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## Optimization of Gradient Chromatofocusing for the Protein Separation and LC-MS/MS Determination of NGP1-01 in Mouse Serum, Brain, and Retina

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**OPTIMIZATION OF GRADIENT CHROMATOFOCUSING FOR THE  
PROTEIN SEPARATION**

**AND**

**LC-MS/MS DETERMINATION OF NGP1-01 IN MOUSE SERUM, BRAIN, AND  
RETINA**

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**Master of Medicinal Chemistry**

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**Cleveland State University**

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**OPTIMIZATION OF GRADIENT CHROMATOFOCUSING FOR THE  
PROTEIN SEPARATION AND LC-MS/MS DETERMINATION OF NGP1-01 IN  
MOUSE SERUM, BRAIN, AND RETINA**

**HARINI JOGIRAJU**

**ABSTRACT**

Gradient chromatofocusing (GCF) is a chromatographic technique developed by our laboratory that generates linear pH gradients using weak anion-exchange HPLC with inexpensive low-molecular buffer components. This method shows significant advantages over conventional chromatofocusing and salt gradient ion-exchange chromatography in protein separation on DEAE column. In GCF an elution buffer (consisting of multiple acidic buffers with evenly spaced  $pK_a$  values) is mixed in successively greater proportions with application buffer (composed of multiple basic buffers with evenly spaced  $pK_a$ s), to generate a smooth linear pH gradient. GCF has the advantage of giving an estimate of the protein pI and being able to be directly interfaced to a mass spectrometer when volatile buffers are used.

Although GCF yields considerable success in protein separation, extensive study has not been done yet. Further characterization and method optimization studies in GCF are done in the present work. Chapter 1 describes the characteristics of the conventional ion-exchange chromatography, chromatofocusing, and gradient chromatofocusing for protein separation.

In the second chapter, a new method with smooth linear pH- gradients on self packed DEAE anion-exchange column was developed by introducing “bridging” buffers between application and elution buffers. This was employed for the chromatographic separation of a mixture of conalbumin, rat albumin, and  $\beta$ -lactoglobulin which resulted with good resolutions.

In the third chapter, the effect of the number of buffer components on protein separation in high-performance gradient chromatofocusing ion-exchange using linear pH- gradients was studied. The results showed that the number of buffer components has significant effect on protein separation. Results showed the increased resolutions and decreased peak widths at half height were obtained with increased number of buffer components. This optimized GCF technique was applied to the separation of prolactin isoforms by a PEI weak anion exchange column.

In chapter four, the development and validation according to FDA guidelines of a sensitive quantitative LC-MS/MS method for the determination of a neuroprotective compound, NGP1-01, in mouse serum is described. The developed method was applied to preliminary pharmacokinetic studies to quantify NGP1-01 in limited number of dosed mouse serum samples to show the suitability for undertaking full NGP1-01 pharmacokinetic studies.

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# **CHAPTER I**

## **OPTIMIZATION OF GRADIENT CHROMATOFOCUSING FOR THE PROTEIN SEPARATION**

### **1.1. Background and significance**

Proteins are important large biological molecules that play critical roles in almost all biological processes. They are building blocks for living cells and serve as functional, structural and regulatory agents of the body's processes. Types of proteins include antibodies, enzymes, hormones, messengers, structural components, transporters, and others. There are about 30,000 proteins in human bodies translated from the estimated 30,000 human genes. These proteins undergo post-translational modifications. So it is estimated that the human body may contain over two million proteins, coded for by only 20,000 – 25000. With this enormous diversity of proteins, separation techniques are essential in the analytical determination of a particular problem. To perform in vitro analysis, a protein must be purified away from other cellular components [1, 2].

High performance liquid chromatography (HPLC) is emerging as an essential technology in the purification and separation of proteins due to its high efficiency and high resolution capabilities. Protein HPLC can be categorized based on the separation mechanism into ion-exchange, reversed phase, size exclusion, hydrophobic interaction, affinity, and metal interactions [3-5]. Due to its non-denaturing characteristics and charge based separation, high resolving power, versatility, high protein binding capacity, and ease of operation, ion-exchange HPLC is one of the important and most utilized techniques in protein separation and purification [6, 7].

## **1.2. Ion-exchange chromatography**

### **1.2.1. Overview**

Ion-exchange chromatography is the most widely used HPLC technique that separates and purifies proteins based on their overall charge either salt-gradient or pH-gradient elution [8]. The principle of ion-exchange chromatography in the salt gradient elution is based on the competitive interaction between sample molecules and salt ions for the charged functional groups on the stationary phase [9]. Ion-exchange chromatography retains the molecules by the reversible interaction of electrostatic charges of the solute molecule functional groups with an opposite charge of the ion-exchanger functional groups [10]. To maintain neutrality, the charges on both the analytes of interest and the ion exchanger are associated with ions of opposite charge, termed counter ions [11].

The ion-exchange process can be described [12] by taking into account an anion-exchange resin, where  $R-X^+$  is fixed charge group and  $A^-$  is a counter ion or the analyte ion for anion-exchanger and  $B^-$  is a different counter ion present in the mobile phase introduced onto the ion-exchanger which displaces the counter ion or the analyte ion of ion-exchanger .

Equilibrium is established between two ions  $A^-$  and  $B^-$  as follows:



In ion-exchange HPLC, the ion-exchangers consist of three parts: a stable base support, fixed charge groups which are attached to base support, and counter ions. The base support for HPLC should meet the following requirements: particle size should be small and uniform, must provide high physical strength to resist the high pressure, chemically stable toward wide range of mobile phase conditions, and should provide access to a greater number of retention sites. The general types of base supports used in ion-exchange chromatography include modified Silica supports, other inorganic oxides and organic polymers such as polystyrene divinylbenzene based. Apart from these, silica modified with organic polymers has also been used [13, 14].

### **1.2.2. Stationary phases in ion-exchange HPLC**

Ion-exchange chromatography can be subdivided into cation-exchange chromatography and anion exchange chromatography. In cation-exchange chromatography positively charged ions bind to a negatively charged ion-exchange sites whereas in anion- exchange chromatography the binding ions are negative and the stationary phase is positive. Stationary phases for both ion-exchange chromatographies can be further classified as either strong or weak. Strong ion-exchangers are totally ionized across the complete operational 2-12 pH range, while weak ion-exchangers are only charged with in a more limited pH-range [15, 16]. Table 1.1 lists the most common functional groups on ion-exchange packing materials.

**Table 1.1. Commonly used functional groups in ion-exchange chromatography**

<b>Name</b>	<b>Functional group</b>	<b>Type</b>
Diethylamine	$-\text{N}^+\text{H}(\text{CH}_2\text{CH}_3)_2$	WAX
Diethylaminoethyl (DEAE)	$-(\text{CH}_2)_2-\text{N}^+\text{H}(\text{CH}_2\text{CH}_3)_2$	WAX
Dimethylaminoethanol	$-\text{O}-(\text{CH}_2)_2-\text{N}^+\text{H}(\text{CH}_3)_2$	WAX
Polyetheleneimine	$-(\text{NH}-\text{CH}_2-\text{CH}_2-)_n-\text{NH}_3^+$	WAX
Carboxymