEL OD-R column will increase with an increase in concentration of perchlorate counter-anion.

The increase in concentration of acidic modifier has also been shown to increase the retention of basic solutes by other researchers. This increase has been attributed to ion-pairing, ion [43]. Experimental results show that the relationship between the capacity factor and the counter-anion concentration can be parabolic or hyperbolic. The increase in the in the retention factor was observed with an increase in the counter-anion concentration and was attributed to an ion-interaction mechanism [42,44].

Ionized basic analytes in buffer/organic mixtures are solvated [45]. These solvated protonated basic analytes interact electrostatically with the counter-anions, which leads to an increase in the analytes desolvation and consequently increase in its hydrophobicity. Therefore an increase in the concentration and type of counter-anion in the mobile phase will have an effect on the retention of these protonated bases.

Nagy et al [46], have shown that basic analytes such as amines are solvated, and the degree of hydration stabilizes the protonated molecule. The author has also shown that increasing water molecule will increase the stabilization of the amine. Basic analytes with increasing substituents around the nitrogen atom on the amino group can sterically hinder the hydration of these analytes. Therefore these analytes will by solvated to different degrees, depending on the size and number of constituents adjacent to the amino group.

In a separate article, Nagy [47], have applied electrostatic potential calculations to investigate the hydration effects on the basicity of amines. It has also been shown that the pK_a of amines is influenced by the conformational changes in solution as well as by the water molecules attached to the analyte. It has also been shown those organic solvents such as acetonitrile and methanol can also affect the analyte solvation.

Since the aqueous and the organic eluents influence the analyte solvation, the composition of the mobile phase surrounding the analyte may be different from that of the bulk mixture. This may be explained by preferential solvation. Preferential solvation is attributed to the presence of a molecular excess of either of the eluents components immediately surrounding the analyte [48] and this may be influenced by the structural features of the aqueous-organic mixture [49,50]. It has been shown that preferential solvation exist between hydrogen, acetate ions [51], fluoroquinolones [52], and buffer species such as tartrate, citrate, phthalate and phosphate ions in acetonitrile-water mixtures [53,54].

Protonated basic analytes are solvated with water molecules are hydrophobic and will prefer to be in the mobile phase. Therefore, they will elute early from the column. An increase in counter-anion concentration in the mobile phase will disrupt the analyte solvation and form ion pairs with the positively charged analyte. The disruption of the analyte solvation will render the positively charged analyte more hydrophobic and will result in an increase in the retention. The degree to which the protonated base is

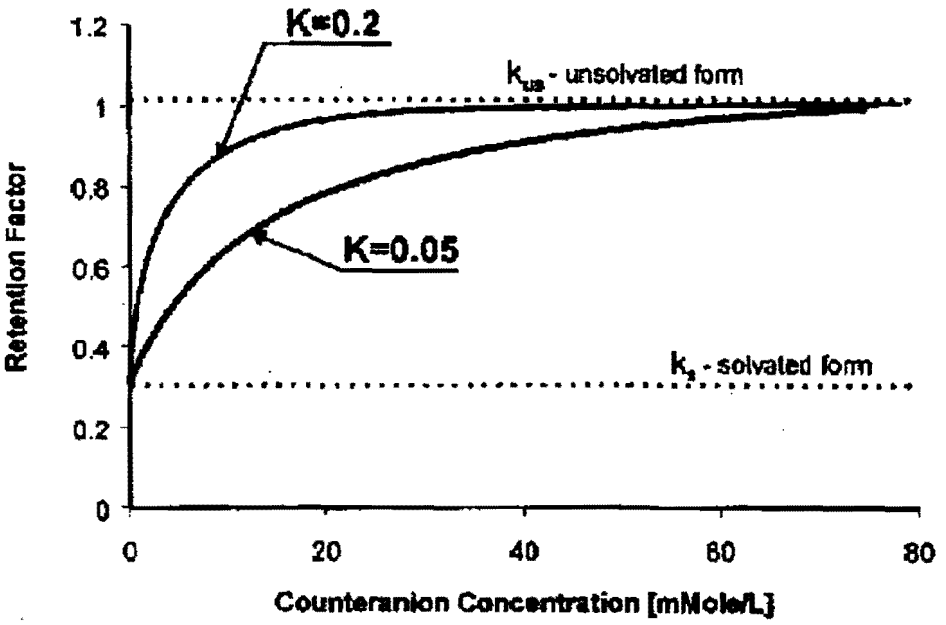
desolvated was determined to be a function of the concentration and type of the counter-anion in the mobile phase.

Based on this theory, Lo Brutto et al [55] have developed a theoretical model based on the solvation/desolvation equilibrium of basic analytes retention in HPLC. The model is described by the following equation:

$$k = \frac{k_s - k_{us}}{K.[A^-]+1} + k_{us} \qquad [1\text{-}6]$$

Where k is the retention factor, k_s and k_{us} is the limiting retention factor of the corresponding solvated and unsolvated forms of the basic analyte, K is the analyte solvation constant, and $[A^-]$ is the concentration of the counter-anion. This model was applied to explain the dependencies of the retention factor of the basic analyte versus counter-anion concentration. Fully protonated basic analytes maybe solvated by the eluent components in the mobile phase. LoBrutto et al [55] have shown **(Figure 1-5)** the degree of analyte solvation / desolvation could be controlled by the concentration of counter-anion in the mobile phase. An increase in the counter-anion concentration disrupts the solvation shell of the protonated analyte. This resulted in the increase in the hydrophobicity of the protonated analyte, which results in a parabolic increase in the retention factor.

Figure 1-5: Theoretical Dependence of Retention Factor on Counter-Anion Concentration. [from reference 55]



When developing a RPLC method for the analysis of these ionogenic analytes all of the above parameters must be taken into account. As described earlier, chromatography is a process by which analytes in a mixture is separated. The evidence of the separation is monitored by the use of a sensitive detection system. With conventional UV/VIS detection systems, solvent purity is important. Impurities may be detected, and show up on the chromatogram as ghost peaks. Also, a wide variety of inorganic salts are available to be used for buffer preparation. However, the effect of the mobile phase composition has to be delicately controlled when considering electrospray ionization detection of these separated ionogenic solutes in RPLC.

Chapter 2

Factors Affecting the Electrospray Ionization Efficiency

Mass Spectrometry is generally considered a universal detection system that offers greater sensitivity over other detection systems such as ultraviolet/visible (UV-VIS), fluorescence and electrochemical detectors. Today, mass spectrometry [56] is a microanalytical technique requiring only a few picomoles of sample to obtain characteristic information regarding the molecular weight and the structure of the analyte. In all cases, some form of energy is required is transferred to the analyte molecules to effect ionization. In the classical technique of electron impact ionization, some of the emergent molecular ions of the analyte "explode" into a variety of fragment ions; the resulting fragmentation pattern together with residual molecular ions, generating a mass spectrum. In principle, the mass spectrum of each analyte is unique and can be used as a chemical "fingerprint" to characterize the analyte.

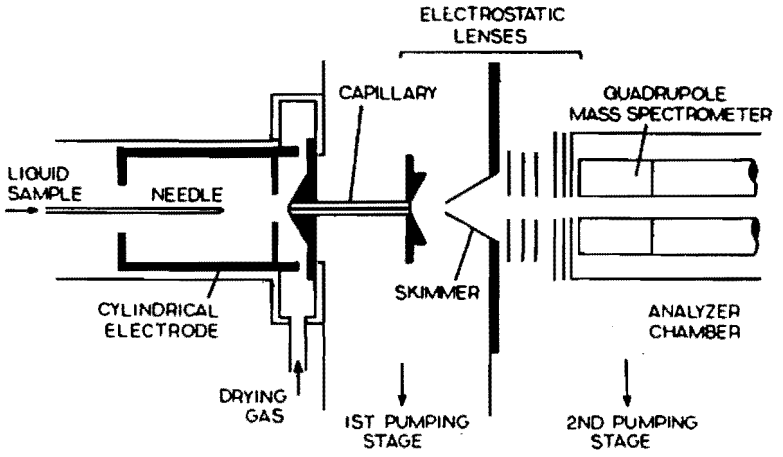
Mass Spectrometry has become an effective tool for the quantitative and qualitative, and the structural characterization of drugs and their metabolites in biological fluids. LC/MS was first used for the analysis of drugs and their metabolites a decade ago [57]. However, widespread application of LC/MS in the field of drug metabolism and toxicology has developed only in the past few years [58]. This development is mainly due to the introduction of LC/MS systems based on atmospheric pressure ionization (API) sources coupled to quadrupole mass analyzers. New API methods include

atmospheric pressure chemical ionization (APCI) and, in particular electrospray ionization (ESI).

The use of electrospray ionization as a means of introducing a liquid into the mass spectrometer was first pioneered by [59,60] in the late 1960's and early 1970's. Their research was continued by Yamashita and Fenn [61] in 1984. This technique, which resulted in the description of an LC-MS interface in 1985 [62], has made a significant commercial impact in the last decade [63]. ESI is a soft ionization technique and has the unique ability to transfer ions from the liquid phase to ions in the gas phase. This feature makes ESI well suited for the detection of compounds such as nucleic acids, peptides, proteins, carbohydrates and other thermally labile compounds. This distinguishes ESI from other mass spectrometric techniques.

ESI has become one of the most important ionization techniques for the on-line coupling of liquid phase separation techniques such as high performance liquid chromatography (HPLC) and capillary electrophoresis (CE) with mass spectrometry (MS). HPLC-ESMS is being increasingly used for the identification and determination of organic analytes of biological significance. A schematic diagram [62] of an early electrospray LC/MS interface is shown in **Figure 2-1**.

Figure 2-1: Schematic Diagram of an Early Electrospray LC-MS Interface and Ion Source. [from reference 62]



When coupled to High Performance liquid Chromatography (HPLC), electrospray ionization mass spectrometry detection (HPLC/ESI/MS) is a powerful technique for the analysis of drugs, peptides, DNA bases and other compounds in complex matrices [64]. HPLC/ESI/MS offers high selectivity, sensitivity and identification of compounds in a mixture, which cannot be achieved by either HPLC or ESI alone. The acceptance of ESI/MS as a sensitive and selective universal detection system has driven instrument manufacturers to develop mass spectrometers that are more user-friendly and affordable. With the reduced cost of these systems, ESI/MS has become the detector of choice for most HPLC systems.

Mechanism of Electrospray Ionization

Electrospray is an efficient method developed for transferring ions from the solution phase into ions in the gas phase. This technique is well suited for compounds that either exist as ions in solution, can be ionized at an appropriate pH or polar neutral molecules that can associate with small ions such as Na^+, K^+ or NH_4^+. This process is strongly endothermic since in solution the ion is solvated and is not in the gas phase. For the process

$$Na^+(aq) \rightarrow Na^+(g)$$

$$\Delta G^0_{sol} = 98 kcal/mol$$

$$\Delta H^0_{sol} = 106 kcal/mol$$

where ΔG^0_{sol} is Gibbs free energy of the transfer of ions from the liquid phase and ΔH^0_{sol} is the reverse process, i.e. the transfer of the ion from gas phase to the solution phase.

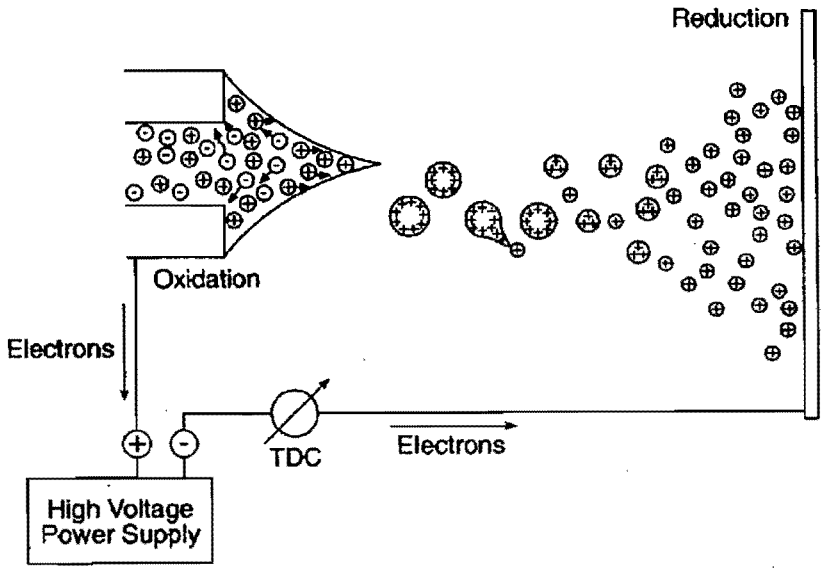
The path from analyte neutrals/ions in solution to gas phase ions in ESI/MS has been subject to a large number of investigations and controversial discussions [65]. However, two processes are proposed. Both include three major steps in the production by electrospray of gas phase ions from solution phase ions as illustrated in **Figure 2-2.** First, the production of charged droplets at the ES capillary tip (the electrophoretic mechanism). Secondly, the shrinkage of the charged droplets by solvent evaporation and repeated droplet disintegration, leading ultimately to very small highly charged droplets capable of producing gas phase ions, and third, the production of gas phase ions from the very small and highly charged droplets.

In step 1, a voltage V_c of 2-4 kV is applied to the metal capillary which is typically 0.2 mm o.d. and 0.1 mm i.d. and located 1-3 cm from the counter electrode. Because the electrospray capillary tip is very thin, the electric field E_c in the air at the capillary tip is very high and can be evaluated by the approximate relationship as follows, when the counter electrode is large and planar by:

$$E_c = 2V_c / r_c \ln(4d / r_c) \qquad [2\text{-}1]$$

Where r_c is the capillary outer radius (~ 10^{-4} m) and d is the distance from the capillary tip to the counter electrode (~ 0.02 m).

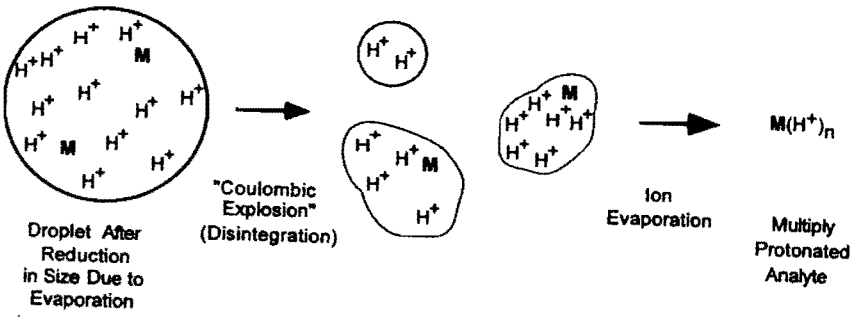
Figure 2-2: Schematic Representation of an Electrospray Source as an Electrochemical Cell [from reference 66]



This is also known as the charge separation process or the electrophoretic mechanism. Here the positive charges move towards the surface of the droplet. When the mutual repulsion between the positive ions at the surface overcomes the surface tension of the liquid a cone is formed. This is followed by the emergence of a fine jet from the cone tip and the production of charged droplets. This process of nebulization generally uses the assistance of a high velocity gas flow with the pneumatic nebulization forming the droplets and the electric field charging them. In addition to the use of pneumatic nebulization, dilution of the aqueous solutions prior to electrospray with organic solvents reduces surface tension of the droplet.

After the droplet separates from the liquid front at the tip of the capillary, electrical repulsion has become larger than the cohesive force that keeps the liquid together. As the droplet travels through the gas at atmospheric pressure, shrinkage occurs by evaporation of solvent, so that charge density at the droplet surface increases. When electrostatic repulsion exceeds the surface tension, the droplet becomes unstable and falls apart. The radius of primary droplets in ES is of the order of 0.5–1 μm and this will reduce ultimately to a radius of approximately 10 nm, prior to release of sample ions (ion evaporation, step 3) which can then be taken into the mass spectrometer itself **(Figure 2-3).**

Figure 2-3: Sketch Showing Macroscopic Droplets Containing the Analyte and an Excess of Protons Followed by a Size Reduction Due to Evaporation [from reference 67a]



The total number of ions that escape from droplets is related to the charge on droplets, which can be derived from spray current measurement. Kebarle et al. [67], have proposed equations for a two electrolyte system.

$$I_A = f.p.\frac{k_A[A^+]}{k_A[A^+]+k_B[B^+]}.I_{spray} \qquad [2\text{-}2]$$

$$I_B = f.p.\frac{k_B[B^+]}{k_A[A^+]+k_B[B^+]}.I_{spray} \qquad [2\text{-}3]$$

Where I_A=A^+ ion signal at the MS detector, I_B=B^+ ion signal at the MS detector, f=fraction of charges on droplets that are converted to gas phase ions, p=fraction of gas phase ions transported into the mass analyser, k_A=sensitivity coefficient for A^+, k_B=sensitivity coefficient for B^+ and I_{spray}=total droplet current (electrospray current).

According to these equations, the abundance of a sample ion is proportional to the amount of charge on the droplets and proportional to a sensitivity coefficient k, which is dependent on the ion structure. Ionic surface-active species have a high k value (~10) and are observed with high sensitivity in ESMS. Alkali metal ions have a low k value (ca 1)

and the k values for protonated organic bases are in between (ca 3-6) [67]. Equation 2 can be extended for a multi-electrolyte system by extending the denominator with the appropriate number of $k_x[X^+]$ terms. The significance of equation 2 in HPLC-ESMS and CE-ESMS is that at constant I_{spray}, the A^+ ion signal is proportional to $[A^+]$ if $[A^+]<<[B^+]$. This condition is usually met in HPLC-ESMS if B^+ is an electrolyte present in the eluent at the mM level while the analyte concentration is at the μM level.

The same sequence of evaporation and Coulomb fission steps is proposed in the ion desorption (evaporation) model [68,69]. However, at some intermediate stage before the droplets are so small that they contain only one analyte molecule, the surface field is sufficiently intense to cause expulsion of a charged analyte ion from the droplet surface.

The fundamental difference between the two theories is in how the analyte ions become separated from other species in the droplet prior to the ionized species entering the mass analyzer through a skimmer cone.

Mass Analyzers

To date, a wide variety of detectors such as single and triple quadrupole, ion trap, time-of-flight, magnetic sector and Fourier Transform are available with ESI interface. By far, most of the mass analyzers used for ESI are the quadrupoles, which can still

operate efficiently in spite of the relatively high pressure associated with the ESI interface [70].

This nonmagnetic mass spectrometer employs a combination of direct-current (DC) and radiofrequency (RF) fields and acts as a "mass filter". Mechanically, the quadrupole consists of four longitudinal parallel rods with a hyperbolic cross section surface **(Figure 2-4).** The symmetrical arrangement allows idealized hyperbolic fields to be produced according to quadrupole theory [71]. The opposing surfaces (i.e those diagonally opposite) are connected together electrically and to RF and DC voltage sources.

The ions are extracted from the ion source and are accelerated (5-15 V) into the central space of the quadrupole towards the detector. The ion trajectories through the central space between the rods at a given set of DC and RF voltages of a specific m/z (mass to charge ratio) value avoid collision with the surface of the quadrupole and will reach the detector **(Figure 2-5).**

The resolving power of the instrument is established by the ratio of the constant RF and varying DC voltages. Quadrupole instruments can scan from the order of a few milliseconds to provide the convenience of "real time" spectra to several hundred milliseconds to provide better ion counting statistics for better sensitivity for analytes present at the subpicomole level.

Figure 2-4: Schematic Diagram Showing Arrangement of Quadrupole Rods and Electrical Connection to RF Generator. [from reference 67a]

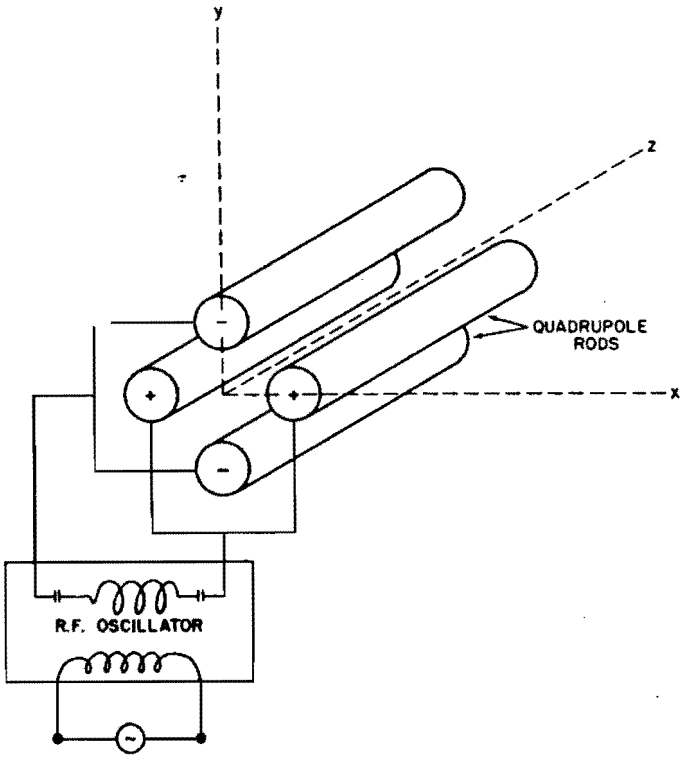
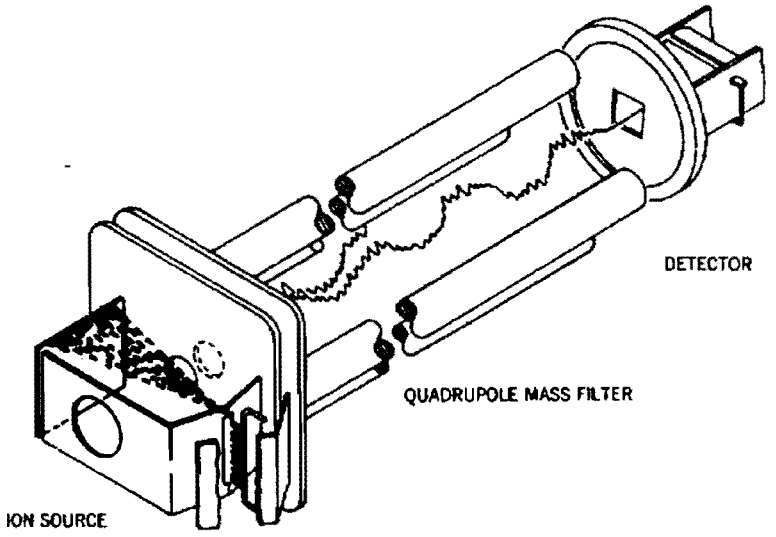


Figure 2-5: Schematic Diagram of quadrupole Mass Spectrometer Illustrating the Irregular Fight Path of Ions from the Ion Source Through the Central Space Between the Quadrupole Rods to the Detector. [from reference 67a]



Operation and Performance of the ESI Interface

The performance of the LC/MS system is dependent on various interrelated parameters. Most importantly is the nature of the analyte, which determines the type of solvent and the composition of the mobile phase used to achieve separation in the HPLC system. Some researchers have shown that parameters affecting the ESI efficiency include mobile phase additives, solution pH, flow rate, solvent composition and analyte concentration [72-78]. Further optimizations of instrumental parameters that can affect the sensitivity of the ESI response include position of the spray probe, gas flow, needle potential, and the operational voltage in the ion-transfer region.

The position of the spray needle in most commercial systems is relatively fixed. However, systematic studies performed on a home-built system by Ikonomu et al [77,79] have shown that the position of the spray needle relative to the sampling orifice positioned approximately 1 cm off-axis provides optimum response and signal stability in the analysis of small molecular ions.

One of the most important instrumental parameters in optimizing the ESI response is the application of the needle potential. In general the needle potential should be set a few hundred volts higher than the electrospray onset voltage. A typical value is ± 3 kV, depending on the location of where the voltage is applied and on the mode of ionization (positive-ion or negative-ion mode).

Improvements in ESI Interface

Improvements in HPLC/ESI/MS analysis have focused mainly on the MS instrument. Particularly on approaches that facilitate the desolvation process. Desolvation depends on heating during the transport of the droplet through the metal or glass capillary [80,81]. The heated capillary is placed within 3-10 mm from the spraying to efficiently capture the charged species from the plume of the spray.

The addition of heat significantly promotes the desolvation process during transport through the capillary. Systematic studies of the mechanism of ion transport from the spray plume have shown that space and the charge dominate the process [77, 82, 83], thus requiring delicate optimization for the efficient operation. Others have shown [84,85] that some interfaces that rely on a heated channels through multiple differential pumped vacuum stages will assist the desolvation and declustering, where a small fraction of the ions are then transported through the skimmer into the optics of the mass spectrometer.

Effects of Chromatographic Parameters on ESI Efficiency

When considering HPLC with ESI MS detection, the analyte ionization must be taken into account because the degree of analyte ionization in solution will have an effect on the ESI sensitivity. Electrospray ionization (ESI), is one of the most efficient and

successful interfaces used in high performance liquid chromatography with mass spectrometry detection (LC/MS). ESI serves as an efficient means of producing ions from nonvolatile compounds in the liquid phase into ions in the gas phase and is well suited for the analysis of proteins, peptides, carbohydrates and other thermally labile compounds. LC-ESI-MS provides an excellent combination technique to introduce separated analytes into the mass spectrometer for molecular weight and structural determination. This technique allows the use of only volatile mobile phase modifiers thus imposing limitations on the choice of the acid modifiers used to achieve separation in HPLC.

HPLC mobile phase type and composition exhibit noticeable effects on the spray performance, ion species formation, ionization equilibrium in solution and gas phase ion production and hence has a major impact on the ESI ionization process. The type of organic solvent and the composition of the organic solvent will affect the viscosity, polarity, surface tension, density and the dielectric constant of the mobile phase. Other parameters such as the concentration of the analyte, matrix affects and the flow rate of the mobile phase will play an important part in the ESI response. These parameters will be discussed in detail in the following sections of this chapter.

Recently, work by Brown and Needham [86,87] have demonstrated the importance of mobile phase additives and the stationary phase in improving analyte sensitivity in LC/MS. In addition, others have shown the importance of sample preparation in obtaining a successful HPLC/MS/MS analysis [88]. One of the limitations of HPLC with

ESI/MS detection is that the mobile phase must be compatible with the mass spectrometer. In order to achieve the maximum MS signal, the choice of acidic modifiers used to prepare buffers for the aqueous portion of the mobile phase is limited to the use of volatile components at very low concentrations.

The Effect of Sample Matrix on ESI Efficiency

Contrary to common perceptions, the reliability of quantitative assays for the determination of drugs in biological fluids using high-performance liquid chromatography with tandem mass spectrometric (LC/MS) detection methods and the integrity of resulting pharmacokinetic and toxicokinetics data may not be absolute. Results may be adversely affected by lack of specificity and selectivity due to ion suppression caused by the sample matrix, interferences from metabolites, and "cross-talk" effects [89].

In their work the authors have shown the effects of the sample matrix on the determination of finasteride in human plasma. The ion suppression effect was studied by analyzing standards injected directly in mobile phase and comparing the response of the analytes spiked *before* extraction into five different plasma pools and standards spiked into the plasma *after* extraction.

The absolute peak areas for drug and internal standard in different plasmas were calculated, and the slopes and peak area ratios at all concentrations within the standard curve ranges were compared. When analyses were performed under conditions of *minimal* HPLC retention, the slope of the standard line for one set of plasma samples was substantially different (about 50% higher) from that from other plasma sources. The precision of the assay, expressed as coefficient of variation (CV, %) was also inadequate and varied from 15 to 30% at all concentrations within the standard curve range. When the same experiments were repeated using *high* HPLC retention, the slopes from different plasma sources were practically the same, and the CV was improved to 6-14%.

By increasing retention factor and providing more chromatographic retention of analytes, the "unseen" interferences from plasma matrix were mostly separated from analytes, practically eliminating the ion suppression. In addition, by eliminating a number of the endogenous components through more selective extraction, the ion suppression was also minimized.

The ion suppression phenomenon was originally described by Kebarle and Tang [90] who showed that electrospray ionization response of organic bases decreased with an increase in concentrations of other organic bases. Coeluting, undetected matrix components may reduce the ion intensity of the analytes and affect the reproducibility and accuracy of the assay. Plasma matrix effects on an electrospray ionization response

were also demonstrated by [91] in the analysis of SR 27417, a platelet-activating factor receptor antagonist.

The Effects of pH and Analyte Ionization on ESI Efficiency

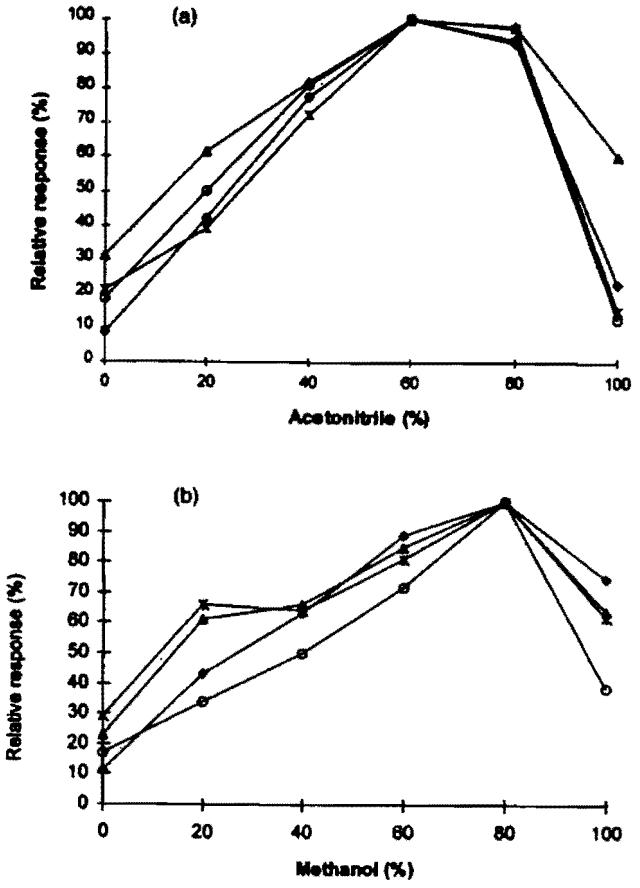
The equilibrium of basic analytes in pure water is not the same in aqueous/organic mixtures. Barcella [28] has also shown that the pK_a of basic analytes actually decreases upon the addition of organic solvents. The effect of organic solvents shifts the ionization equilibrium to the less ionized form and also shifts the pH of the aqueous mobile phase to greater values. These effects must also be accounted for in the proper description of basic analytes ionization efficiency in LC/MS.

Effect of Solvent Concentration on ESI Efficiency

In LC/MS, aqueous/organic mixtures are often used to achieve separation of analytes in a mixture. Moderate concentrations of solvents such as methanol and acetonitrile are commonly used. Lower viscosity and higher vapor pressure compared to water characterize these solvents and provide a more enhanced MS signal [90]. ESI response in various mobile phase combinations has also been reported [92] showing that the higher the percent of organic composition in the solvent system the higher the ESI response. Solvent properties such as surface tension, conductivity, viscosity and dielectric constant were determined to be important parameters in the success of the ESI/MS process [93,94].

Zhou and Hamburger [95] , have studied the effects of organic solvent content on the ESI response on a series of structurally diverse compounds. **Figure 2-6** shows the relative [M+H]+ ions response for the four compounds from 0 to 100 % water/acetonitrile and water/methanol. An almost linear response was observed with an increasing proportion of organic solvent, reaching a maximum around 60 % and 80 % for acetonitrile and methanol respectively, and decreasing towards 100 % organic solvent.

Figure 2-6 Relative Response for Molecular Ions in Positive Electrospray Ion Mode as a Function of Organic Solvent.



Relative response for $[M+H]^+$ ions with (panel A) 0 to 100 % acetonitrile and (panel B) 0 to 100 % methanol [from reference 95]

Effect of Flow Rate on ESI Efficiency

Mobile phase flow-rate is another important parameter in LC/MS. In early electrospray interfaces, the flow-rate was restricted to 10 μL/min, which was sufficient for protein characterization, but insufficient for LC/MS. With the popularity of LC/ESI/MS, there is a move towards a high-flow electrospray interface, but the best electrospray performance is still achieved at low flow-rate. At a lower mobile-phase flow-rate, the mass flow of the analyte relevant ions is relatively large.

The importance of low flow-rates and the advantages of the use of miniaturized LC columns were investigated. The effect of flow rate on the droplet size was investigated by Vestal [98]. He derived an equation for the droplet radius R (in μm) at the Rayleigh limit as a function of the liquid flow-rate (in μL/min) and the total spray current I (in A):

$$R = 1.73x10^{-5}\sqrt[3]{\left(\frac{F}{I}\right)2} \qquad [2\text{-}4]$$

For a typical spray current of 100 nA, this equation predicts droplet diameters in the order of 1-2 μm at 1 μl/min and of 100-200 μm at 1 ml/min.

Base on experimental measurements of droplet size and charge, Fernandez de la Mora *et al* [99] have proposed a relation for the droplet radius R in electrospray:

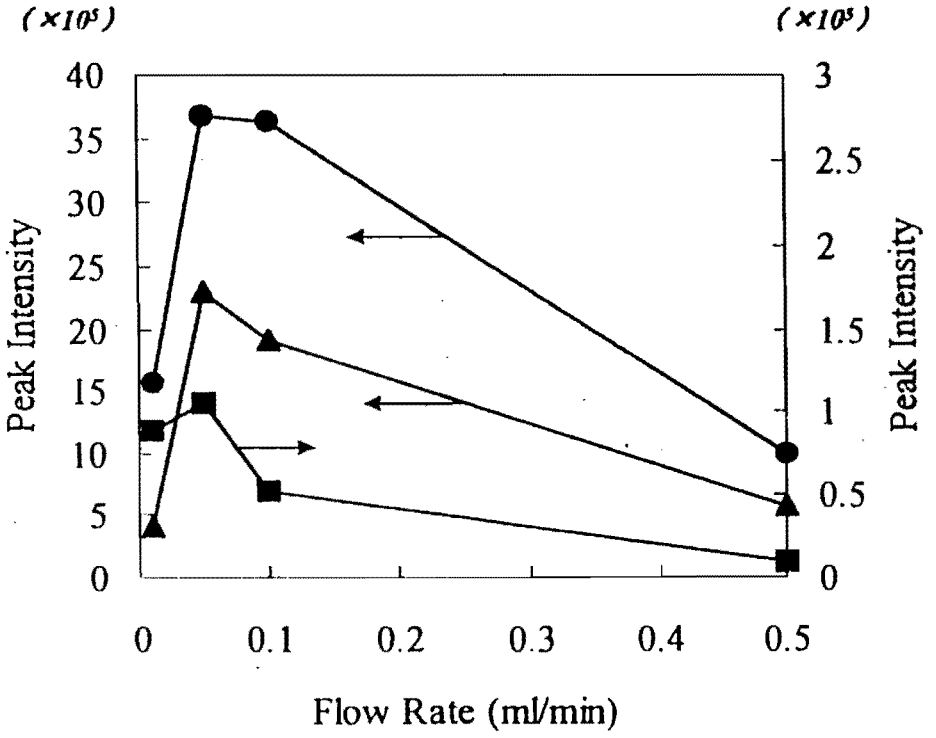
$$R\alpha\sqrt[3]{PF^2\sigma} \qquad [2\text{-}5]$$

Where F is the liquid flow-rate, ρ is the liquid density, and σ is the surface tension. The equation indicates that increasing the spray current by increasing the liquid flow-rate is counterproductive, as it results in larger droplets. At higher flow rates the fraction *f* of the droplets charge that is converted to gas-phase charge decreases.

Recent work by Nishikawa and Tsuchihashi [100] have shown the effects of flow rate on the ESI efficiency. In their study, the authors chose three analytes of varying polarity. As shown in **Figure 2-7**, the optimum ESI efficiency was achieved when a flow rate of 50 μl/min was used.

In LC/MS, solvents such as methanol and acetonitrile are often used. In addition to the effect of flow-rate on droplet size, these solvents can contain significant concentrations of impurity electrolyte ions such as NH^+_4, Na^+. The pH of the solution exiting the capillary for flow rates of 10, 1.0 and 0.1 ml/min would be 5.2, 4.2 and 3.2 respectively. These changes in pH are quite dramatic and would be expected to alter the ES response of a weakly basic analyte through an increase in the degree of protonation in solution [101].

Figure 2-7 Electrospray Ionization Intensity as a Function of Flow Rate. [from reference 100]



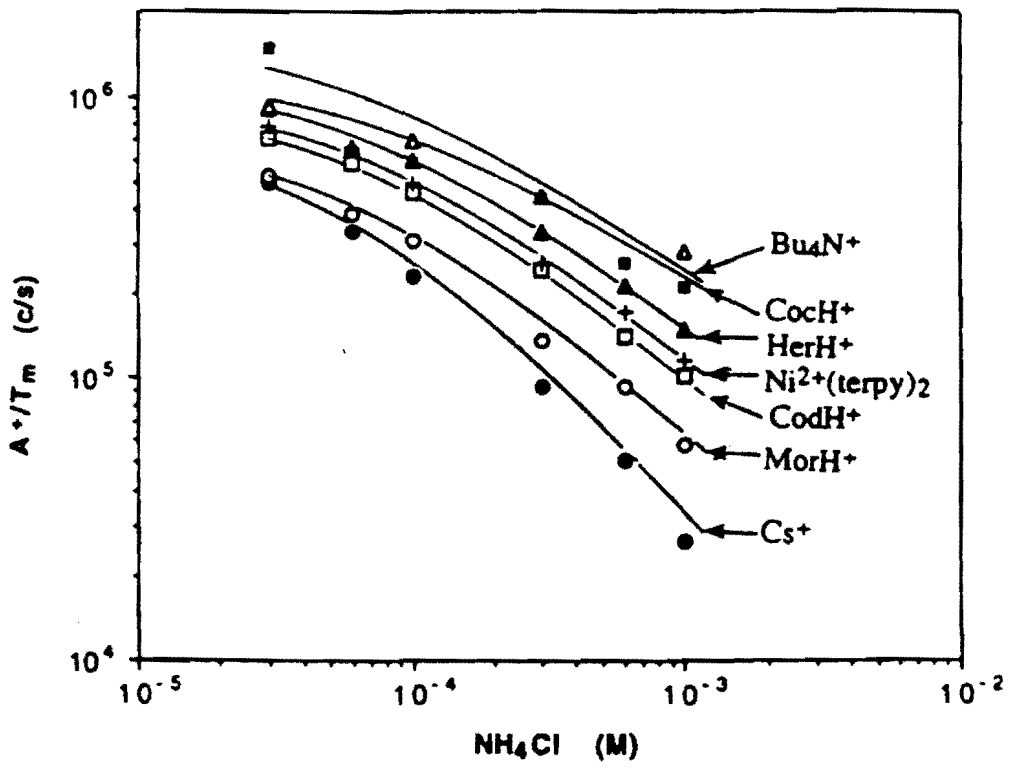
Effect of Counter-anion Concentration on ESI Efficiency

LC/ESI/MS detection is advantageous for the analysis of many basic, acidic and zwitterionic compounds [64]. However, the analysis of basic analytes by RPHPLC has been very difficult due to secondary equilibrium of these analytes and the unreacted residual silanol groups on the column. This type of interaction between the analyte and the unreacted residual silanols results in asymmetric peak shapes, which is demonstrated by fronting or tailing of the chromatographic peaks. To overcome this undesired effect of secondary equilibria, high ionic strength buffers and /or ion-pairing reagents are used. Buffer concentrations of 100 mM or greater are often employed to decrease peak tailing. The concentration and the type of buffer used to prepare the mobile phase will affect the ESI response.

Tang and Kebarle [75] have shown that if the concentration of the analyte is held constant and a second electrolyte such as NH_4Cl is present in increasing concentration, this will result in gradual decreasing ESI response. Their results are shown in **Figure 2-8**. An addition of increasing concentration of NH_4Cl decreased the ion intensity of all analytes studied. In addition the authors have shown that the relative decrease in ion intensity is dependent upon the evaporation rate constant of the analyte by:

$$k_1 = (kT/h)e^{-\Delta G^0 / RT} \qquad [2\text{-}6]$$

Figure 2-8: Analyte Response as a Function of the Ammonium Acetate Concentration in Solution [from reference 75]



This equation expresses the transition-state energy theory and $\Delta G^{\#}$ represents the free energy of the transition state, where an ion-solvent molecule leaves a small droplet. Further examination of $\Delta G^{\#}$ has suggested that this numerical value is dependent upon the sum of the electrostatic potential energy due to the repulsion between the ion cluster and the charged droplet, the potential due to the attraction between the charge of the ion and the polarizable drop, and the solvation energy of the ion.

The ESI/MS interface allows only the use of volatile organic modifiers. The greatest signal is observed when modifiers such as acetic or formic acids are used for pH adjustments with the addition of less than 10 mM of their corresponding ammonium salts. To achieve the greatest signal intensity in ESI/MS, low buffer concentrations of 10 mM or less and no ion pairing reagents must be used.

The effect of salt concentration on analyte response using electrospray ionization mass spectrometry (ESI-MS) was measured and compared to a predicted value based on an equilibrium partition model previously developed by Enke [102,103]. The model predicts that analyte response will be proportional to the concentration and that the response factor will decrease with increasing electrolyte concentration. The measured analyte response was proportional to the concentration over four orders of magnitude when electrolyte concentration was below 10^{-3} M, as the model predicted.

The authors have shown that most of the deviations form the predicted value are due to the fact that ion desolvation and/or transmission are significantly decreased at the

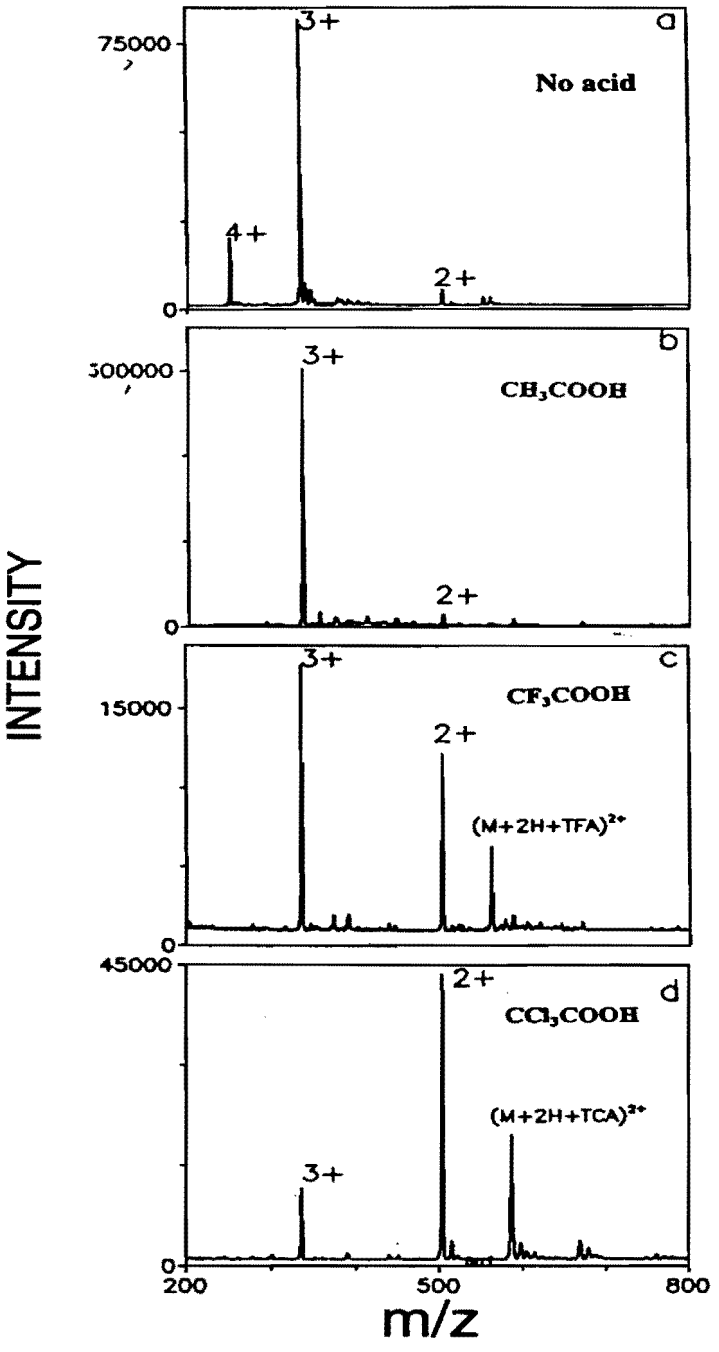
highest salt concentrations. The authors also observed changes in the shape and diameter of the spray as a function of salt concentration. This was attributed to the fact that the increase in charge density at high salt concentrations results in repulsive forces that cause the ions to spread out more.

The increase in spray diameter reduces the density of ions at the center of the spray and thus fewer ions enter the mass spectrometer causing the decrease in response. This also indicates that a smaller fraction of the ions reach the detector than at lower salt concentrations. An increased current was observed at the highest salt concentrations, which indicates that more excess charge ions are produced in the solution phase. Thus the decrease in response indicates a loss in ion transfer efficiency or desolvation efficiency (or both).

Effect of Acidic Modifier on ESI Efficiency

Mirza and Chait [104] have studied the effects of different acid modifiers on ESI efficiency of a basic peptide in three different acidified solutions of 50 % aqueous methanol containing acetic, trifluoroacetic and trichloroacetic acid where each solution was carefully adjusted to pH 2.2. In addition, a spectrum of the peptide was obtained when no acid was added (**Figure 2-9**)

Figure 2-9: The Effect of Different Types of Acidic Modifiers on the Ion Formation in Positive Electrospray Ionization [from reference 104]



In a separate study the authors have shown, that by the addition of 3mM of the conjugate sodium salt (pH was 6.3) for the corresponding acids, the ESI response decreases similarly to the decrease in ESI response when the acids were used as modifiers. The decrease in ESI response was found to be dependent solely on the basicity of the anionic species and was independent of the source of the anion (salt or acid).

The authors propose a two step mechanism for the observed signal reduction. The first step occurs in the solution, where the anion pairs with the positively charged basic group on the peptide. The second step occurs during the desolvation step in the gas phase. In this step the ion pair associates to yield the neutral acid and the peptide with a reduced charged state. Different anions were observed to have different propensities for charge neutralization following the order of:

$$CCl_3COO^- > CF_3COO^- > CH_3COO^- \sim Cl^-$$

This propensity for charge neutralization of the different anionic species is reflected in the avidity of the anion-peptide interaction.

Trifluoroacetic acid (TFA) is a commonly used acidic modifier for pH adjustment of aqueous buffers used in the mobile phase. This strong acid is notorious for its signal suppression effects in LC/MS. Optimization of the ESI performance in the presence of TFA for peptide mapping by LC/MS was reported by Apffel et al [74,105]. The signal

suppression of TFA is due to a combination effect of ion-pairing and surface tension effect.

The signal suppression occurred for a variety of analytes as shown in (**Figure 2-10**). It is assumed that the TFA anion more-or-less masks the positive charge on the analyte molecule at the droplet surface and thereby prohibits the ion evaporation/desolvation of the analyte. As shown in **Figure 2-10**, the addition of a post-column sheath liquid of propionic acid in 2-propanol (75:25, v/v) was used to counteract the ion suppression.

Influence of Analyte pK_a on ESI Ionization Efficiency

The influence of analyte pK_a and gas-phase proton transfer on ESI Ionization sensitivity of nucleotides and nucleosides was studied [106]. Specifically, the authors have investigated the influence of ammonium acetate and tripropylamine (TPA) in solution on the $[M+H]^+$ ion signal of thymine, guanine, and adenine. The physiochemical properties for the compounds studied are summarized in **Table 2-1**.

The authors demonstrated the relationship between the pK_a of the analyte and the influence of the presence of ammonium acetate in solution (**Figure 2-11**).

Figure 2-10: Signal Suppression by TFA and Solution to the Problem with a Sheath Liquid of 10% Propionic Acid. [from reference 105]

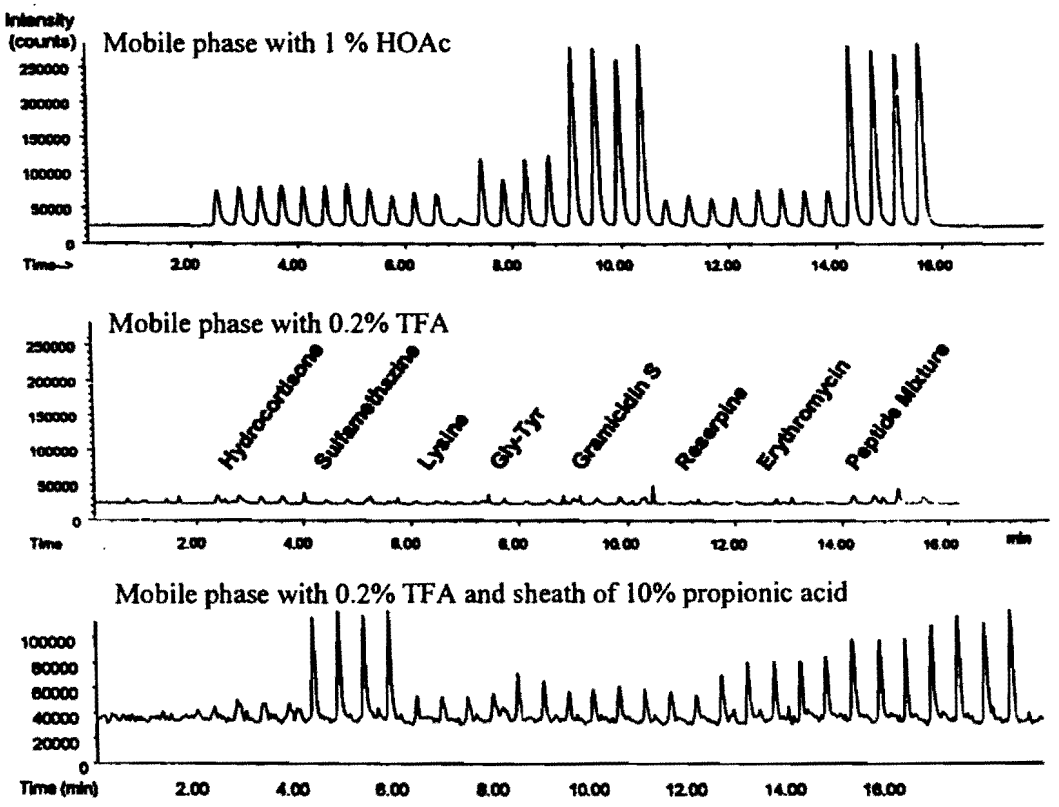


Table 2-1: Gas Phase Basicity and pK_a Values for Some Nucleosides [from reference 105]

Analyte	PK_a	Gas Phase Basicity
NH_3	9.3	195.6
TPA	10.4	226.2
Adenine	4.1	215.7
Guanine	3.2	215
Thymine	0	201

Figure 2-11: The Affects of Analyte pK_a and the Influence of Ammonium Acetate Counter-anion on $[M+H]^+$ Intensity. [from reference 106]

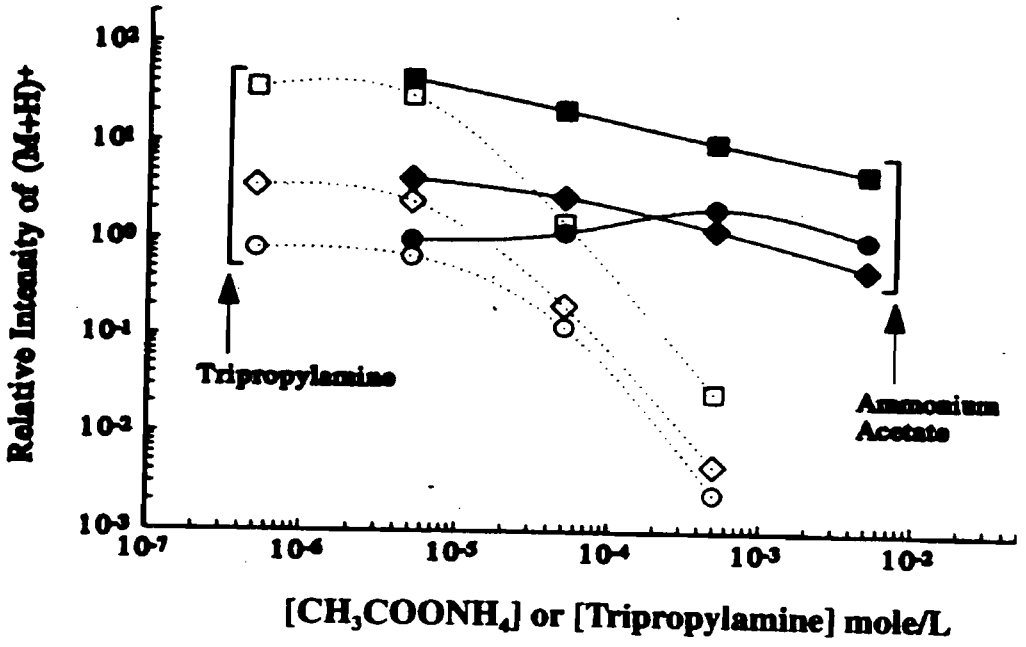
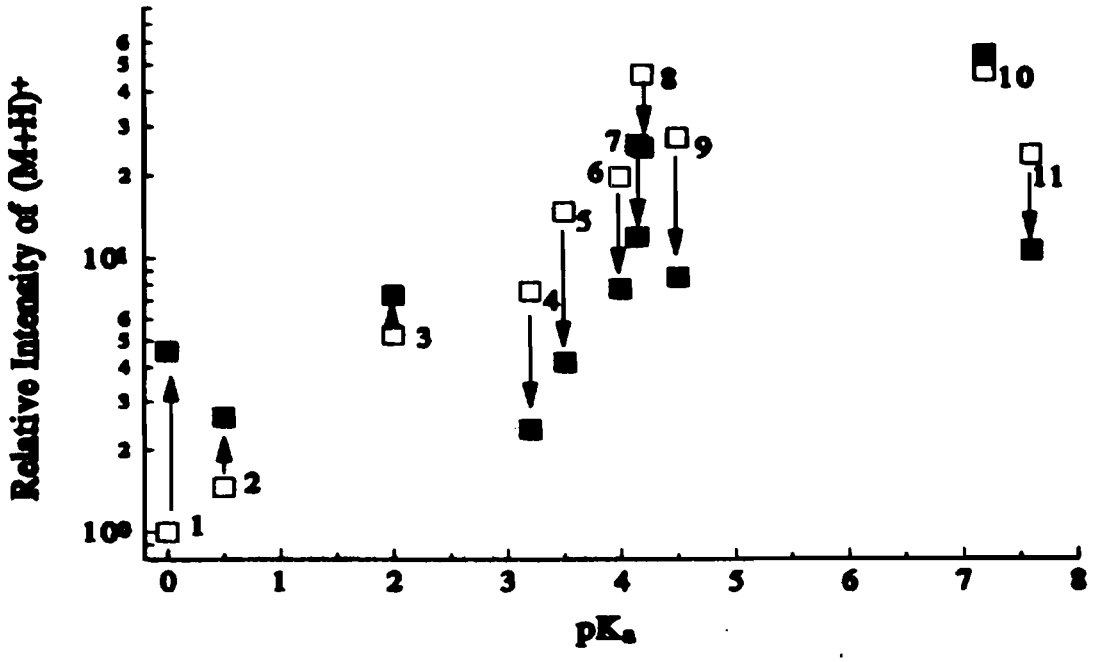


Figure 2-11 shows a plot of the relative intensity of the [M+H]+ ions versus the concentration of ammonium acetate and TPA. The [M+H]+ ion intensities for adenine and guanine exhibit a linear decrease with increasing concentration of ammonium acetate. The signal reduction was attributed to the presence of high concentration of electrolyte in the solution. For thymine, they observed an initial increase in the ion signal intensity of [M+H]+ from 5-500 μM of CH_3COONH_4 which was followed by a slight decrease at a concentration of 0.005M. The increase supports the idea that gas-phase protonation results in the formation of $[M+H]^+$ from the reaction of $[B+H]^+ + M \leftrightarrow B + [M+H]^+$, where M is the neutral thymine molecule and B is NH_3. For compounds with $pK_a < 3$ the addition of ammonium acetate (0.5 mM) can promote the gas-phase proton transfer reactions to increase the $[M+H]^+$ ion intensity.

In **Figure 2-12** Voyksner *et al* [106], show a plot of the ion intensity of $[M+H]^+$ ions of 11 nucleobases and nucleosides (50 μM) in solutions that contain 500 μM ammonium acetate, versus the analyte pK_a. These data indicate that the solution chemistry plays an important role in the signal of the analytes, but gas-phase proton transfer chemistry may be important for compounds with $pK_a < 3$. The relative significance of such processes depends on the pK_a and the gas-phase basicity of the analyte, and the nature of the electrolytes in solution.

Figure 2-12: The Effect of Analyte pK_a on ESI Intensity at Constant (500 μM) Ammonium Acetate Concentration. [from reference 106]



M. G. Ikonomou, et al [77] have examined the sensitivities observed for a variety of bases with known aqueous basicity constants K_B. The observed BH^+ intensities obtained from 10_5 M solutions of the pure bases in methanol. A decrease in BH^+ intensity with increasing pK_B value was observed and that trend reflect the decreasing solution concentration of BH^+ with increasing pK_B. Some "scatter" of the data was observed and was attributed to the difference in ion intensity for different BH_+ that were electrosprayed need not be the same even when the solution BH^+ concentrations are the same. This is due to different desorption efficiencies from the charged droplets to the gas phase for BH^+ with different molecular structure. Also as a result of differences in structural features and volatilities of different analytes may lead to different desorption efficiencies for the BH^+ ions.

In the same article the authors show the strongest bases with $pK_B < 5$ are nearly completely ionized, ie $[BH]^+ \sim 10\text{-}5$ M. These analytes show a rapid decrease in the ESI ionization intensity. Examples are given for strong bases such as cocaine and tripropylamine which are essentially completely ionized in methanolic solutions, the addition of acidic electrolytes will decrease the ESI response. However, for weak bases which are not fully ionized in these solutions, addition of acidic electrolytes will lead to an increase in BH^+ concentration in solution. This will lead to an increase in BH^+ intensity.

Effect of Analyte Concentration on ESI Efficiency

Banks and coworkers [107] have studied the linear dynamic range of the SIM ESI response for adenosine in 20 % methanol water mixture. A plot of the ESI response versus the concentration of adenosine yields a linear response from 100 amol to 1.6 pmol. Above 1.6 pmol, no substantial increase in ESI signal was observed. This plateau in ESI response at the high concentration level was attributed to the formation of clusters or the precipitation of the analyte in solution.

The dependence of ion intensity on the concentration of the analyte in methanolic solutions investigated by [77]. The results obtained showed the slope of the plot of concentration versus the ESI intensity was close to unity (linear) in the low concentration range up to 10^{-5} M. This means that the ion intensity in this range is close to proportional to the ion concentration in solution. At concentrations above 10^{-5} M, the ion intensity flattened out.

The Effect of Gas Phase Basicity on ESI Efficiency

Gas phase basicity of analytes, pK_a and the nature of the electrolyte in solution have been reported to play an important role in the electrospray ionization of selected nucleobases and nucleosides. The intensity of the MH^+ ion for a compound with $pK_a \leq 4$ was proportional to its pK_a values. For compounds with $pK_a < 3$, the addition of ammonium salts was reported to promote gas phase proton-transfer reaction and increase

the MH^+ ion intensity [108]. Banks [107] has also reported the formation of dimer and trimer ions of nucleosides at a concentration of ~ 100 μM, and the ion signals of the protonated species increased with increasing pK_a of the nucleosides.

Ikonomou *et al* [77] have shown the effects of gas phase basicity on the ESI intensity for organic bases. The experiment was performed by mixing gaseous bases B were mixed at known partial pressures into the air supplied to the atmospheric ionization chamber. Simultaneously methanolic solutions containing known concentration of a second base B' was increased. The authors have shown that the stronger the gas phase base added to the atmospheric ionization chamber would cause a larger decrease in the ESI intensity at constant analyte concentration. According to the authors, this provides additional evidence for the importance of gas phase proton transfer when analytes at solution concentrations above 10^{-5} M are coelectrosprayed.

Recent Applications of HPLC-ESMS from the literature

Proteins/Peptides/Carbohydrates

Glycoproteins are fundamental to many biological processes, including fertilization, immune defense, viral replication, cell growth, inflammation, etc. These molecules contain oligosaccharides that are attached to a protein at the hydroxyl group of serine or threonine (O-linked) or to the amide side chain of asparagine (N-linked). Variable oligosaccharide structures can be found at each linkage site and further complexity arises from the partial occupancy at each glycosylation site. Mass

spectrometry has played a valuable role in the elucidation of these complex glycoproteins, including carbohydrate structure and amino acid sequence.

Electrospray ionization mass spectrometry (ESMS) was used to determine the heterogeneity of the carbohydrate composition of glycosylated insulin-like growth factor binding protein 6 (IGFBP-6) [109]. Major glycoforms contained 8-16 monosaccharides, including *N*-acetylhexosamine, hexose, and *N*-acetylneuraminic acid. Glycosylation sites of IGFBP-6 were identified as Thr^{126}, Ser^{144}, Thr^{145}, Thr^{146}, and Ser^{152} by using a combination of ESMS and Edman sequencing of tryptic fragments separated by reverse-phase high-pressure liquid chromatography.

Nucleic Acids and Their Constituents

Esmans [110], regards ESMS as becoming the HPLC detector of choice for the analysis of both high and low molecular mass nucleic acid material in biological samples. In a section of this paper, he described the detection and determination of DNA adducts resulting from DNA's reaction with chemical carcinogens. This is a particularly difficult task in *in vivo* samples where the adduct (in which a new covalent bond is formed) may be present in only minute amounts (1 base modified out of 10^6-10^{11} bases, i.e. ca 10 pg of adduct in 1 mg of DNA).

As an example, Vanhoutte et al [111] have investigated the use of HPLC-ESMS and HPLC-tandem ESMS for the structural identification of the 2'-

deoxynucleotide/bisphenol A diglycidyl ether adducts. Negative ion ESMS low energy collision activated decomposition (CAD) spectra allowed the differentiation between phosphate alkylation and base alkylation. Among other adducts discussed are phenylglycidyl ether adducts (such as (I)), N^6-oxopropenyl-2^1-deoxyadenosine, N^2-3-ethenoguanine isolated in vivo from human and rat liver, hedamycin-d$(CACGTG)_2$ and DNA adducts of 2-amino-1-methyl-6-phenylimidazo[4,5,6]pyridine and malondialdehyde.

Environmental Applications

Manufacture and use of Sulfonated azo dyes are widely used in the textile industry to color natural fibres. These dyes cause an environmental problem if the effluent is not carefully monitored. Azo dyes have been shown to undergo reduction in natural waterways and the degradation products include amines, which are known to be carcinogenic. There is therefore an interest in sensitive techniques to monitor and identify low levels of azo dyes. Oxspring et al [112] have reported the separation and determination of reactive textile dyes, which included sulfonated azo dyes, by HPLC using visible spectrometric detection.

The interfacing of HPLC with a sensitive mass spectrometric detector is therefore seen as a viable hyphenated technique for the identification and quantification of such sulfonated azo dyes, and their degradation and metabolic products in samples such as effluents, river waters, etc.

Several studies of the ESMS behaviour of sulfonated azo dyes, a necessary precursor to ESMS coupling to HPLC, have appeared in the literature. Smyth *et al* [113] have examined the negative ion ESMS of four Remazol textile dyes and their hydrolysis products. They have assigned the major MS signals to species such as $[M-Na]^-$, $[M-2Na+1H]^-$ and $[M-2Na]^{2-}$. It was possible to use this information to directly monitor some of these dyes in an effluent sample although separation procedures such as HPLC coupled with ESMS were recommended for a more complete analysis of such samples. Ballantine and coworkers [114] have also investigated the negative ion ESMS of polysulfonated azo dyes and have found that certain amine bases such as diethylamine enhance the sensitivity of ESMS towards these molecules.

Low Molecular Mass Organic Bases

HPLC-ESMS is now widely used in the pharmaceutical industry for the detection and determination of drugs and their metabolites. This is because many drugs contain basic nitrogen atoms that can be protonated in the electrospray process and hence are detectable by ESMS. Smyth et al [113] have surveyed recent applications of HPLC-ESMS and CE-ESMS to drug analysis. Zavitsanos and Alebic-Kolbah [115] have developed a sensitive and selective HPLC-ESMS method for the enantioselective determination of the α-adrenoreceptor antagonist terazosin (III) in human plasma following a 5 mg single oral dose. The chromatography was based on normal phase chiral separation with post-column solvent addition of 2-propanol-5 mM ammonium acetate (3:1 v/v). Positive ion ESMS utilised the intense $[M+H]^+$ peak at *m/z* 388.2 and there was a relatively small peak at *m/z* 410.2 corresponding to $[M+Na]^+$. The method was compared to

HPLC-fluorescence detection and was found to be more sensitive allowing useful information to be obtained about enantiomeric ratios from subject plasma samples at later time points post dose.

McLean et al [116] have recently compared CE-ESMS and HPLC-ESMS for the detection and determination of 15 1,4-benzodiazepines and their metabolites. The former technique displayed a superior selectivity but HPLC-ESMS, utilizing a quadrupole ion trap mass spectrometer, possessed superior LODs and was able to detect and quantify diazepam and its metabolites in a hair sample of a patient on therapeutic dosage of this drug.

Low Molecular Mass Organic Acids

Chiron *et al* [117] have used automated on-line liquid-solid extraction, followed by HPLC-ESMS for the determination of acidic herbicides such as 2,4-D, MCPA, MCPP, MCPB, benzolin, bentazone and hydroxybentazones. Their proposed method required only 50 cm^3 water, had an LOD of 0.01-0.03 $\mu g\ dm^{-3}$ and utilised SIM on the $[M-H]^-$ species.

The maximum allowable concentration of the herbicide glyphosate (IV), molecular mass 169, in drinking water set by the EC is 0.1 $\mu g\ dm^{-3}$ and can be assayed by derivatization with 9-fluorenyl methoxycarbonyl chloride (FMOC-Cl) (V) followed by solid phase extraction and finally determination by HPLC with fluorescent detection. The structure of the derivative is given in (VI), MW391. The major degradation product,

aminomethylphosphonic acid (AMPA) (VII) (MW111), can also be analysed by this method. This method, however, is relatively non-specific and requires MS confirmation to determine the identity of the glyphosate and AMPA peaks.

Recently, Vreeken [118] published a paper with this end in mind producing a fully automated procedure using HPLC-MS and HPLC-MS-MS. Their scan routine resulted in three analytically usable signals, i.e. the $[M-H]^-$ ion signal from the derivative during the HPLC-MS scan and two product ion signals formed upon CID. Quantification and identification based on the ratios of these three signals and the retention time were very specific thus reducing the number of false positives.

Molecules That do not Contain Acidic or Basic Functional Groups

Molina et al [119] have determined organophosphorus pesticides in water by solid phase extraction followed by HPLC-ESMS using SIM of the $[M+Na]^+$ ion with a method detection limit of 0.01 $\mu g\ dm^{-3}$. Hydrocarbon carotenes are another example of a class of compounds that will not ionise under ESMS conditions. However, because of the low ionisation potential of the electrons in the conjugated chain of carotenoids, molecular ion radicals, $M^{\bullet+}$, have been generated for carotenes during ESMS by adjustment of the electrospray conditions to facilitate electrophoretic charging and electrochemical ionisation at the metal-liquid interface of the electrospray capillary [120]. Ionisation was enhanced by post-column addition of a halogenated oxidant (0.1% heptafluorobutanol).

Metabolism and Pharmacokinetics of Illicit Drugs

Because lysergic acid diethylamide (LSD) continues to be a popular drug of abuse in many countries, efforts to find more effective analytical methods for detecting LSD use also continue. Immunoassays are available for the determination of LSD in biological fluids [121,122]. They offer the advantage of speed and relatively low cost, but do not provide the specificity and quantitative accuracy of LC/MS [123, 124]. Also, Immunoassays are generally substantially higher than concentrations determined by mass spectrometric methods in the same samples, presumably due to cross-reactivity of the antibodies with LSD metabolites [122, 125].

The major difficulty in identifying an LSD user by analysis of body fluids is the very low concentration of LSD in blood and urine following ingestion of the drug. It is necessary either to develop analytical methods capable of reliably detecting and measuring LSD at substantially lower concentrations, or to identify a metabolite that is excreted in urine at higher concentrations for a longer time period.

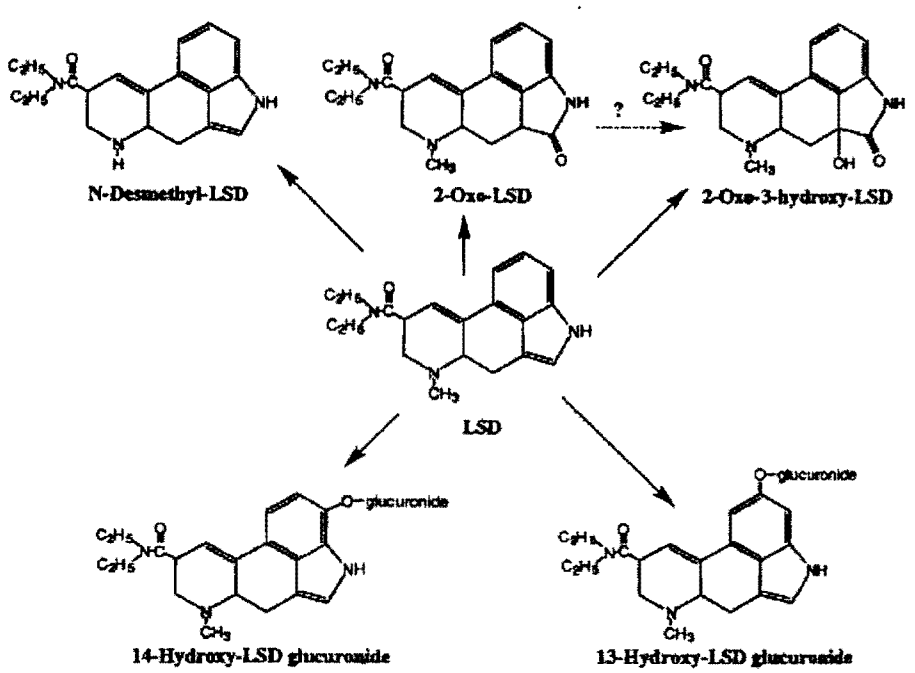
Because LSD is rapidly metabolized and only a small fraction of a dose is excreted in the urine as unchanged LSD, there is continued interest in identifying metabolites of LSD that may be detected in urine for a longer time period. The following five metabolites

have been identified in urine or blood from human users (**Figure 2-13)** *N*-demethyl LSD ("nor-LSD"), 2-oxo-LSD, 2-oxo-3-hydroxy-LSD, 13-hydroxy-LSD and 14-hydroxy-LSD [126,127]. The 13- and 14-hydroxy-LSD metabolites are excreted in urine as glucuronide conjugates [126]. 2-Oxo-LSD has been reported as the major human metabolite of LSD.

The first LC-MS assay to be used for routine analysis of forensic samples was described by Webb and co-workers [128,129]. This resulted in structurally significant fragment ions at *m/z* 281 and 223, which were monitored along with the MH^+ ions. The LOD for confirmation of LSD in urine was set at 1.0 ng/ml.

The most recently reported LC-MS assays for LSD have included analysis for 2-oxo-3-hydroxy-LSD which, as mentioned above, should permit determination of LSD use over a longer time period. Slawson and co-workers [130] evaluated an LC-MS system for determination of LSD, iso-LSD and 2-oxo-3-hydroxy-LSD in urine. ESI gave abundant protonated molecule ions (MH^+) for each of the analytes and internal standards. Quantitation was based on the MH^+ peak areas of the analytes relative to the MH^+ peak areas of the corresponding internal standards. Calibration curves were linear from 25 to 5000 pg/ml with correlation coefficients greater than 0.99.

Figure 2-13: Five known metabolites of LSD in human urine. [from reference 126]



In addition to increasing the applicability of LC/ESI/MS, major interest lies in achieving a better understanding of the ESI processes: the mechanism of ionization, as well as the effects of experimental parameters on the ions that are produced. Parameters that affect the ESI response produced include mobile phase pH, flow rate, solvent composition, analyte pK_a and concentration. As we see, these are the same parameters that affect the retention of basic analytes in HPLC also have an impact on the ESI response in LC/ESI/MS.

High Performance liquid Chromatography with electrospray ionization mass spectrometry detection (HPLC/ESI/MS) is a powerful routine analytical technique used for the analysis of drugs, peptides, DNA bases and other compounds in biological and other complex matrices. The liquid flow required for ESI-MS makes this technique an ideal ionization method for HPLC detection by MS. The advantages of an ESI/MS detection system in reversed phase HPLC-ESI-MS have been demonstrated [131-133]. The ESI process is not fully understood. However, recent improvements in HPLC/ESI/MS analysis have focused mainly on the MS instrument instead of fully understanding how the use of different acidic modifiers in the mobile phase affects the spray performance and the sensitivity of the analysis.

The majority of drug, peptides, DNA bases and other compounds in biological fluids analyzed in the pharmaceutical industry and clinical laboratories today contain ionogenic functional groups. These compounds are routinely analyzed by reversed phase HPLC. In order to achieve maximum selectivity and reduce band broadening due

to secondary equilibrium, high concentration of counter-anion is used to prepare the aqueous portion of the mobile phase. These mobile phase additives, although necessary for the separation and selectivity in HPLC, have a deleterious effect on the ESI sensitivity. Therefore, for optimal sensitivity, it will be necessary to work within a system that would accommodate both techniques.

The research presented in this dissertation focuses on addressing how the use of volatile acidic organic modifiers used in HPLC affects the electrospray ionization efficiency. The stationary phase used in all cases in this research was octadecylsilane (C18), which is one of the most commonly used stationary phases in RP-HPLC. The mobile phase used was comprised of various compositions of acetonitrile as the organic modifier. Our investigation utilized various acidic modifiers at different concentrations to demonstrate their affects on retention in HPLC and their affects on the overall electrospray ionization efficiency. It is our hope that the insight gained by these investigations will result in further understanding the effects of this important analytical technique.

Chapter-3

Results and Discussion - Effects of Acidic Modifiers in Reverse Phase HPLC

Summary

The objective of the first part of this dissertation was to investigate the effects of various volatile mobile-phase additives, mobile phase pH, analyte ionization constant, concentration of counter-anion concentration and solvent composition on the retention of a series of structurally related compounds. Mobile-phase additives are used to adjust the retention and selectivity of the analytes, to improve sensitivity and ultimately to improve chromatographic separation efficiency of structurally related compounds in a mixture.

Optimal conditions for chromatographic separations monitored by UV/VIS detection systems may not be the same as those for LC-ESI-MS analysis. In HPLC with ESI detection the analyst is restricted to the use of volatile organic modifiers. Therefore, the mobile-phase additives under investigation are suitable for reversed-phase chromatography, volatile and therefore deemed suitable for mass spectrometric detection. The less volatile salts, ammonium formate and ammonium acetate, were used to prepare aqueous buffers as mobile-phase additives to deduce secondary equilibria commonly associated with the retention of basic analytes in reversed phase chromatography. Either the addition of formic, acetic, propionic acids or ammonium hydroxide was used to adjust the pH of the aqueous portion of the mobile phase.

A wide range of basic analytes possessing pK_a values ranging from 4.6 to 10.5 was evaluated. Dependencies of capacity factor on the pH of the mobile phase were determined. The effect of the concentration of acetonitrile in the mobile phase on the dissociation constant and the retention of the analyte was studied. Experimental results obtained obeyed the predicted theoretical sigmoidal dependencies of capacity factor on the pH of the mobile phase. An addition of increased concentration of acetonitrile demonstrated the characteristic shifts of capacity factor versus the pH of the mobile phase, are in good agreement with the theory.

Introduction

Reversed phase liquid chromatography continues to be the method of choice for the analysis of basic compounds in the pharmaceutical research industry. The use of alkyl-bonded stationary phases such as C-8 and C-18 are most commonly employed for the separation of these compounds from their impurities and degradation products. These modified silica based columns have good chromatographic properties and neutral compounds can be successfully analyzed with high efficiency and reproducibility. However, separation of basic analytes on these systems can be quite challenging. The major drawback to using reversed phase columns for the analysis of basic analytes is that the analyte will interact with residual silanol groups on the stationary phase, resulting in distorted peak shape and relatively long retention time.

Suppression of the interaction between the analyte and residual silanol groups will improve the peak shape and enhance the efficiency of the separation. Successful

optimization of HPLC separations of ionizable compounds requires, in many instances, the use of mobile phase additives and organic solvents for the accurate measurement and control of the mobile-phase pH [134]. These two parameters (mobile phase additives and organic solvent) are interdependent because the pH value of the buffered aqueous component of the mobile phase varies with the composition of the organic solvent added.

Bosch et al [135], have derived equations that allow calculation of the pH value of some of the most commonly used HPLC buffers for any methanol-water composition and that relate the retention of a weak acid with the pH value of the buffer and solvent composition [7]. The authors have also shown that the use of the pH value of the buffer in the particular methanol-water mixture that constitutes the mobile phase gives better relationships with the solute retention than the traditional method that uses the pH value of the buffer in water. The effects of different volatile acidic modifiers in various compositions of aqueous buffered-acetonitrile on the retention of basic analytes are presented.

Experimental

High-Performance Liquid Chromatography - Instrumentation

The retention data for the solutes were measured using isocratic conditions with a flow rate of 1 mL/min. Zorbax Eclipse XDB-C18, (Hewlett Packard, Little Falls, DE), 150 x 4.6 mm id., particle diameter 5μm column was used in all studies. The chromatographic equipment consisted of a Perkin Elmer model 250 binary pump, an ISS 100 autosampler

with a 10-μL loop valve. (Perkin Elmer, Norwalk, CT) was used. A Kratos spectroflow 783 Programmable Absorbance Detector set at 254 nm was used to monitor the retention time of each analyte. The chromatographic data acquisition and analysis was performed using Turbochrom-4 (Perkin Elmer, Norwalk, CT).

The retention times of these solutes were obtained by averaging the results of three independent injections. All experiments were performed at ambient temperature, which was approximately 25 ^{0}C. pH measurements of each aqueous buffer solution was performed using a Beckman μ40 pH meter. The pH meter was calibrated with pH 1.0, 2.0, 4.0 7.0 and 10.0. A test mixture of aniline and 2,6-Lutidine was used as a system suitability check before and after each set of experiments to determine the stability of the column and system performance.

Reagents

HPLC grade acetonitrile was obtained from Sigma Chemical Co. (Milwaukee, WI). For mobile phase additives formic acid, acetic acid, propionic acid, ammonium acetate and ammonium formate were purchased from Sigma-Aldrich (Milwauke, WI). The following basic analytes were used as controls: aniline, pyridine, 2,6-dimethyl pyridine and 3,4-dimethyl pyridine Sigma-Aldrich (Milwauke, WI). The anti depressants: promipramine, desipramine, imipramine and trimipramine were purchased from Sigma-Aldrich (Milwaukee, WI).

Sample Preparation

For the pH dependency study, a stock solution of 10 mM ammonium acetate buffer was prepared. The initial pH of this solution was 6.24. This solution was used to prepare various mobile phase compositions. The pH of the aqueous portion of each mobile phase composition was either acidified with acetic or formic acid, or basified with ammonium hydroxide. The pH was measured before the addition of acetonitrile and ranged 1.02 – 8.16. All aqueous mobile phase was filtered under vacuum using nylon 66-membrane filter obtained from Fisher Scientific (Pittsburgh, PA).

Stock solutions (1 mg/mL) of all analytes were prepared in acetonitrile. Test samples were prepared by further diluting each stock solution to prepare 1 μg/mL in various mobile phase compositions. Samples were stored at 4 ^{0}C when not in use. The t_0 value obtained for the Zorbax XDB-C18 column with the minor disturbance method at 25 ^{0}C was 1.40 minutes. A test mixture of aniline and 2,6-Lutidine was used as a system suitability check before and after each experiment to determine the stability of the column and system performance.

Data Analysis

The theoretical model describing the dependence of retention factor, k', on the pH of the mobile phase in reversed phase chromatography has been derived by taking into

account the ionization equilibria of the compound. The tricyclic antidepressants studied contain two ionizable nitrogen groups. A search of the literature provides only one pK_a value for each analyte.

To determine which nitrogen group was associated with the quoted pK_a value, the electrostatic potential of each nitrogen group on the molecule was determined using the Spartan software (Irvine CA.) and the results are shown in **Table 3-1**. The electrostatic potential is defined as the energy of interaction of a free proton with a molecule. The magnitude of the sign of the energy is an indication of the molecules susceptibility to electrophilic attack [136]. A region of the molecule where the electrostatic potential is comparatively large and negative is a region susceptible to electrophilic attack. A region with a large positive energy is subject to a nucleophilic attack.

The proton is placed on the electron density isosurface at the van der Waals radii of the molecule and the energy of the proton is calculated. The calculated electrostatic potential generated is representative of the relative basicity of atoms on the molecule. The proton is under vacuum, therefore the initial energy is zero. Apart from the numerical calculated electrostatic potential generated, the data is displayed by drawing the positive and negative isovalue surface partitions of the molecule into regions subject to electrophilic and nucleophilic attack. Figure 4-1 shows the electrostatic potential for imipramine and protriptyline. The blue surface envelops the negative electrostatic potential region and the red envelops the positive electrostatic potential region.

The pK_a values for the solutes were also calculations using ACDlab software (Advanced Chemistry Development Inc., Toronto, Ontario, Canada) software. The pK_a values for both nitrogen atoms, analyte structure and molecular weight are (shown in **Table 3-2**).

Results and Discussion

Chromatographic Parameters

In chromatography, the distribution constant (K) is usually determined in terms of the ratio of the analyte concentration in the stationary phase and the mobile phase as:

$$K_c=C_s/C_m \qquad [3\text{-}1]$$

Determination of the distribution requires measurements of mobile phase and stationary phase volumes, which are very complicated. Therefore retention parameters such as retention factor (k), adjusted retention time (t'_R), and retention time (t_m) are often used.

The retention factor (k) is given as:

$$K=t'_R/t_m \qquad [3\text{-}2]$$

The t_m value was determined for the Zorbax XDB-C18 150 x 4.6 mm column by the minor disturbance method, at 25 ^{0}C to be 1.40 minutes.

Table 3-1: Electrostatic Potential Showing the Relative Basicities for Nitrogen Groups in Antidepressants

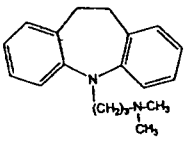
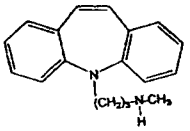
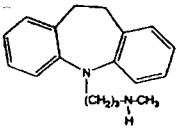
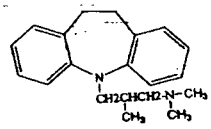
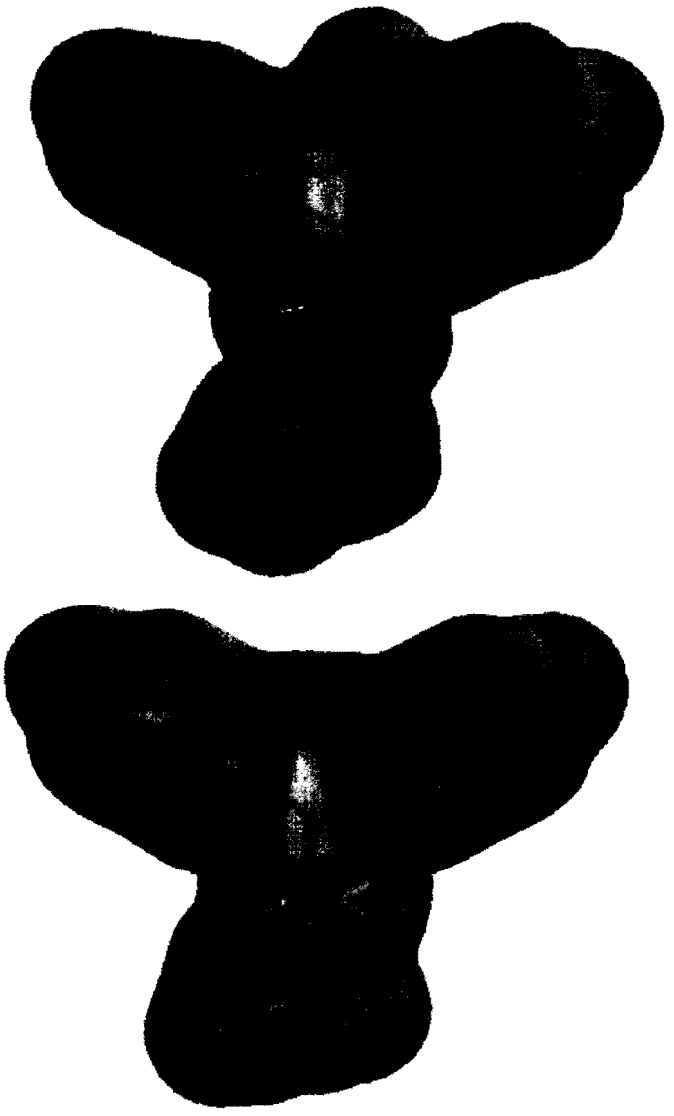
Analyte	Chemical Structure	Molecular wt	Electrostatic Potential	
			Aliphatic "N" Atom	Aromatic "N" Atom
Imipramine		280.4	-66.3	-33.2
Protriptyline		263.4	-70.9	-48.6
Desipramine		266.4	-67.3	-34.3
Trimipramine		294.4	-69.1	-32.5

Figure 3-1: Display of Comparison of Electron Density for Imipramine and Protriptyline



Imipramine
pKa = 9.5

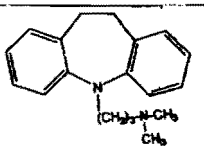
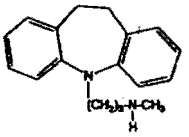
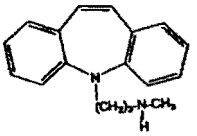
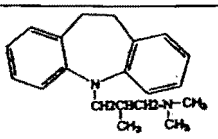
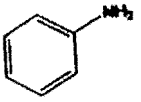
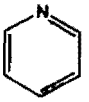
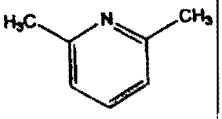
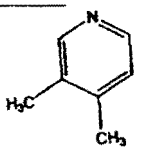
Protriptyline
PKa =8.2

The retention factor, k', for any ionizable solute as a function of mobile phase pH can be expressed by considering that the observed k'. The observed k' is a weighted average of the k' for the ionized and neutral forms of the solute according to the mole fraction of these forms in the mobile phase [17]. Hovarth *et al* have described the sigmoidal dependence of the ionizable analyte and have proposed that the interaction between the analyte and the solvent is controlled by their hydrophobicity. Deviation from the theoretical sigmoidal model may be evidence of non-hydrophobic interactions with the solvent [137].

The basic solutes chosen for this study cover a wide range of pK_a constants and hydrophobicity. A list of the studied analytes, their chemical structure, molecular weight and pK_a values are listed in **Table 3-2.** The stationary phase chosen is C-18, which is by far the most commonly used sorbent support used in reverse phase chromatography. Mixed acetonitrile-ammonium acetate or ammonium formate buffers have been chosen as mobile phases in this experiment because of their volatility and their appropriate use in LC-ESI-MS.

Experiments were carried out to determine the retention factor for all analytes studied. The pH of the mobile phase ranges from 1.02 to 8.16. The octadecylsilane, ODS, stationary phase use allowed for a pH limit of 8.5, therefore pH dependency studies were limited to a pH value less than pH 8.5. The mobile phases used were various mixtures of acetonitrile-water, with the composition of acetonitrile of 20, 30, 40 and 50 % (v/v).

Table 3-2: Structures and Ionization Constants of Basic Analytes

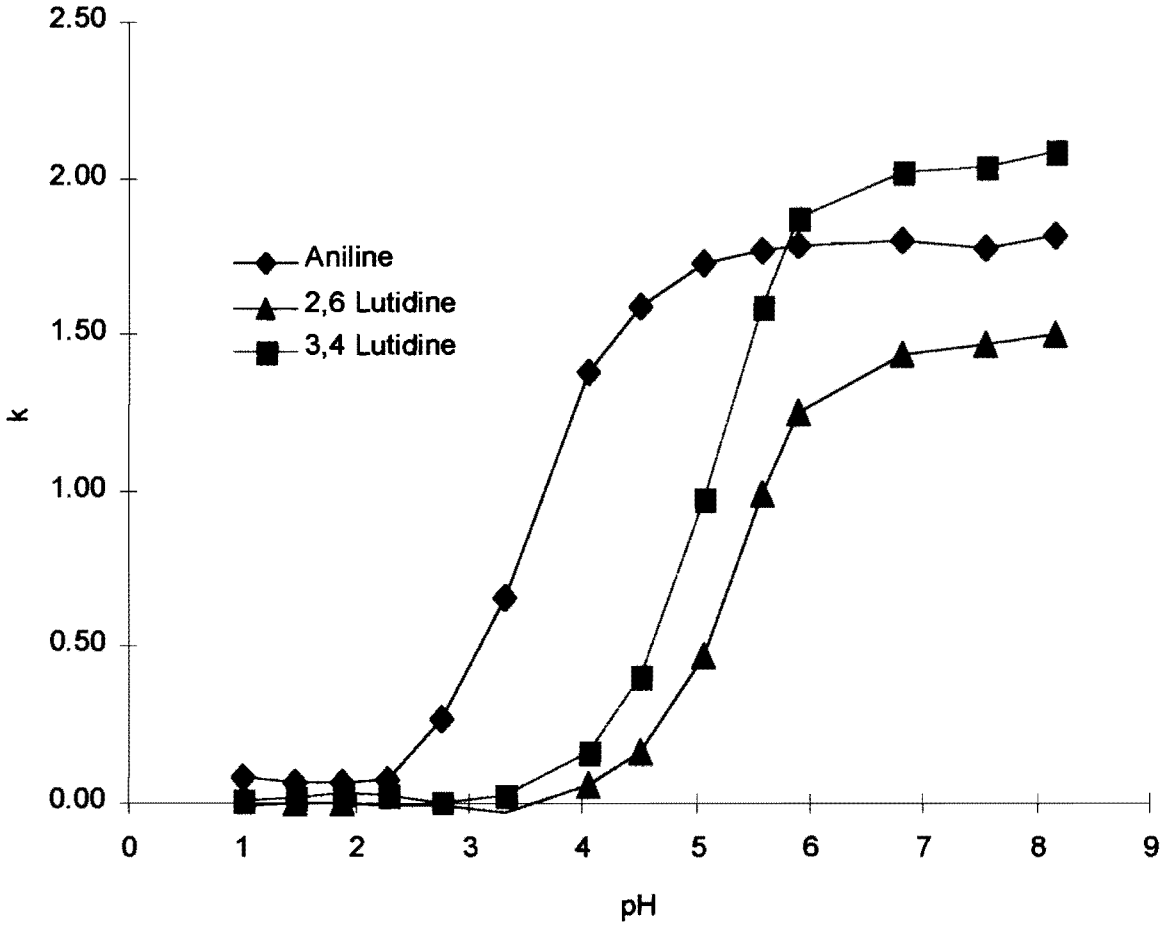
Analyte	Chemical Structure	Molecular wt	Literature pK_a Values	AcD pK_{a1} Values	AcD pK_{a2} Values
Imipramine		280.4	9.5	9.5	1.3
Desipramine		266.4	10.2	10.4	1.3
Protriptyline		263.4	10.4	10.4	0.7
Trimipramine		294.4	10.5	9.4	1.0
Aniline		93.1	4.60	4.6	-
Pyridine		79.1	5.17	5.3	-
2,6-Lutidine		108.1	6.71	6.67	-
3,4-Lutidine		108.1	6.47	6.2	-

Study 1 – Effect of pH on retention factor

The retention factor of each analyte was measured as a function of the pH of the aqueous portions of the mobile phase. Plots of the retention factors for several basic control analytes and antidepressants versus pH are shown in ***Figure 3-2*** and ***Figure 3-3*** respectively. These figures show the expected sigmoidal dependence of retention factor versus pH. The inflection point of the retention dependency on the mobile phase pH represents the analyte's apparent pK_a value under a given set of chromatographic conditions [17]. As can be seen in ***Figure 3-2***, the retention factor for aniline, at pH above 5.5 shows a plateau effect. This area of the sigmoidal curve represents aniline in its neutral form.

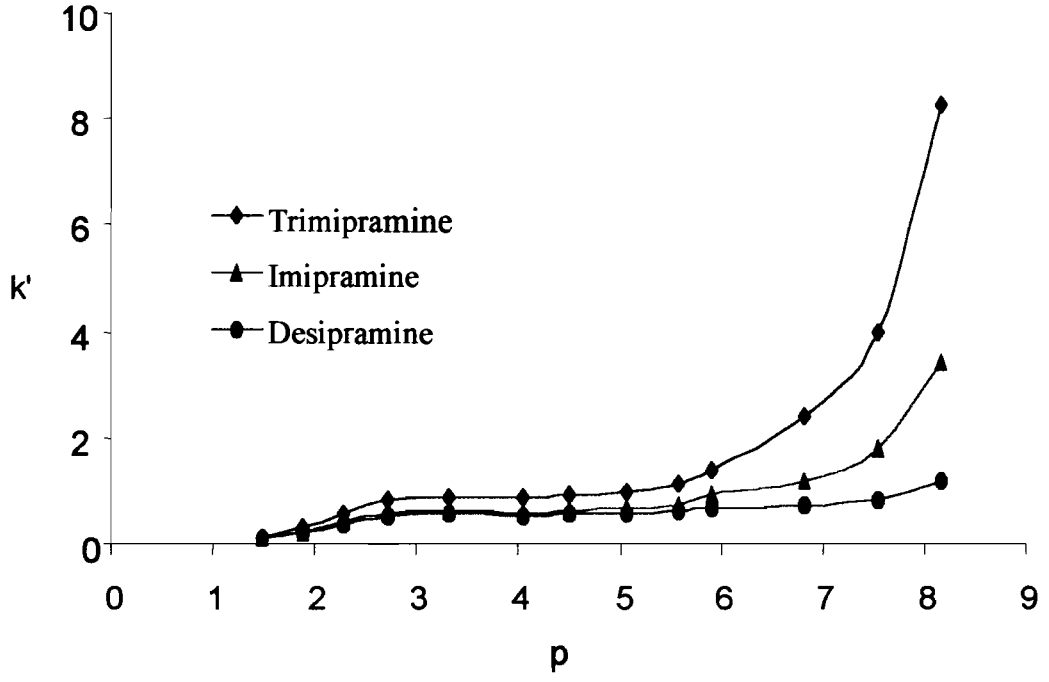
The data generated from the retention dependencies of the analyte versus pH of the mobile phase was used to calculate the apparent pK_a of the analyte under a given set of mobile phase conditions. This was accomplished by using MathCad non-linear regression analysis. A list of the theoretical and apparent pK_a values is shown in Table 2. Aniline has an aqueous pK_a value of 4.6. The observed pK_a shift was due to the influence of the organic modifier (30 percent acetonitrile) in the mobile phase [22]. In the pK_a region of the curve, small changes in the pH resulted in the greatest change in the retention factor. Below pH 2.0 with further addition of acid, the plateau effect was once again observed. This region represents the retention of the fully ionized basic analyte. In 100 % aqueous conditions a basic analyte is fully protonated at 2 pH units below the pK_a value. Here we notice that aniline is fully protonated at approximately pH 2.0

Figure 3-2: Retention Factor Dependencies of Control Basic Analytes as a Function of Mobile Phase pH



Chromatographic Conditions: column: 15x0.46 cm Zorbax XDB-C-18; mobile phase: acetonitrile-10 mM ammonium acetate buffer pH adjusted with acetic acid. PH =1.38 – 8.16 (30:70); flow rate, 1.0 mL/min; 254 nm; 2 μL injection volume.

Figure 3-3: Retention Factor Dependencies of Tricyclic Antidepressants as a Function of Mobile Phase pH



Chromatographic Conditions: column: 15x0.46 cm Zorbax XDB-C-18; mobile phase: acetonitrile-10 mM ammonium acetate buffer pH adjusted with acetic acid. PH =1.38 – 8.16 (30:70); flow rate, 1.0 mL/min; 254 nm; 2 μL injection volume

The apparent chromatographic pK_a values determined in eluent systems modified with either acetic or formic acid were compared with literature values (***Table 3-2***). As shown in ***Table 3-2***, the chromatographic pK_a's determined in aqueous/organic mixtures are lower than those measured by potentiometric titration. This was attributed to an actual increase in the mobile phase pH upon the addition of the acetonitrile and/or the suppression of the pK_a of the basic compound.

The pK_a values calculated for the basic control analyte studied in 30/70 acetonitrile/buffer mobile phase were about 0.8 – 1.4 pH units less than their literature values. The apparent pK_a shift for 2,6-Lutidine ranged from 1.3 – 1.4 which was consistently higher than the other control analytes. When the apparent pK_a's are corrected for the effect of the acetonitrile composition, there are still some discrepancies in the apparent pK_a values for these analytes. This may be attributed to steric effects or shielding of the nitrogen atom by the methyl groups on the molecule.

For Pyridine these effects were more dramatic. This may be attributed to accessibility of the nitrogen atom on the molecule. Similar results were obtained by [30,138,139]. The author attributed this effect to the interaction of pyridine with the residual silanol groups.

The Zorbax-C18 XDB stationary phase use allowed for a pH limit of up to 8.5, therefore pH dependency studies for the tricyclic antidepressants were limited to a pH value less than pH 8.5. **Figure 3-3** shows two inflection points were observed for each of

the antidepressants studied. The inflection points represent the two ionizable nitrogen groups in the molecule. From the electrostatic potential studies we are confident that the inflection points at pH >8.5 represent the nitrogen group on the alkyl side chain, and the inflection point at pH ~ 1.75 represent the less basic nitrogen within the cyclic part of the molecule.

Table 3-3: Comparison of Titrimetric and Chromatographic pK_a Values using Ammonium Acetate and Ammonium Formate Buffer in 30 % Acetonitrile

Analyte	Titrimetric pK_a Values	Chromatographic pK_a Values	
		NH_4 Acetate	NH_4 Formate
Aniline	4.61	3.55	3.80
Pyridine	5.17	3.91	4.31
2,6-Lutidine	6.71	5.32	5.47
2,4-Dimethylaniline	4.89	3.86	3.99
4-tert-Butylpyridine	5.99	4.94	5.01

Study 2 - Effect of Acetonitrile Concentration on Apparent pK_a

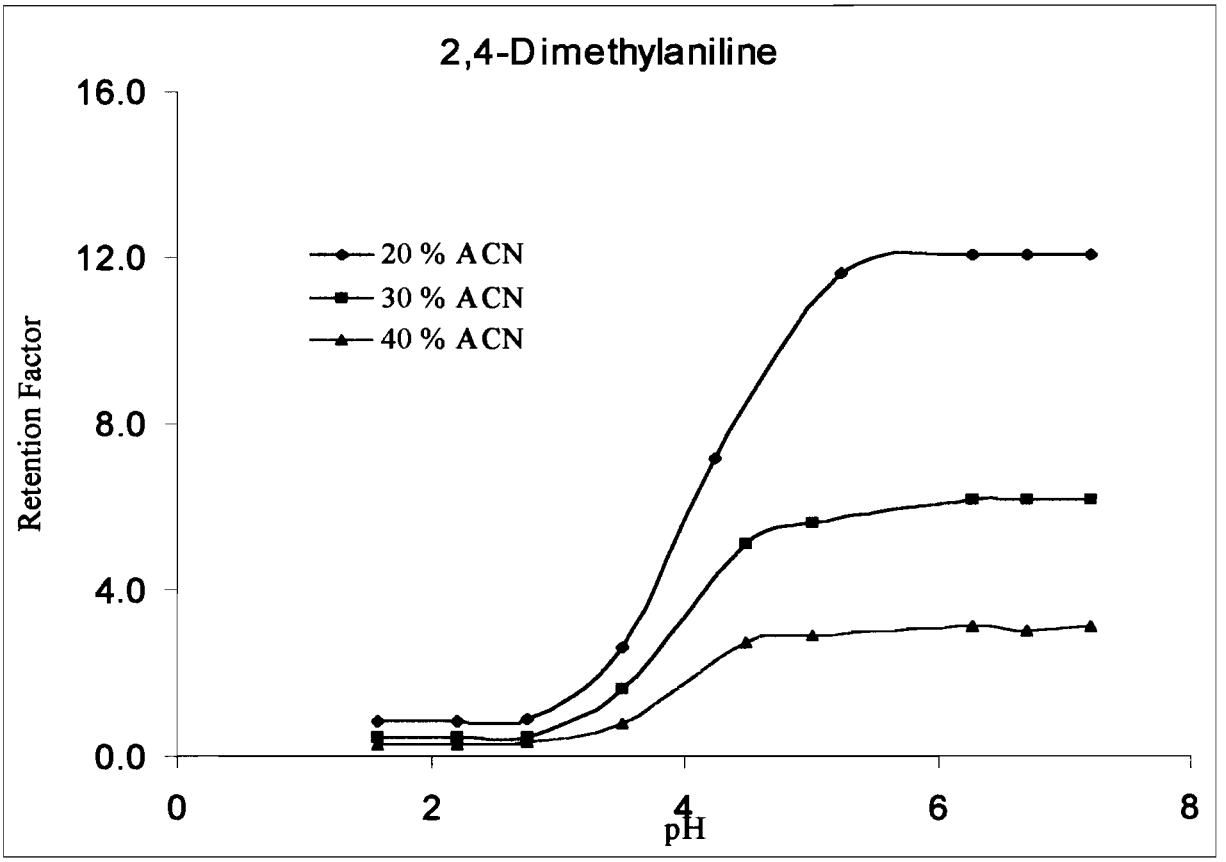
To further investigate the relationship between the composition of acetonitrile in the mobile phase on the ionization and the solvation constants of these analytes a second study was conducted. The aqueous portion of the mobile phase used comprised of 1 mM of ammonium formate and the pH was acidified with formic acid or basified by the addition of ammonium hydroxide. The pH of the aqueous portion of the mobile phase ranged from 1.58 to 8.16 and was determined prior to the addition of acetonitrile.

The apparent pK_a of the control analytes studied were measured at 10, 20, 30, 40 and 50 percent buffer-acetonitrile mixtures. The analytes apparent chromatographic pK_a values were determined in eluent systems described above and compared to literature values, and are shown in **Table 3-3**. The apparent pK_a values of the analytes studied decreased linearly by approximately 0.3 pH units with the addition of every 10 % of acetonitrile added to the mobile phase. Again, this is attributed to an actual increase in the mobile phase pH upon the addition of acetonitrile. A plot of the apparent pK_a values for 2,4-Dimethylaniline under different mobile phase compositions is shown in **Figure 3-4**. The observed shifts in the sigmoidal curve of retention factor versus the pH of the mobile phase are related to the influence of the organic modifier concentration on the dissociation of the basic analyte.

Table 3- 4: Chromatographic pK_a Determination of Basic Analyte with Increasing Percent Acetonitrile in Mobile phase

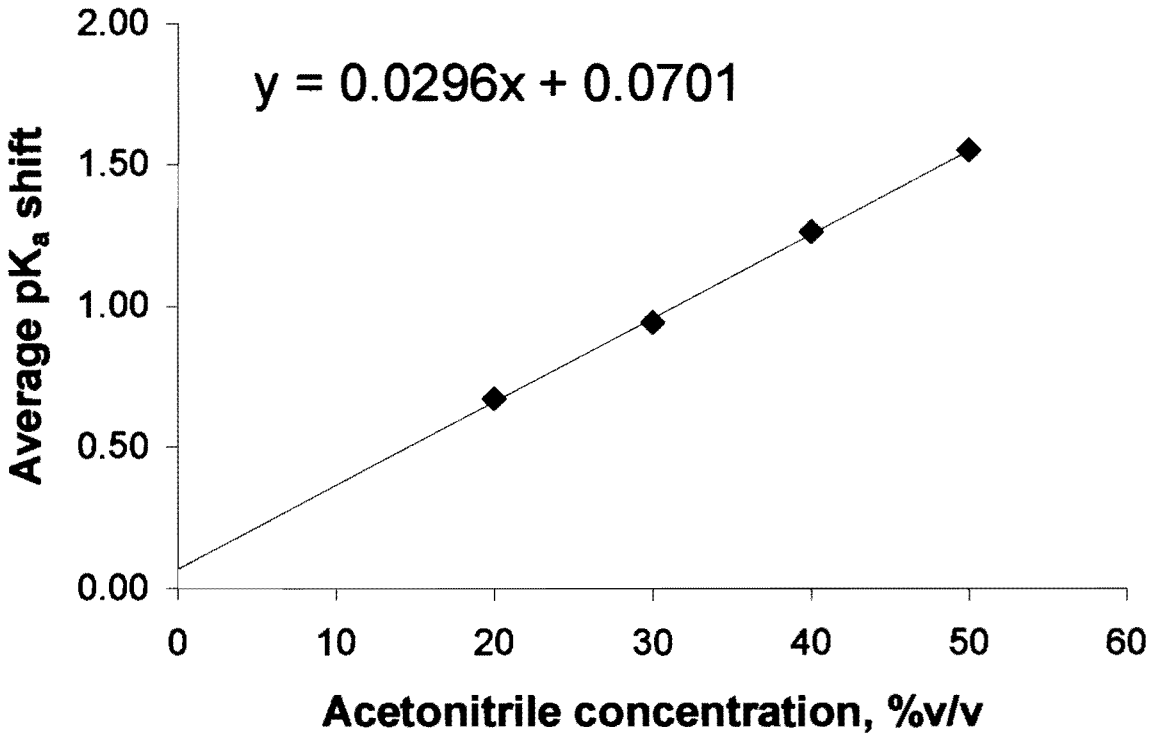
		Percent Acetonitrile			
Analyte	Titration	20	30	40	50
Aniline	4.60	3.90	3.64	3.40	3.15
Pyridine	5.17	4.24	3.97	3.46	3.18
3,4-Lutidine	6.47	6.01	5.90	5.43	5.19
2,6-Lutidine	6.71	6.04	5.84	5.61	5.23
2,4-Dimethylaniline	4.89	4.24	3.94	3.64	3.36
4-tert-Butylpyridine	5.99	5.37	4.89	4.72	4.42

Figure 3-4: Dependence of the Chromatographic pK_a Shift for 2,4-Dimethylaniline as a Function of Organic Modifier Concentration in the Mobile Phase.



Chromatographic Conditions: column: 15x0.46 cm Zorbax XDB-C-18; mobile phase: acetonitrile-10 mM ammonium acetate buffer pH adjusted with acetic acid. PH =1.38 – 7.5 with 20, 30, & 40 % acetonitrile; flow rate, 1.0 mL/min; 254 nm; 2 µL injection volume.

Figure 3-5: Dependence of Average Chromatographic pK_a Shift for Six Basic Analytes on the Organic Concentration in the Mobile Phase.



Study 3 - Effect of Counter-anion Concentration on Retention Factor

Based on the difference in dissociation constants of the weak volatile acids used to adjust the pH of the buffered aqueous phase in the previous study, we expect the concentration of the counter anion in the mobile phase to change with further addition of acid. The negatively formed counter anion of the acid will interact with the positively charged basic analyte in the low pH region and may form ion-associated complexes. The effects of the increase in counter-anion concentration on the retention of basic analyte were studied.

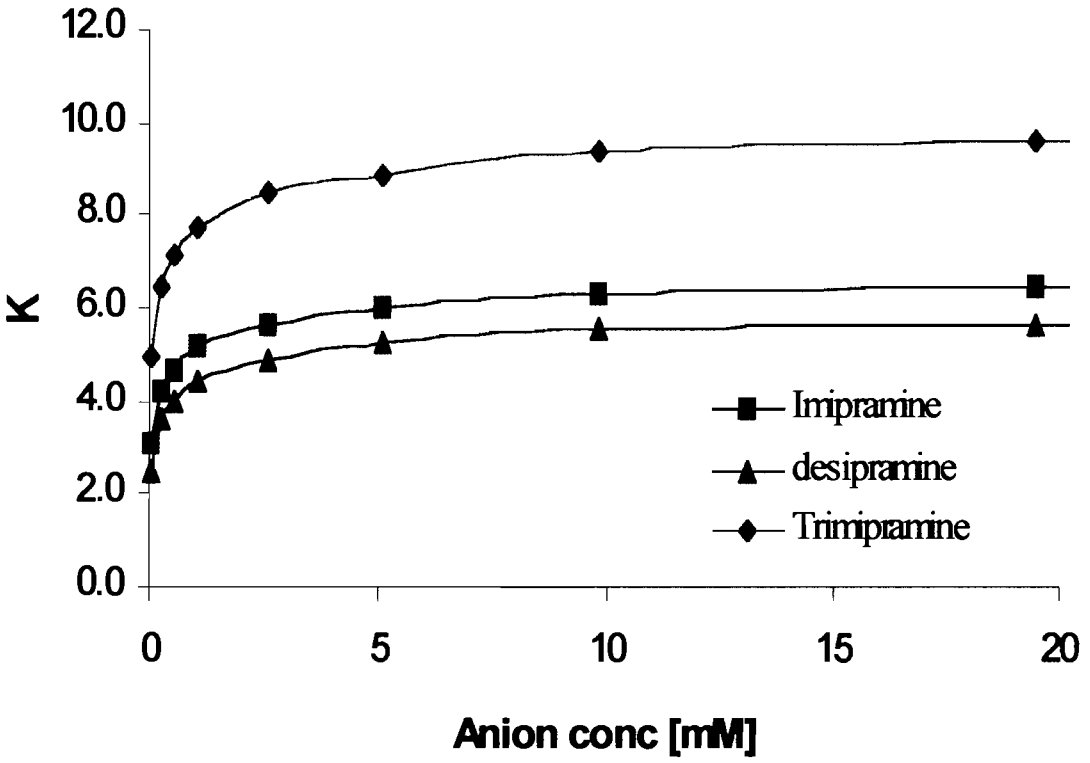
Based on the chromatographic pK_a values determined and the electrostatic potential determined by the Spartan software, it was confirmed that there are two pK_a values for the antidepressants studied. From the retention factor dependency on pH (Figure 3-3), we know that at pH 4, the nitrogen group outside the tricyclic group will be fully protonated and the nitrogen group within the tricyclic group will be in its neutral form. Therefore we chose pH 4 to study the effects of counter-anion concentration on the retention factor of these analytes.

This experiment was performed at pH ranging from 4.02 to 4.05, with an increase in acetate counter-anion concentration. The mobile phase composition of 70/30 aqueous buffer acetonitrile mixture was used. The counter-anion concentration was changed by addition of the ammonium acetate salt. Due to the dissociation of the ammonium salt, which resulted in an introduction of ammonium in the mobile phase, there was a slight

increase in the pH of the aqueous portion of the mobile phase. The pH was maintained at 4.02 ~ 4.05 by the addition of a small volume (~3-5 μL) of acetic acid. The total anion concentration was calculated from the summation of anions generated from both the acid and the salt form.

The retention dependencies for three antidepressants are shown in **Figure 3-6**. At low counter-anion concentration, an increase of the analyte retention is more pronounced since at that stage the analyte desolvation rate is the greatest. When concentration of the counter-anions in the mobile phase is high enough for complete desolvation of all analyte molecules the analyte retention remained constant with further increase of acidic modifier (plateau effect). **Figure 3-6** shows that, for most studied analytes, complete desolvation was achieved at 10-20 mM of acetate counter-anion in the mobile phase.

Figure 3-6: Retention Factor Dependencies of Tricyclic Antidepressants at pH 4 with Increase in Ammonium Acetate Counter-anion in the Mobile Phase.



Chromatographic Conditions: Column: 15x0.46 cm Zorbax XDB-C-18; mobile phase: acetonitrile:aqueous pH adjusted with acetic acid (30:70); flow rate, 1.0 mL/min; 254 nm; 2 µL injection volume.

It has been shown [45], that different acids will affect the retention of basic analytes to different degrees in the low pH region due to the formation of ion-association complexes. The interaction changes the retention profile of the basic analyte due to changes in the charge density, polarizability and solvation. Counter-anions that have less localized charge, high polarizability and a lower degree of hydration usually result in a greater effect in the retention of basic analytes. This is known as the chaotrophic ion [42]. The chaotrophic ions are known to change the arrangement (hydrogen bonding) of water molecules to more disordered form [44]. Since perchloric acid is a known chaotrophic ion, we compared the effects of perchlorate, acetate and formate counter anions on the retention dependencies of the studied analytes.

This study was conducted using increasing concentrations of counter anions in the mobile phase. The mobile phase composition was again set at 70/30, aqueous buffer/acetonitrile at pH 4. When sodium perchlorate was used, no further addition of acid was needed to maintain the pH value. However, when ammonium acetate and ammonium formate were used, addition of either acetic or formic acid was added to maintain a constant pH value.

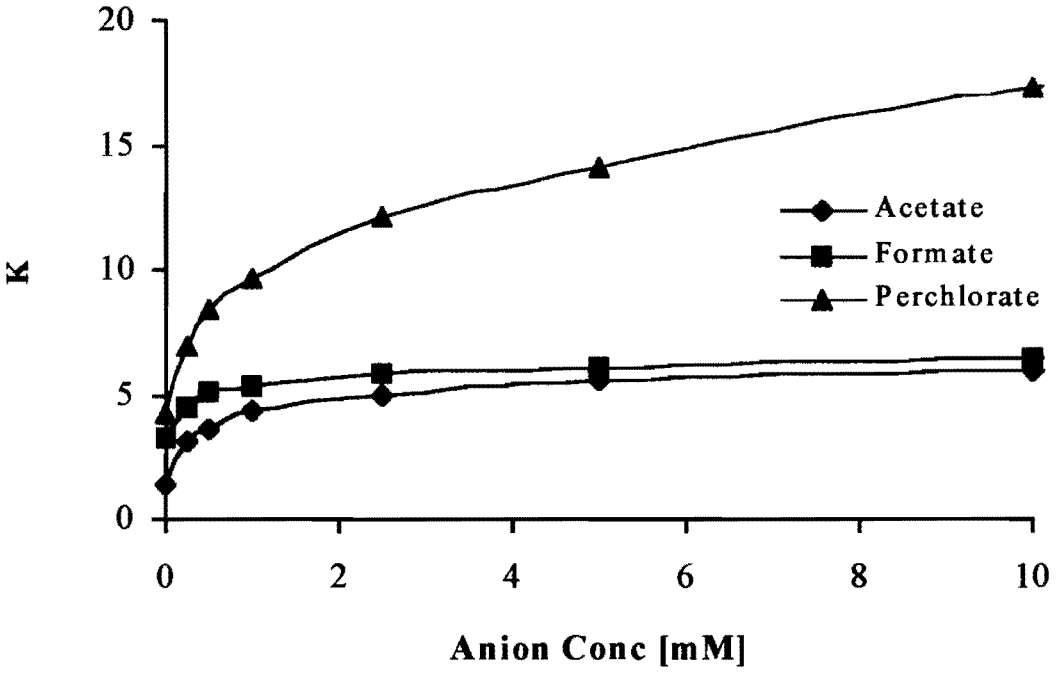
The effects of the retention dependencies of the protonated bases were compared using acetate and formate counter-anion to a strong inorganic counter-anion such as perchlorate **Figure 3-7**. The differences in the retention dependencies of the analytes may be explained in terms of the relative strength of the chaotropic counter-anion formed in solution. Perchlorate counter-anion has a single negative charge that is delocalized over

four oxygen atoms and is highly unlikely to form hydrogen bonds. Acetate and formate counter-anions are weaker counter-anions bearing a single negative charge delocalized over two oxygen atoms. Formate counter-anion is a slightly stronger counter-anion than acetate. Compared to acetate, formate has a single hydrogen atom, which is electron withdrawing compared to the electron donating properties of the methyl group of acetate counter-anion.

A mathematical model developed by Kazakevich et al [55] for the description of the influence of counter-anion concentration on analyte retention was applied to the data obtained from the previous study. The equation used for the description of the model was detailed in Chapter 2 of this dissertation. The authors suggest solvation-desolvation equilibrium for the description of the counter-anion concentration effect on the retention of basic analytes in HPLC.

The retention dependencies of the basic analytes on the concentration of perchloric, formic and acetic counter-anions are shown in **Figure 3-7**. The retention factor for each analyte as a function of counter anion concentration was fitted to a nonlinear regression program, MathCad 8. This data treatment allowed for the calculation of the desolvation parameter, K, and the limiting retention factors k_s and k_{us}, which represent the solvated and the unsolvated forms respectively. The calculated desolvation parameters are shown in **Table 3-5.** As shown in **Table 3-5**, the use of perchlorate counter anion, resulted in a greater fraction of the unsolvated form of the analyte.

Figure 3-7: Retention Factor Dependencies of Imipramine at pH 4 with Increasing Concentration of Acetate, Formate and Perchlorate Counter-anion in the Mobile Phase.



Chromatographic Conditions: Column: 15x0.46 cm Zorbax XDB-C-18; mobile phase: acetonitrile:aqueous pH adjusted with acetic acid (30:70); flow rate, 1.0 mL/min; 254 nm; 2 µL injection volume.

Table 3-5 Comparison of Retention Factor and Solvation Parameters of Basic Analytes using Different Acid Modifiers

Solvation Parameters	Aniline	2,6-Lutidine	3,4-Lutidine	Imipramine	Desipramine	Protriptyline	Trimipramine
			Perchlorate Counter-anion				
k_s	0.911	0.028	0.031	6.323	5.608	5.425	9.252
k_{us}	1.068	0.274	0.361	28.865	24.978	24.33	42.567
K_c	4.06	0.207	0.716	0.106	0.112	0.125	0.103
			Formate Counte-anion				
	Aniline	2,6-Lutidine	3,4-Lutidine	Imipramine	Desipramine	Protriptyline	Trimipramine
k_s	0.679	-0.074	-0.067	2.546	2.319	2.213	3.736
k_{us}	1.275	0.013	0.147	6.657	5.808	5.915	9.731
K_c	12.321	1.179	2.538	2.84	2.222	2.909	3.312
			Acetate Counter-anion				
	Aniline	2,6-Lutidine	3,4-Lutidine	Imipramine	Desipramine	Protriptyline	Trimipramine
k_s	1.031	-0.069	-0.075	1.002	0.917	0.861	1.58
k_{us}	1.391	0.026	0.141	6.263	5.455	5.57	9.21
K_c	2.356	1.053	9.366	1.611	1.672	1.521	1.566

Conclusion – HPLC

The retention dependencies of basic analytes on the pH, composition of organic solvent and counter anion concentration were measured. A plot of the retention dependencies of the retention factor versus the pH of the mobile phase showed the predicted theoretical sigmoidal curve. The measured chromatographic pK_a values of studied analytes all showed an apparent decrease upon further increase of acetonitrile in the mobile phase.

The increase in concentration of acetonitrile led to an increase in the pH of the mobile phase. This increase in the pH of the mobile phase reduced the analyte to the less ionized form. Therefore changes in the pH upon addition of acetonitrile in the mobile phase resulted in decreased apparent pK_a shift of the basic analyte. The change in the apparent pK_a is approximately 0.3 pH units for every 10 % of acetonitrile added to the mobile phase.

An increased concentration of acetate, formate and perchlorate counter anions to the mobile phase disrupt the analyte solvation shell to different degrees. The desolvation effect is more pronounced at the low counter anion concentrations. Further increasing the counter anion concentration will increase the retention of the basic analytes. Once the analyte is fully desolvated the retention factor levels off.

Chapter-4

Results and Discussion - Effects of Acidic Modifiers on LC/ESI/MS

Summary

The effects of various volatile and acidic mobile-phase additives on parameters pH, analyte pK_a, and concentration of organic on electrospray ionization mass spectra of a series of structurally related antidepressants were studied in positive ion mode. Particularly, the response of the $[M+H]^+$ ions observed upon addition of formic, acetic, propionic, ammonium formate and ammonium acetate were studied. The sensitivity as $[M + H]^+$ of the test analytes at various pH values were dependent upon their ionization in the mobile phase. At a constant pH, where the analyte was fully protonated in the mobile phase the $[M+H]^+$ ion decreased with increasing concentration of counter-anion. A relative $[M+H]^+$ increase with increasing pK_a value of the analyte was observed.

The utility of mobile phases containing of 80 mM ammonium formate adjusted to pH 2.6 with formic acid at 60/40 (buffer / acetonitrile) was demonstrated for chromatographic separations. Because acetonitrile provides better desolvating properties than aqueous buffers, an increase in the concentration of acetonitrile in the mobile phase resulted in an increase of ESI/MS intensity.

Introduction

The objective of the second part of this dissertation was to systematically investigate the effects of various mobile-phase additives, solution pH, ionization constant, and concentration of acetonitrile on the ESI mass spectra and molar responses (or sensitivities) of a series of structurally related compounds. Mobile-phase additives are used to improve chromatographic separations of complex mixtures, to increase the solubilities of the analytes, to improve the ESI responses of the analytes, or to improve ESI performance. Optimal conditions for chromatographic separations may not be the same as those for ESI-MS analysis.

Although HPLC/MS is well suited for these analyses, most previously reported LC conditions are not compatible with ESI mass spectrometry, because they require complex mobile phases containing nonvolatile buffers or ion-pairing reagents to improve chromatographic resolution. These complex mobile phases result in clogging at the interface, buildup of deposits in the ion source, and drastic reduction in ion production. The mobile-phase additives under investigation are suitable for reversed-phase chromatography, volatile and therefore deemed suitable for mass spectrometric analysis.

A large number of basic pharmaceuticals have been developed and are currently being used for a variety of physiological therapies. Some of these drugs include tricyclic antidepressants such as imipramine and desipramine, which have been used for the treatment of major depression for over 30 years. A large number of HPLC methods have

been developed mainly for the therapeutic monitoring of the compounds in biological systems [140-142]. These methods have been characterized by poor sensitivity for both imipramine and desipramine with detection limits of 10-50 ng/ml.

Other HPLC methods employing the use of electrochemical detection have been described [143] resulting in an improved sensitivity of 1 ng/ml for imipramine and desipramine. Chen *et al* [144] have separated imipramine, desipramine and their 2 and 10-hydroxylated metabolites in human plasma and urine using phosphate buffer at pH 6, resulting in a detection limit of 3 ng/mL for the parent drug.

Due to the importance of regulatory constraints for greater sensitivity and structural elucidation of drug metabolites in pharmacokinetic and toxicokinetic studies LC/MS is becoming an invaluable tool in the pharmaceutical industry. Antidepressants and other basic compounds and their metabolites can be quantitatively analyzed with structural identification by LC/MS, however the choice of mobile phase additives imposes certain limitations on the ESI ionization efficiency. The use of the buffer systems described earlier for the analysis of these antidepressants employs inorganic buffers and is not suitable to be used in LC/MS. Therefore HPLC methods utilizing the appropriate volatile buffers suitable for LC/MS should be examined.

In this investigation we demonstrate the effects of different acidic modifiers, pH, ionization constant and the composition of acetonitrile used in the mobile phase on ESI

efficiency of basic analytes in LC-ESI-MS. These mobile phase additives are generally used to improve separation of basic analytes and their metabolites in biological matrices.

Mass Spectrometry and Sample Introduction.

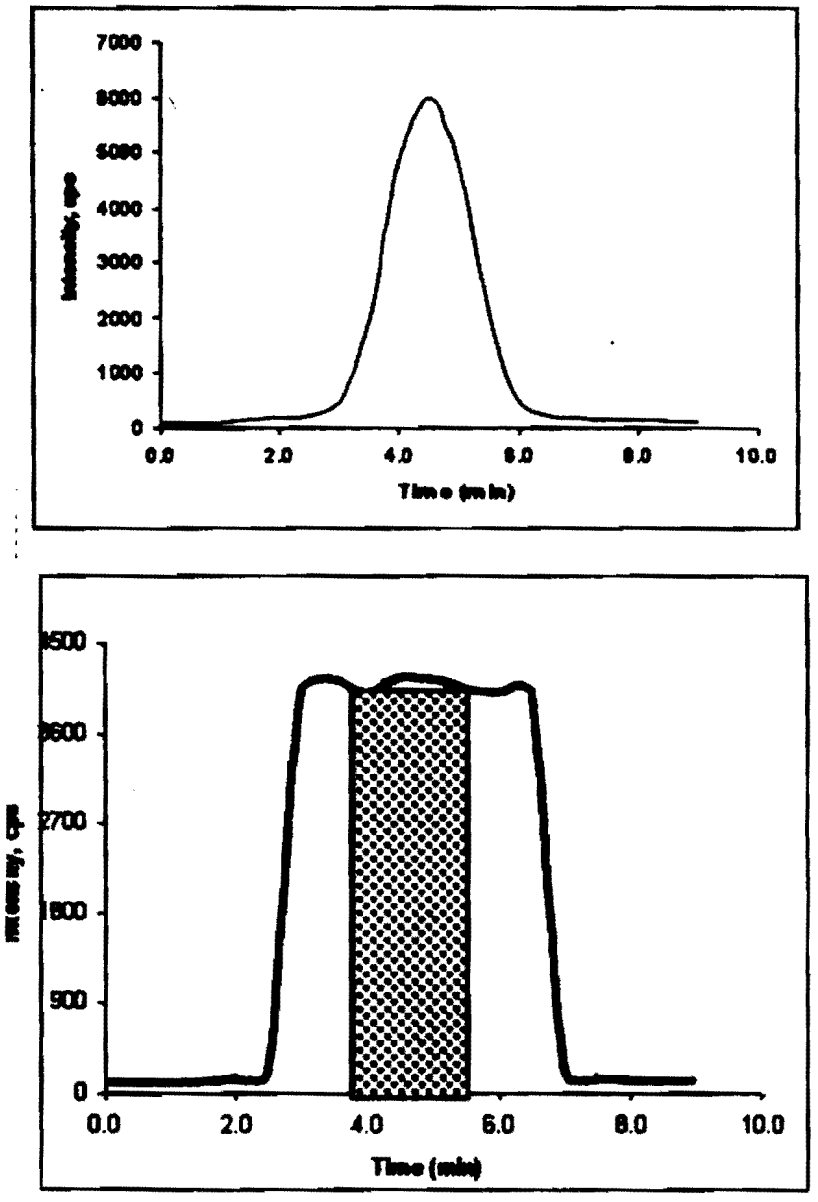
Mass spectral analyses were performed with a Finnigan Mat, Navigator single-stage quadrupole mass spectrometer (San Jose, CA.). The mass spectrometer was equipped with an electrospray interface set at a nebulizer gas pressure of nitrogen of 60 psi. The nitrogen curtain gas was adjusted to a constant flow rate of 1.2 L/min. Positive or negative ions formed at atmospheric pressure were sampled into the quadrupole mass filter via a 0.0045-in.-diameter aperture.

To evaluate the effects of mobile-phase additives and concentration of acetonitrile composition on ionization efficiency, analytes were infused into the ESI source at a constant flow rate of 20 μL/min, using a Harvard 200-infusion pump (South Natick, MA). Electrospray ionization mass spectra were acquired in positive ion modes by single ion monitoring (SIM) and full scan scanning over the *m/z* range 150-700 in steps of 0.1 with a 2-ms dwell time. Prior to each set of analysis the mass analyzer was calibrated with a poly(propyleneglycol) (PPG) solution.

Following the calibration with PPG, the system was monitored for approximately 10 minutes to observe any residual ions from the PPG. Background ions <3000 cpm observed when the mobile phase was infused, was considered to be acceptable. Each analyte was infused into the ESI source until the ESI gave the maximum response or a

plateau profile. This was accomplished within 5 minutes (**Figure 4-1**). The ESI response was integrated over one minute interval, and counts per second were calculated based on the response. The ESI signal reported was averaged over three separate scans. This method was chosen in order to minimize any dilution effect (concentration gradient) that would occur if samples were introduced into the source by loop injection.

Figure 4-1: Comparison of Peak Profile using Different Sampling Techniques



Top panel represents loop injection and the bottom panel represents sample integration over time interval at continuous infusion.

Sample Preparation

The mobile-phase additive ammonium acetate or ammonium formate was added to deionized water to prepare buffers of various concentrations and the pH was measured. The aqueous portion of the mobile phase was prepared from these buffers and the pH was adjusted with either formic, acetic or propionic acid. The pH of the aqueous portion prior to the addition of acetonitrile ranged from 5.26 to 2.48.

Stock solutions (1.00 mg/mL) of each analyte were prepared in acetonitrile and stored under refrigeration. For the experiments on sensitivity or ionization efficiency, samples were further diluted to a final concentration of 100 nM /ml in a mobile phase composition (70:30) aqueous/acetonitrile mixture. The analytes were infused into the mass spectrometer interface at a constant rate of 20 uL/min. Single ion monitoring (SIM) and full scan data were collected using Xcalibur software. Solutes were also analyzed when no acid was added to the mobile phase.

Results and Discussion

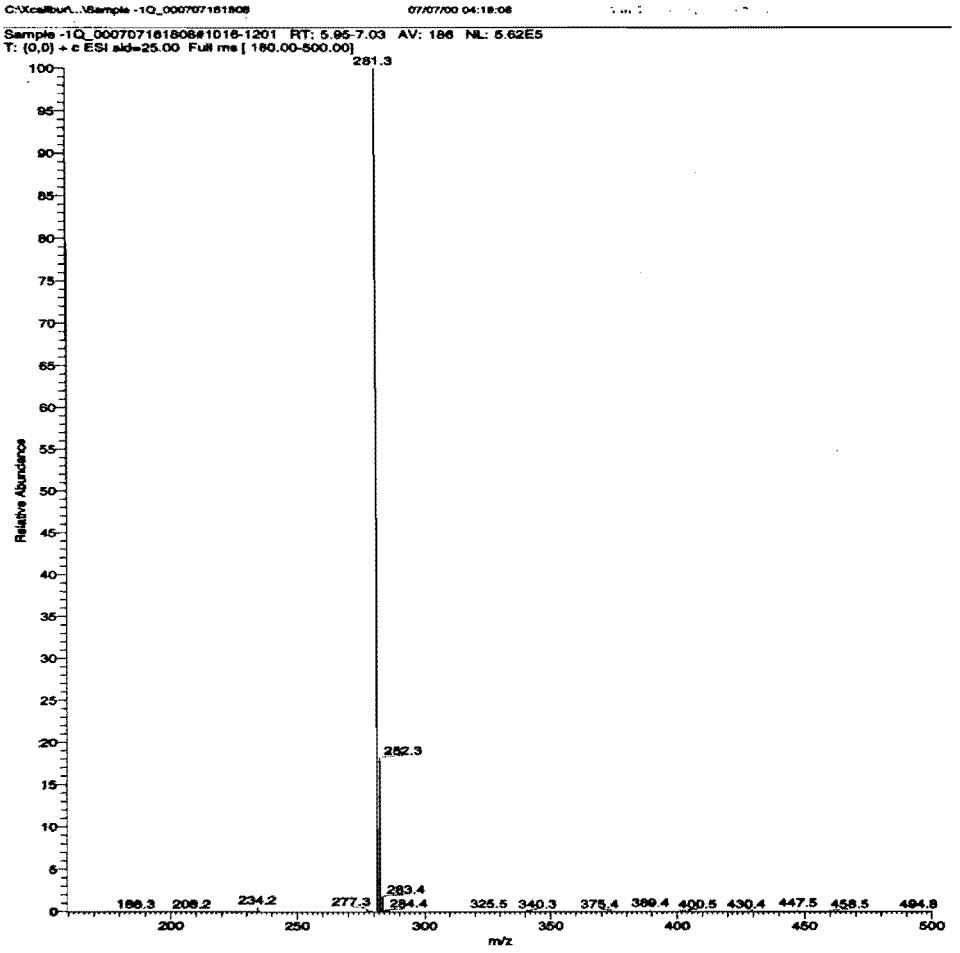
The chemical structures of four antidepressants and four control analytes examined in this study are given in **Table 4-2**, together with their molecular weights and ionization constants, as pK_a. The pK_a values listed were obtained from the literature and represent their ionization constant obtained in 100 % aqueous medium; however we have

demonstrated in Chapter 4, that the composition of acetonitrile in the mobile phase will have a tremendous effect on the ionization of the analyte. An increase in acetonitrile composition in the mobile phase will increase the pk_a of the acidic modifier, thus increasing the pH of the mobile phase. This will result in a decrease in the apparent pK_a shift of the analyte.

The compounds chosen represent a wide range in basicities ranging from 10.2 to 4.6. The antidepressants are much more basic than the aniline and pyridine derivatives, as indicated by the pK_a values for the dissociation of the monoprotonated species.

Since we were primarily interested in the ionization efficiency (sensitivity) of the $[M+H]^+$ ions formed in ESI, great attention was paid to ensure that the ESI conditions did not contribute to the formation of any fragmentation of the analyte. The conditions mentioned earlier were found to be appropriate as demonstrated by full scan analysis of all analytes. **Figure 4-2** shows full scan spectrum data for imipramine in a mobile phase composition of 10 mMol ammonium acetate aqueous-acetonitrile (70/30) composition. Similar spectra were obtained for the other studied analytes. These spectra showed that no significant abundance of multiply protonated ions were observed. Cluster ions, $(M+NH_4)^+$ and $(M+NH_4+CH_3CN)^+$ are some of the possible species that can be formed in this solution mixture. Zhou and Matthias [95] have shown formation of such cluster ions with 0.25 mM NH_4OAc in 60 % acetonitrile, with digitoxin.

Figure 4-2: Full Scan ESI Spectrum for Imipramine



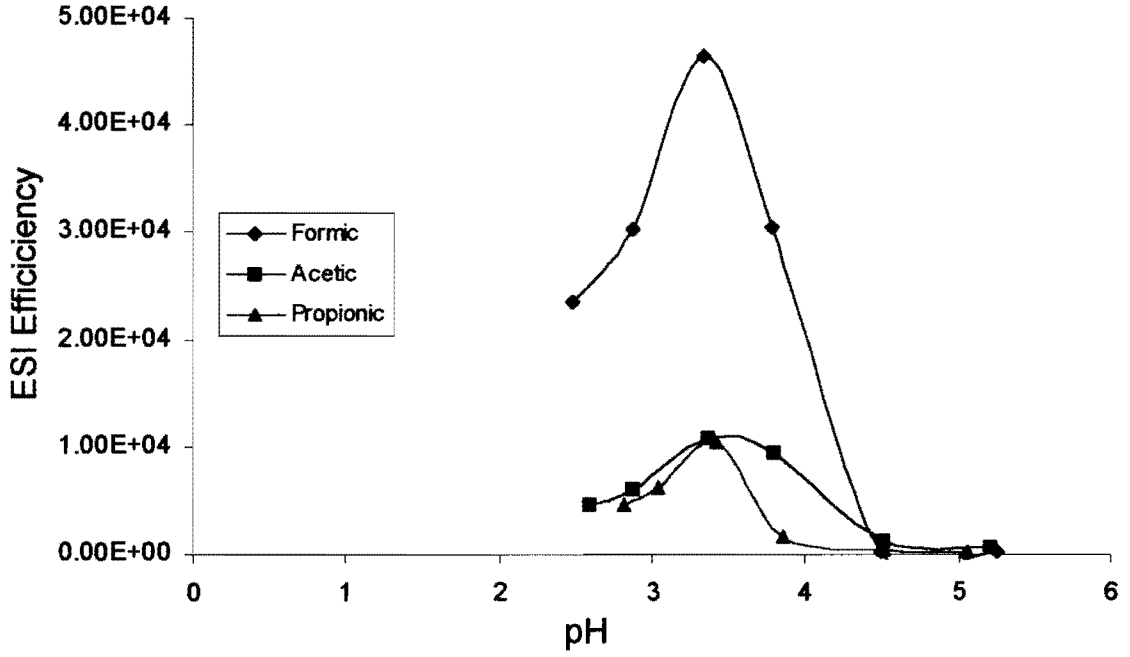
Mobile phase composition: ammonium acetate-acetonitrile (70/30), pH 4.5 with acetic acid. Sample was infused in to the ESI source at 20 μL/min.

Study 1- Effects of PH on ESI Efficiency

The positive ion full scan ESI mass spectra for one of the antidepressants, imipramine (100 μMol/mL), in a mobile phase composition of 10-mMol ammonium acetate / acetonitrile (70/30) mobile-phase is shown in **Figure 4-2**. No significant fragment ions were observed and the dominant sample ion was $[M + H]^+$. No significant abundance of multiply protonated ions was observed.

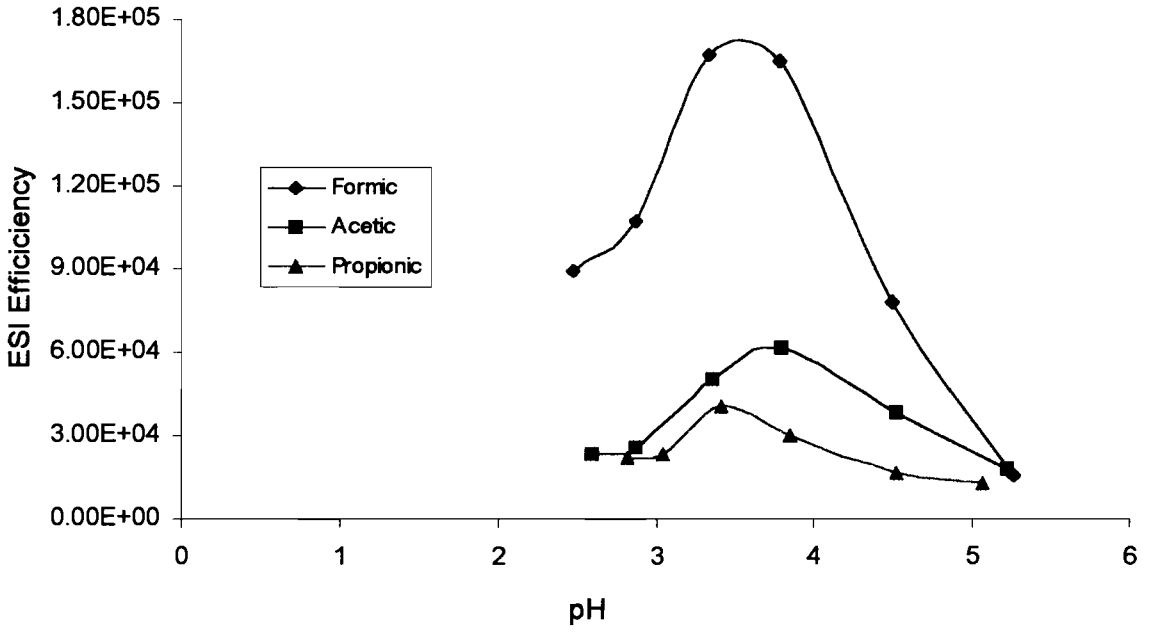
The effects of mobile-phase pH on the intensities of $[M + H]^+$ ions for aniline and 2,6-Lutidine was studied using different acidic modifiers are shown in **Figure 4-3 and Figure 4-4** respectively. The values for ESI intensities were obtained from (100 μMol/mL) solutions of each analyte in a mobile phase composition of 10 mMol ammonium acetate – acetonitrile (70/30). The pH of the mobile phase was adjusted with either formic, acetic or propionic acid. The ESI intensities are the averages of three spectra obtained for each analyte.

Figure 4-3: The Effect of Acidic Modifier with Varying pH on the Electrospray Ionization Efficiency for Aniline



Mass Spectrometric Conditions: mobile phase: acetonitrile- 10 mM ammonium acetate buffer (30/70) with various pH values. pH adjusted with acetic acid and ranged from 2.53 to 5.56, infusion rate, 0.2 μL/min.

Figure 4-4: The Effect of Acidic Modifier with Varying pH on the Electrospray Ionization Efficiency for 2,6-Lutidine



Mass Spectrometric Conditions: mobile phase: acetonitrile- 10 mM ammonium acetate buffer (30/70) with various pH values. pH adjusted with acetic acid and ranged from 2.53 to 5.56, infusion rate, 0.2 μL/min.

The sensitivity profile of the $[M + H]^+$ for two control analytes, aniline and 2,6-Lutidine, are similar but the sensitivity for each analyte is reflected in their respective pK_a values. The pK_a values for aniline and 2,6-Lutidine are (4.6 and 6.71 respectively). In this mobile phase composition (70/30) ammonium acetate – acetonitrile mixture, the apparent pH is ~ 6.9. Consequently, at pH ~6.9, both analytes will exist in solution as the neutral molecular species. The much lower sensitivity for aniline is consistent with its much weaker basicity in aqueous solution, as indicated by the pK_a values for the deprotonation of the $[M + H]^+$ ions.

At pH = 6, when no acid modifier was added to the mobile phase, the minimal ESI intensity was observed. This is attributed to the fact that at this pH value, the analytes exists in their neutral form. Upon further addition of acid, the pH of the mobile phase decreases, and the analyte becomes protonated and solvated. As shown earlier in Chapter 3, the pH of the aqueous-organic mobile will decrease approximately 0.3 pH units for every 10 % of acetonitrile added to the mobile phase. Therefore, the pK_a of aniline and 2,6-Lutidine in this mobile phase composition is expected to be approximately 3.55 and 5.44 respectively.

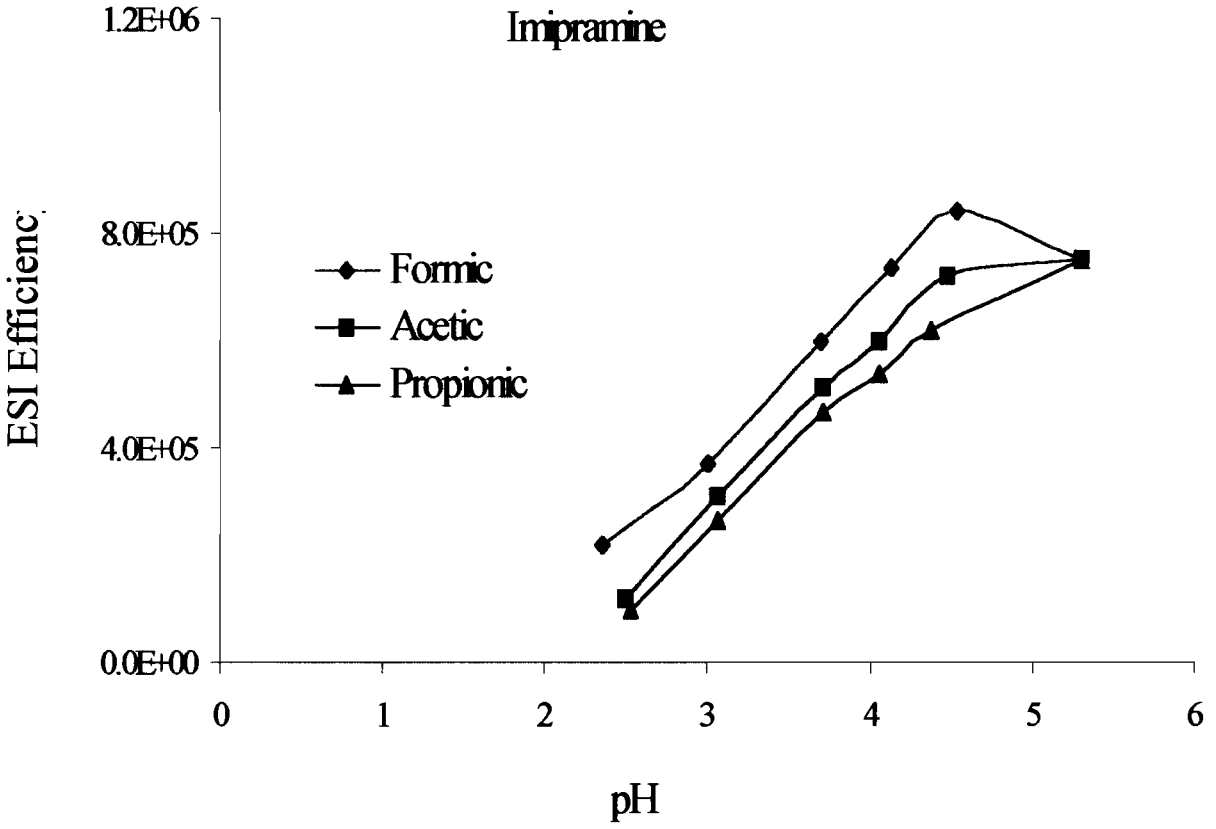
At a pH value slightly below the pK_a (pH = 3.55 for aniline and pH = 5.44 for 2,6 Lutidine) in this mobile phase composition, the ESI intensity showed a significant increased. Beyond this point at the pH value where the analytes are fully protonated, the ESI intensity decreases drastically due to an increase in the free anion concentration.

Ikonomou *et al* [77] have shown that for weak bases that are weakly ionized in methanol, the addition of acidic electrolytes will lead to an increase in $[B+H]^+$ ion intensity. The situation will be complicated by the fact that too much acidic electrolyte will also cause a decrease of the ion intensity. In their work the researchers have also shown that strong bases such as cocaine and tripropylamine, are completely ionized in methanolic solutions.

For these bases the ion intensity of $[B+H]^+$ upon the addition of electrolytes such as ammonium acetate or hydrochloric acid, will decrease by a factor of 10-20 as a result when foreign electrolytes are present at concentrations in the 10^{-2} to 10^{-1} range. Decreases of analyte ion intensity upon addition of foreign electrolytes above 10^{-5} to 10^{-2} M ranges were also observed. The authors attributed this effect to saturation and the decrease in total ion signal at high electrolyte concentrations.

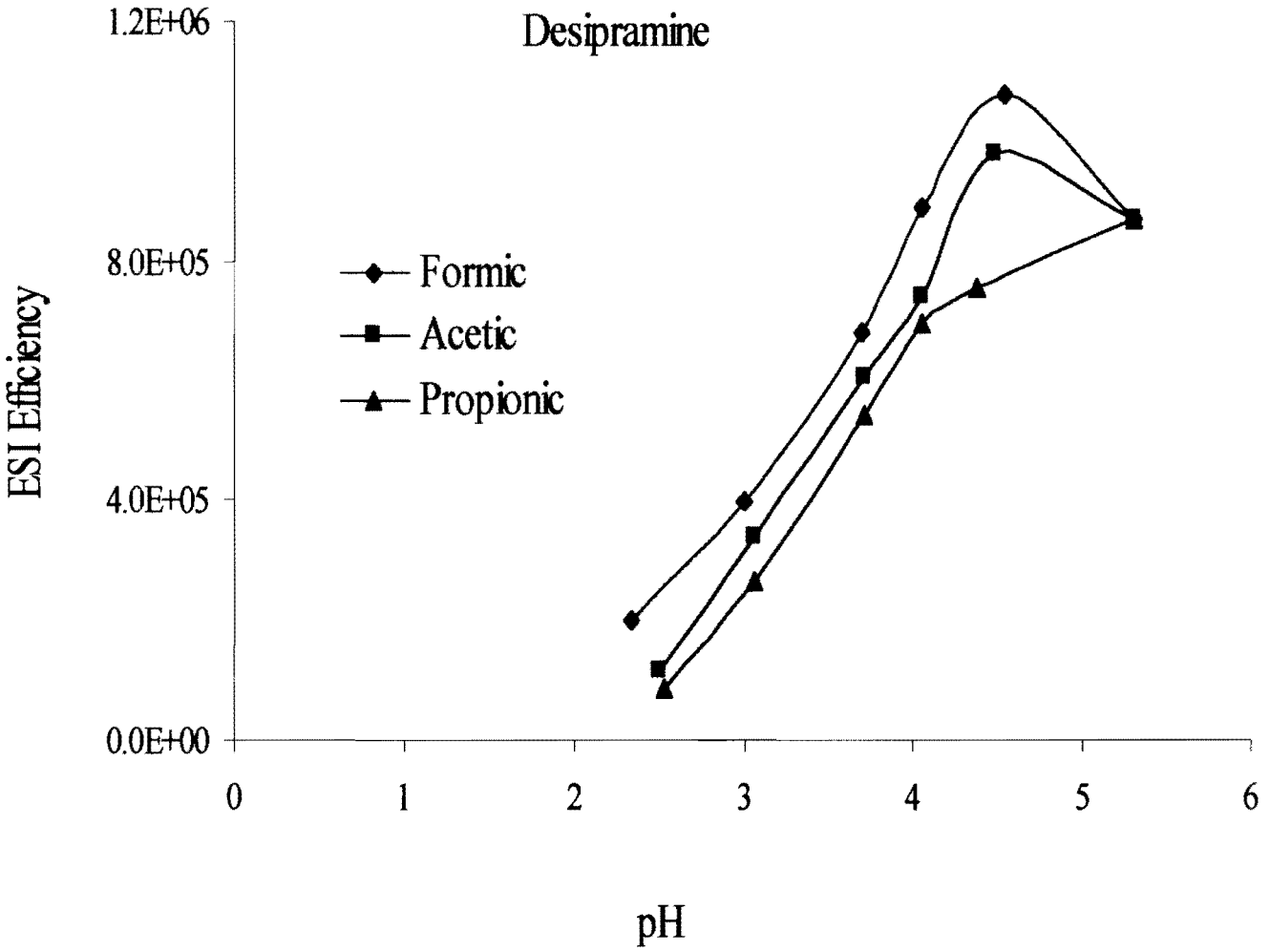
The somewhat expected behavior was observed for the effect of pH on ESI efficiency with respect to the antidepressants. The tricyclic antidepressants are strong diprotic basic analytes, with titrimetric $pK_a \geq 8$ for the aliphatic nitrogen group and $pK_a \leq 2.0$ for the ionizable nitrogen group within the cyclic part of the molecule. At pH ~6 the first ionizable group will be fully protonated and the second ionizable group will exist in its neutral form. Addition of acid will only further increase the ionization of these analytes at a pH value of ≤ 1 and the ion will have a net charge of +2.

Figure 4-5: The Effect of Acidic Modifier with Varying pH on the Electrospray Ionization Efficiency for Imipramine



Mass Spectrometric Conditions: mobile phase: acetonitrile- 10 mM ammonium acetate buffer (30/70) with various pH values. pH adjusted with acetic acid and ranged from 2.53 to 5.56, infusion rate, 0.2 μL/min.

Figure 4-6: The Effect of Acidic Modifier with Varying pH on the Electrospray Ionization Efficiency for Desipramine



Mass Spectrometric Conditions: mobile phase: acetonitrile- 10 mM ammonium acetate buffer (30/70) with various pH values. pH adjusted with acetic acid and ranged from 2.53 to 5.56, infusion rate, 0.2 μL/min.

Once these strong basic analytes are protonated, the addition of foreign electrolytes is subject to ion –pair formation, and the competition of ions reaching the detector will result in a decrease in ESI response. As shown in **Figure 4-5** and **Figure 4-6**, the ESI ionization intensity for imipramine and its demethylated metabolite, desipramine decreased rapidly with the addition of acidic modifiers.

Based on reverse phase chromatographic data presented in Chapter 3, in **Figure 3-3**, we showed the pK_a by the inflection point of the retention dependency study. The pH dependency study was performed using the same mobile phase composition as this study (10 mMol ammonium acetate / acetonitrile 70/30). As indicated by the plateau region in **Figure 3-3**, the antidepressants are all fully protonated in the M^+ state between pH 3 to pH 6.

This decrease in ESI efficiency can also be attributed to the dissociation of the acid modifier in solution. When these weak acids dissociate, their counter-anions form ion-associated complexes with the protonated species in solution. These neutral species will not be detected in the mass spectrometer, therefore resulting in a decrease in ESI efficiency.

One interesting note, the ESI efficiency profile as a function of pH is similar for all acids used for the pH adjustment of the mobile phase. For the more volatile acids, acetic and propionic, the ESI intensity was approximately 50 % less than the ESI

observed for formic acid. Also the viscosity and the relative strength of these carboxylic acids increases from formic to acetic to propionic. These factors all play an important role in the rate at which the analytes are protonated and ultimately the formation of ions in the mass spectrometer.

As shown in **Table 4-1** the proton affinity for formic acid is lower than that of the protonated acetonitrile as well as the other aliphatic (acetic and propionic) carboxylic acids. This suggests that the formic acid will not compete with the analytes for the available protons. The differences in the ESI sensitivities of the basic analytes can be attributed to the ability of the acidic modifiers to protonate the analyte.

Table 4-1: Gas-phase Acidity (to form $[M+H]^+$ ions) and Proton Affinity (to form $[M+H]^+$ ions) for reference compounds [145]

Compound	ΔG_{acid} (AH) (kJ/mol)	Proton Affinity (kJ/mol)
H_3	1657	854
HCOOH	1415	748
CH_3COOH	1429	796
CH_3CH_2COOH	1424	802
CH_3CN	1528	787

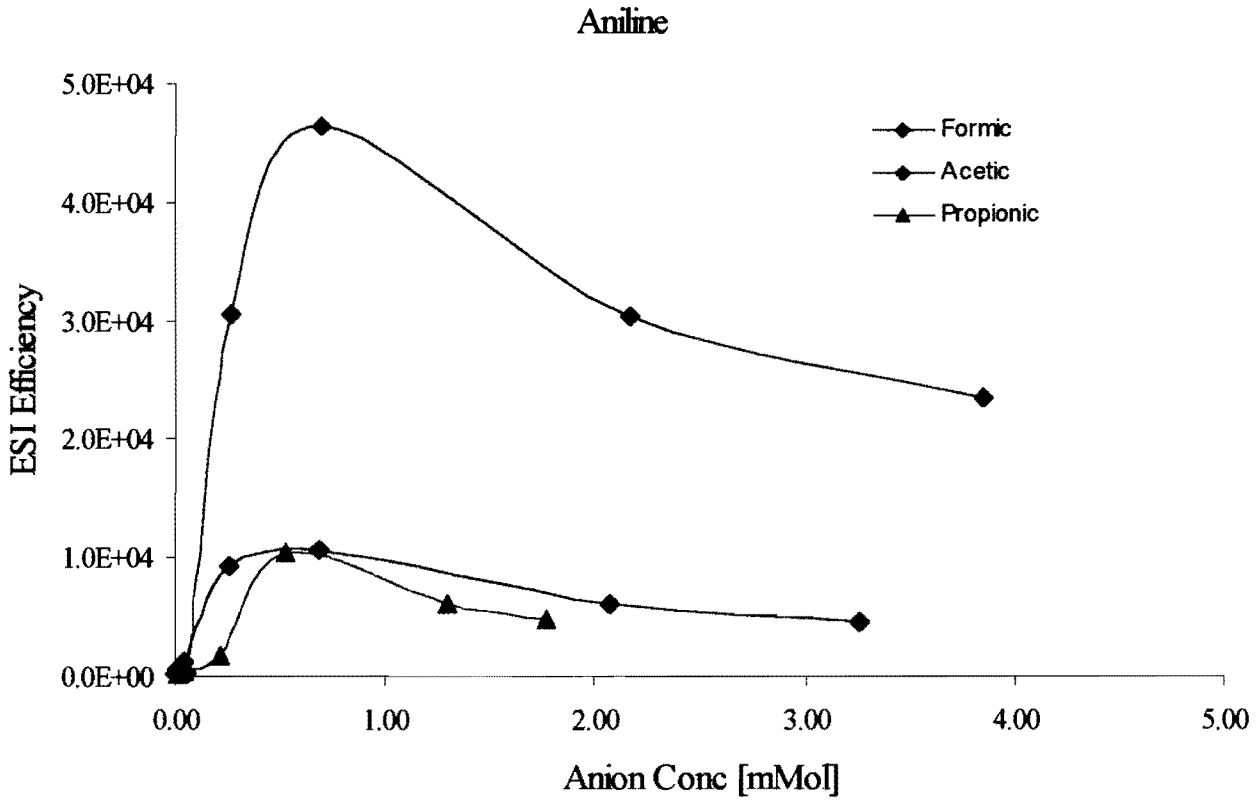
Effect of Counter-Anion Concentration on ESI Efficiency

The Effect of anion concentration on the ESI efficiency was studied. In all previous work performed in this dissertation, we observed that at high acidic modifier concentration the ESI concentration decreased. The concentration of counter-anion was calculated and a plot of the ESI efficiency versus the counter-anion concentration is shown in **Figure 4-7** for aniline. Again, the sensitivity profile was similar when formic, acetic and propionic acids were used as acidic modifiers.

The best or greatest ESI response was observed when ammonium formate / formic acid buffer systems were used. The type and concentration of the different counter-anions have an effect on the ionization efficiency of the analyte. Earlier work by Tang and Kebarle [75] and Enke [103] predicts that the presence of foreign electrolytes will result in a decrease in analyte sensitivity.

This decrease in ESI efficiency may be due to the formation of ion association species. Since ion associated species (neutral species) and carry no net charge, they will not be detected by the Mass Spectrometer. This will result in a decrease in ESI efficiency. In our study, as shown in **Figure 4-7**, a significant increase in the ESI efficiency was observed from a pH range of 5.3 -3.3. The increase observed was independent of the acid modifier used.

Figure 4-7: ESI Efficiency For Aniline as a Function of Counter Anion Resulting from Different Acidic Modifier Used in the Mobile Phase



Mass Spectrometric Conditions: mobile phase: acetonitrile- 10 mM ammonium acetate buffer (30/70) with various pH values. pH adjusted with either formic, acetic or propionic acid and ranged from 2.53 to 5.56, infusion rate, 0.2 μL/min.

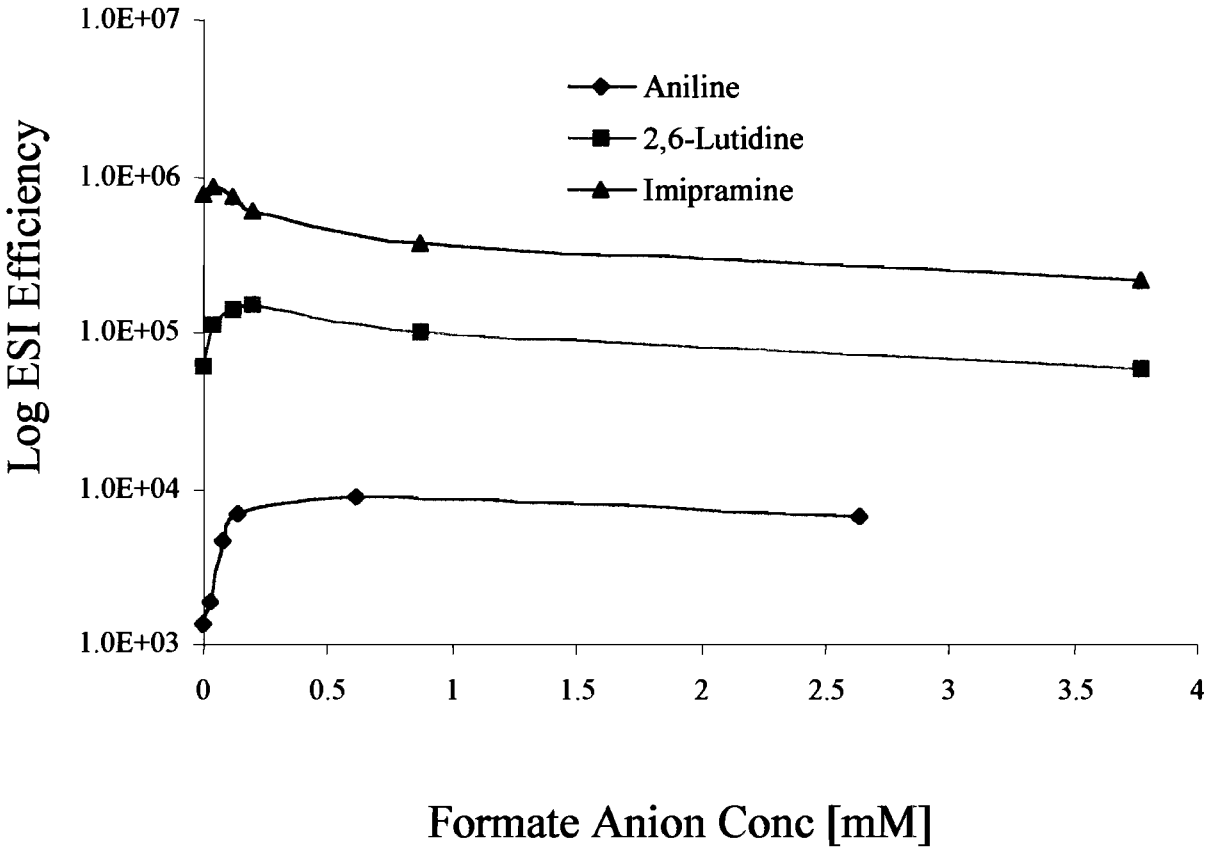
Based on this mobile phase composition 10 mMol ammonium acetate – acetonitrile (70/ 30) accounting for the apparent pK_a shift, we expect aniline and 2,6-Lutidine to be fully protonated at pH 1.55 and 3.44 respectively. Beyond that point, the addition of more acid, and consequently an increase in counter-anion concentration shows a dramatic decrease in the ionization efficiency.

Relative ESI efficiency increased in the order from propionic, acetic to formic acid. This trend in the increase of ESI efficiency is directly correlated to the trend in the strength of the acid. Also, in the ability of the structure of the acid to donate or accept electrons. As we can see, the longer the carbon chain length, the more electron donating the acid and therefore the decrease in acidity which results in a lesser ability to protonate the studied analyte at equivalent concentrations.

A comparison was made between weak bases such as aniline and 2,6-Lutidine, and strong bases such as Imipramine. These strong bases have pK_a values of ~ 9.5. In the mobile phase of 70/30 Buffer-acetonitrile composition, these analytes are completely ionized at a pH of < 6.5, taking into account for the apparent pK_a shift. Decreasing the pH of the mobile phase by the addition of acidic modifiers will cause an increase of the counter-anion concentration as shown earlier. **Figure 4-8** shows the anion concentration will drastically decrease the ESI detection efficiency of these strong bases.

However, weak bases such as 2,6 Lutidine and aniline with titrimetric pK_a values of 6.71 and 4.60 respectively, are completely ionized at pH < 3.81 and 1.7 respectively at this mobile composition. Here we observe that the $[M+H]^+$ ESI signal increased up to an

Figure 4-8: Electrospray Ionization Efficiency Versus Counter-Anion Concentration For Analytes with Different pK_a Values



anion concentration of 1.53 mMol/mL (pH= 3.34), then decreased fairly drastically as shown in **Figure 4-8**. Again, this decrease is attributed to the formation of neutral ion-associated complexes, which cannot be detected in ESI. Also, as the concentration of the counter-anions increase with the addition of more acid, consequently there is an addition of more ions per unit volume. Therefore, there is an increase in the competition between the total amount of [M+H]+ ions reaching the detector.

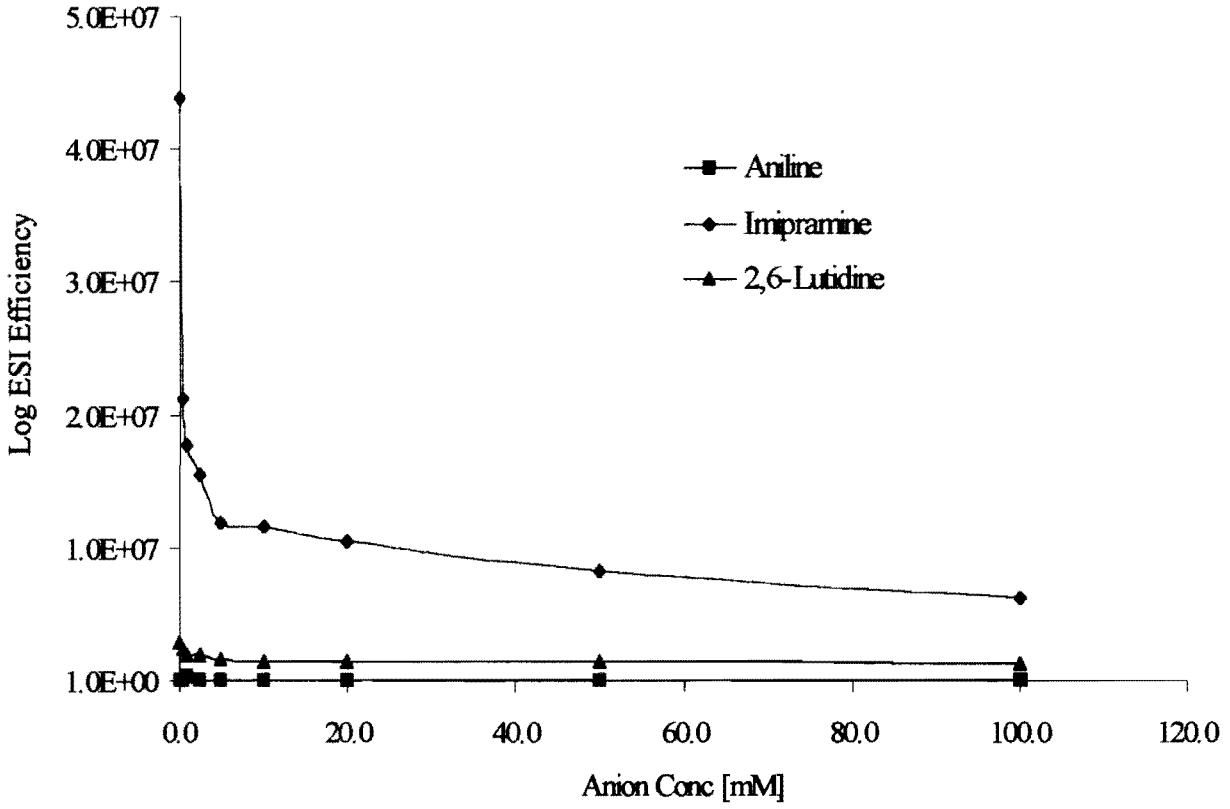
Ikonomou *et al* [77] have shown the ESI sensitivity of a series of basic analtyes in methanolic solutions. After accounting for the pKa shift of the aqueous / organic mixture, the ESI sensitivity of the stronger bases resulted in a greater ESI response. The authors have also shown that the stronger bases resulted in a much larger ESI response. In **Figure 4-8** we demonstrate the impact of analyte basicity on the ESI efficiency for aniline, 2,6-Lutidine and Imipramine. A log scale was used to accommodate large differences in ESI counts. As shown in **Figure 4-8**, we see that the ESI efficiency for imipramine (as reflected by its higher pKa value) resulted in a much greater ESI response compared to 2,6 Lutidine and Aniline. This effect may be attributed to the analyte with the higher pK_a or lower solution basicity resulted in the higher ESI response.

From the retention factor dependency on pH (Figure 3-3), we know that at pH 4, the nitrogen atom on the aliphatic group will be fully protonated and the nitrogen atom within the tricyclic group will be in its neutral form. Therefore we chose pH 4 to study the effects of counter-anion concentration the ESI efficiency of the tricyclic antidepressants.

This experiment was performed at constant pH with an increase in acetate counter-anion concentration in a mobile phase composition of 70/30 aqueous buffer acetonitrile mixture. The counter-anion concentration was changed by addition of the ammonium formate or ammonium acetate salt. Due to the dissociation of the ammonium salt which resulted in an introduction of ammonium in the mobile phase, and the pH of the aqueous portion of the mobile phase was maintained at 4.02 ~ 4.05 by the addition of formic or acetic acid. The total anion concentration was calculated from the summation of anion concentration generated from both the acid and the salt form.

The decrease of ESI efficiency of $[M+H]^+$ ions upon the addition of ammonium acetate for aniline, 2,6-Lutidine and imipramine is demonstrated in **Figure 4-9**. The dramatic decrease by a factor of 10 to 20 for imipramine when counter-anions in the mobile phase ranged from 0 to 120 mM/mL. At pH 4, aniline is not fully protonated, therefore the ESI increased, before showing the expected decrease upon the addition of higher salt concentrations.

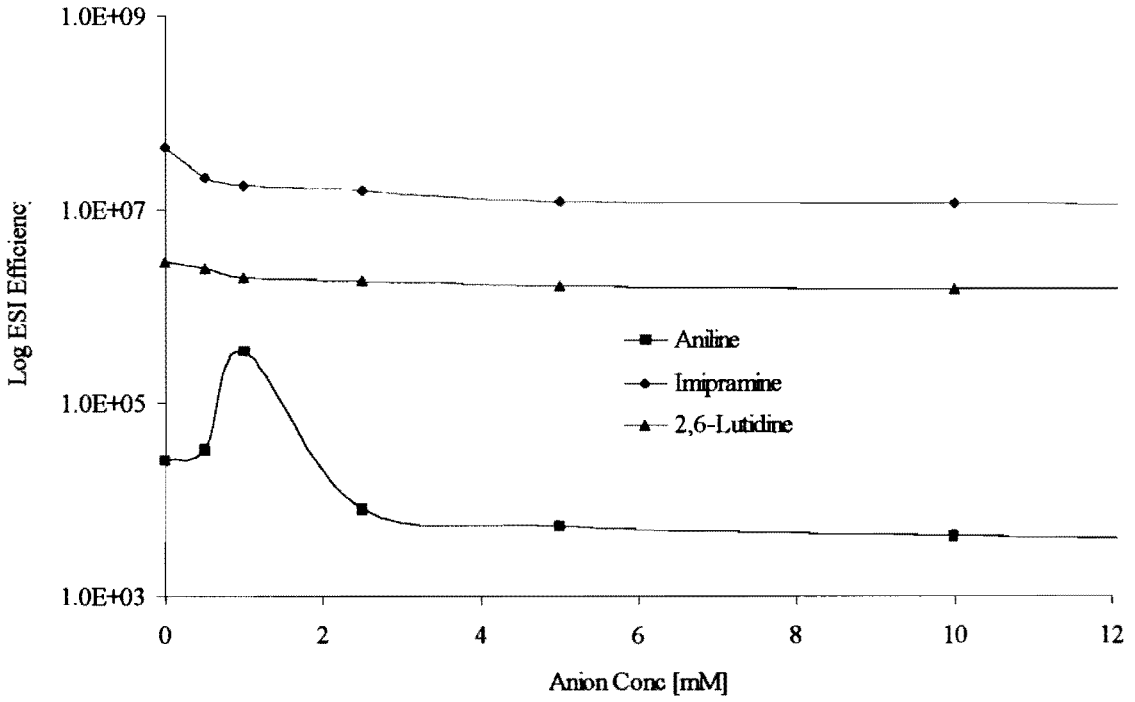
Figure 4-9: Electrospray Ionization Efficiency Dependency of Anion Concentration at Constant pH of the Mobile Phase



Full scan spectra indicated that no adducts were being formed upon addition of either ammonium acetate or ammonium formate in the mobile phase. Therefore, the decrease in ESI efficiency is a result of decreases amounts on $[M+H]^+$ ions reaching the detector. The effect of anion concentration on an analyte with low pK_a as aniline compared to 2,6-Lutidine and imipramine is demonstrated in **Figure 4-10.** A log scale is used to accommodate a large range in ESI efficiency and a smaller anion concentration range is used to show the pronounced effect of anion concentration on aniline. These maybe related to saturation of the ions, which result in a decrease in the total signal at high counter-anion concentrations.

It is well known that analytes with significant nonpolar regions are retained longer in reversed-phase HPLC than more polar analytes [146,147]. The relationship between ESI response and analyte nonpolar character suggests that a correlation might exist between retention time in reversed-phase high-performance liquid chromatography (HPLC) and ESI response. Recent work by Enke et al [148] have shown that the relative responses of small peptides are related to differences in the nonpolar character of the analyte. Here we observe a similar relationship between the relative intensities for aniline, 2,6-Lutidine and imipramine.

Figure 4-10: Log Electrospray Ionization Efficiency Dependency of Anion Concentration at constant pH of the Mobile Phase

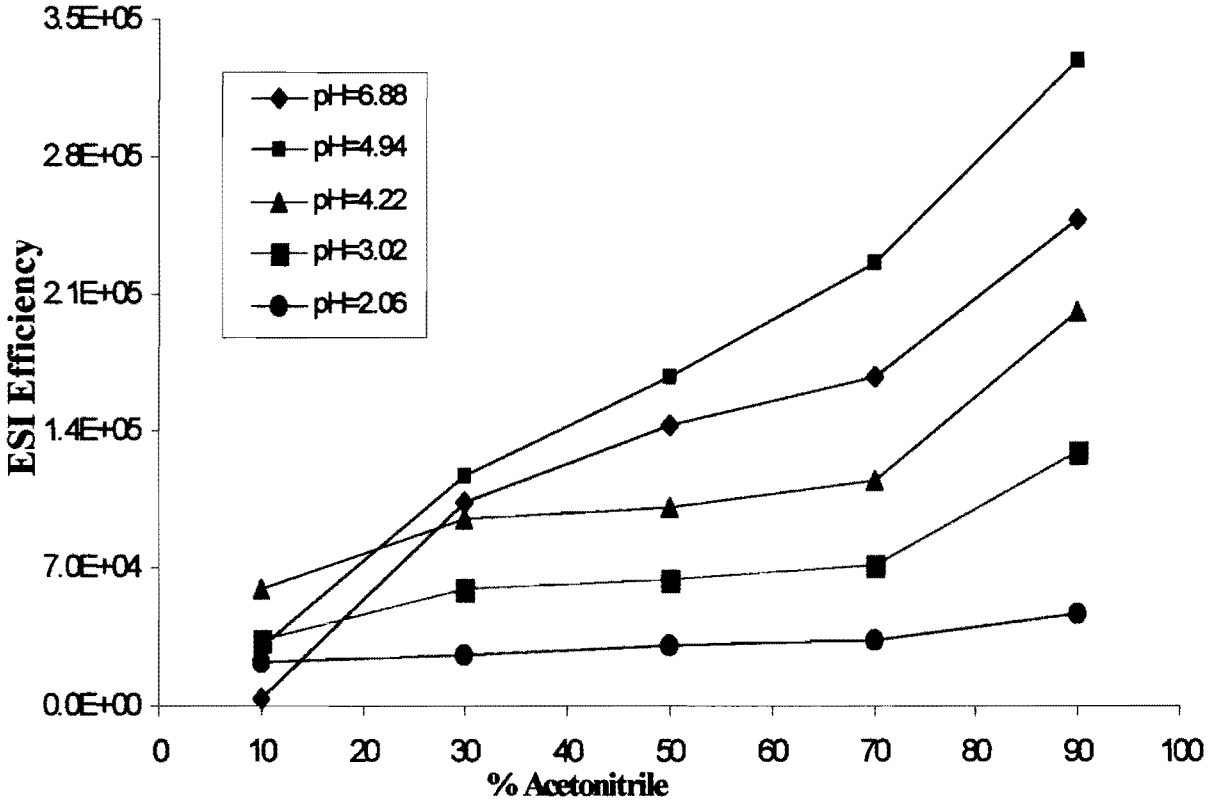


Effect of Acetonitrile Concentration on ESI Efficiency

The use of various water/acetonitrile mixtures is commonly used in reversed-phase chromatography. Therefore, the effect of various aqueous / acetonitrile mixtures on the ESI ionization efficiency was investigated. In Chapter 4 we have shown that the composition of the acetonitrile in the mobile phase will affect the ionization state of the analyte.

In the previous study, it was observed that the use of formic acid as an acidic modifier, resulted in the maximum ESI response of all analytes studied. In this experiment 100 μMol of each analyte was dissolved in different compositions of 1mMol ammonium formate and various acetonitrile compositions. Each analyte was infused separately and directly into the electrospray source at a flow rate of 20 μL/min. An increase in ESI efficiency was observed with the mobile phase composition ranging from 10 to 90 % acetonitrile for all analytes studied. Imipramine was used as a representative analyte to demonstrate the effects of acetonitrile composition in the mobile phase on the ESI efficiency **(Figure 4-11).**

Figure 4-11: Effect of Acetonitrile on the Electrospray Ionization Efficiency of Imipramine



If the analytes in solution exist as preformed ions, the ESI response is expected to increase with increase in organic composition. An increase in organic solvents such as methanol and acetonitrile improves the spray performance, because these solvents lower the surface tension of the droplets [90]. Others [92,149] have reported that the ESI/MS response increases when the concentration of acetonitrile increased

In the studies performed, the ESI efficiency increased with an increase in acetonitrile composition (**Figure 4-11**). The increase may be attributed to two factors of the organic composition employed in the mobile phase. First, as the composition of acetonitrile is increased in the mobile phase, dilution in the concentration of counter-anion will occur resulting in a decrease in counter-anion. The dilution effect was demonstrated when the mobile phase pH was as low as 2.06. With a mobile phase pH of 2.06, the counter-anion concentration is relatively high, nonetheless a slight increase in the ESI efficiency was observed.

Secondly, according to Kebarle and Tang [90] most organic solvents (eg acetonitrile and methanol) have lower surface tension and higher volatility than that of aqueous solvents. The solvent properties increase the desolvation efficiency of the analyte, resulting in a much higher ESI response [150]. Others [151] have shown that post column addition of organic solvent to HPLC effluent (when separation has been achieved under high aqueous mobile phase conditions), will drastically increase the ESI sensitivity. However, this practice can be costly (more instrumentation required) and more labor intensive.

Conclusions

The effect of mobile phase additives on the molecular-ion response for a series of basic analytes with a wide range of solution pH was investigated. Mobile-phase additives had significant effects on the ESI efficiency of all analytes studied. The ESI efficiency was dependent upon the pH of the mobile phase. However, due to the dissociation of the acid in aqueous medium, the counter-anion produced resulted in a gradual decrease in the ESI efficiency. In all cases, formic acid gave the greatest sensitivity for $[M + H]^+$ ions. The ESI response was correlated to the pK_a of the analyte. The strong basic analytes, the antidepressants resulted in a greater ESI response than the weaker basic analytes, aniline and 2,6-Lutidine.

We have also demonstrated that singly charged ions formed in ESI may be due to desolvation and/or transmission to the mass spectrophotometer are significantly decreased at the highest salt concentrations. The decrease in ESI efficiency at the highest salt concentrations (10^{-3} M) may indicate that a smaller fraction of the ions reach the detector than at lower salt concentrations [152]. The increased current that we observed at the highest salt concentrations indicates that more excess charge ions are produced in the solution phase. Thus the decrease in response may indicate a loss in ion transfer efficiency or desolvation efficiency (or both).

The effect of acetonitrile concentrations ranging from 10-90% in the mobile phase on the ESI efficiency was studied. The ESI-MS signal observed increased by approximately 100% when 90% acetonitrile was used. The increase in ESI response was independent of the pH of the mobile phase and may be attributed to the lower surface tension and desolvating properties of acetonitrile.

These observations have significant implications for ESI-MS and can be exploited in methods optimization. Typically, this information could be used for signal enhancement when the use of counter-anions is necessary for methods optimization. The use of mobile phase with high acetonitrile content and low ammonium formate consistently gave the best sensitivity.

Chapter - 5

Correlation of Analyte Retention and ESI Efficiency

High Performance Liquid Chromatography (HPLC) with tandem mass spectrometric detection techniques (LC/MS), and particularly LC/ESI/MS is a very powerful addition to the repertoire of methods available for bioanalytical chemists developing highly sensitive assays in biofluids[153,154]. The continual decrease and general miniaturization and simplification of these instruments have resulted in the general acceptance of mass spectrometer detection in the research industry.

LC/ESI/MS is currently being considered the method of choice for supporting clinical and preclinical studies in the pharmaceutical industry. However, the best ESI conditions may not be optimal from a chromatographic standpoint, i.e. non-optimal separation for the analytes in a mixture. Therefore, a compromise between the HPLC separation and the ESI/MS response has to be made.

In LC/ESI/MS the use of inorganic buffers in the mobile phase is strictly prohibited, therefore, when considering methods involving LC/ESI/MS the use of volatile buffers has to be employed. Matuszewski *et al* [155] have shown that when analyses are performed under minimal HPLC retention and separation, the precision of the analyses was inadequate and varied from 15 to 30% at all concentrations within the standard curve range. Variations in the assay were attributed to ion suppression of endogenous material

or matrix effect. By increasing the retention factor the interferences from sample matrix were separated from the analyte and practically eliminated the ion suppression.

As described in Chapter 3, the retention of ionogenic analytes (acids or bases) can be quite challenging. HPLC retention of fully protonated basic analytes is dependent upon the type and concentration of acidic modifier in the mobile phase. The increase in the strength of the counteranion showed a marked increase in the retention time of the basic analyte. The effects of the various anions on the retention time of basic analytes were dependent upon the type of anion used in the mobile phase. The retention of each analyte decreased from perchlorate to formate to acetate.

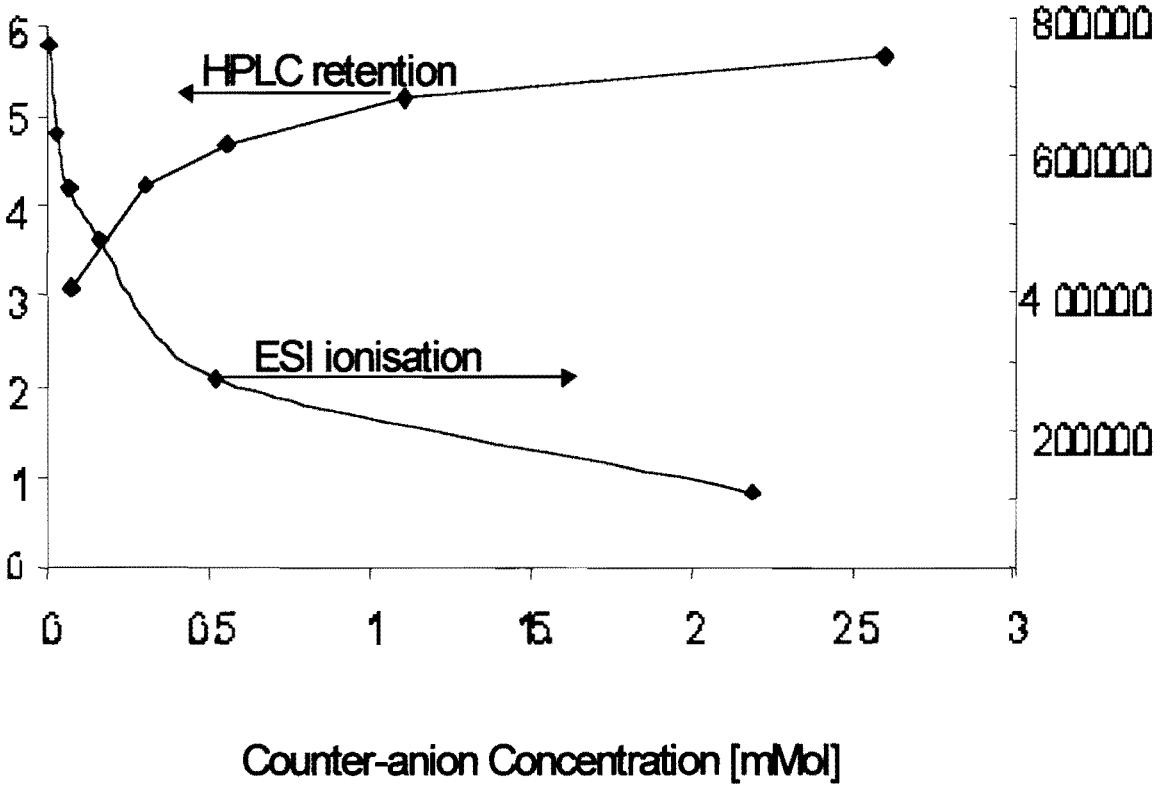
Corresponding dependencies of ESI efficiency on the counteranion concentration for antidepressants have shown a gradual decrease. Analytes with lower pKa values such as aniline and 2,6-Lutidine, on the other hand, show an increase of ionization efficiency in a low counteranion concentration region. This is associated with their partial ionization in the solution at low concentration of acidic mobile phase modifier.

An increase in the concentration of the acidic modifier resulted in complete protonation of the analyte, which results in achieving the ionization efficiency maxima. Further increase of the counteranion concentration leads to the desolvation of the protonated analyte and corresponding decrease of electrospray ionization efficiency. However, when analyte ions are fully desolvated they may form neutral ion association complexes, which will not be detected by the mass spectrometer.

During the LC-ESI-MS methods development for the analysis of basic analytes, acid modifiers are frequently used to promote separation of these compounds from their impurities and their degradation products. These acid modifiers are also used to promote the electrospray efficiency. The choice and concentration of acid modifiers plays a very important role when considering LC/MS with electrospray ionization.

In positive ion electrospray ionization, we found that the ionization intensity varied depending on the type of acid modifier used in the mobile phase. There was an increase in ionization intensity from propionic < acetic < formic acid. ESI intensity is dependent upon the pH and also on the composition of acetonitrile used in the mobile phase. A further decrease in the pH of the mobile phase introduces relatively high counteranion concentrations, which decreases the ESI intensity ***(Figure 5-1)***. As shown earlier, the mobile phase composition shows an apparent pK_a shift on the analytes, and therefore must be taken into consideration considering LC/MS with electrospray ionization. As a rough guide, formic acid gave almost a 20 to 50 % increase in ESI efficiency over acetic and propionic acid respectively.

Figure 5-1: A Comparative Profile of Retention Dependency versus the Electrospray Ionization Efficiency for Imipramine



Addition of protonated mobile phase ions with high affinity for the analyte ions will significantly increase the ESI signal intensity. This response is strictly dependent on the ionization constants such as the pK_a of the analyte. In all cases studied, the addition of formic acid gave the most sensitive $[M+H]^+$ ions in ESI/MS. For analytes that are not fully ionized in solution, the ESI intensity increased to a point where the dissociation of the acid and the eventual increase in counter-anion concentration generated from the acid modifier will result in a gradual decrease in ESI intensity. This phenomenon was independent of the acid modifier used.

The use of high acetonitrile concentrations (up to 90 %) at various pH values of the mobile phase showed a significant increase in the electrospray ionization efficiency for all analytes studied. The ESI increase by over 98 % was observed when the acetonitrile composition in the mobile phase was increased from 10 to 90 %. From the studies performed, the data suggest that in LC/ESI/MS the concentration of the buffers used must be kept at a minimum. However, the use of larger compositions of acetonitrile can be used to counteract the effects of the counter-anion concentration and promote the ESI efficiency.

Chapter 6.

Overall Conclusions

Recent research in LC-ESI-MS has focused primarily on the enhancement of the interface and the mass spectrometer for improved signal and resolution. While improvements in the interface and improvement in the signal and resolution is important, it is our belief that the mobile phase additives play a significant role on the electrospray ionization efficiency. The main goal of this dissertation was to provide a correlation of retention mechanism and the electrospray ionization efficiency of ionizable basic compounds. Here we outlined several studies that offer further understanding and a positive contribution to expanding the research on understanding the effects of mobile phase additives on the electrospray ionization efficiency in LC-ESI-MS operated in the positive mode.

The retention dependencies of moderately and highly basic analytes on the pH, composition of organic solvent and counter anion concentration were determined. Chromatographic pK_a values were measured and compared to those obtained potentiometrically (literature values). The measured chromatographic pK_a values of studied analytes showed a apparent decrease in the pKa values of approximately 0.3 pH units for every 10 % of acetonitrile used in the mobile phase. A plot of the retention dependencies of the retention factor versus the pH of the mobile phase showed the predicted theoretical sigmoidal curve. The increase in concentration of acetonitrile led to an increase in the pH of the mobile phase. This increase in the pH of the mobile phase

reduced the analyte to the less ionized form. Therefore changes in the pH upon addition of acetonitrile in the mobile phase resulted in decreased apparent pK_a shift of the basic analyte. An increased concentration of acetate, formate and perchlorate counter anions to the mobile phase disrupt the analyte solvation shell to different degrees. The desolvation effect is more pronounced at the low counter anion concentrations. Further increasing the counter anion concentration will increase the retention of the basic analytes. Once the analyte is fully desolvated the retention factor levels off.

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Due to the ever-increasing demand by regulatory agencies for the characterization and identification of trace levels of drug and metabolites in biological matrices, LC-ESI-MS has become valuable tool in the analytical laboratory. Clearly an understanding of the effects of HPLC mobile phase additives no the electrospray ionization efficiency can aid in the selection of the appropriate mobile phase to reduce trial and error associated with methods development. It is our hope that the work presented within will provide further insight into the effects of mobile phase additives on the electrospray ionization efficiency of basic analytes.

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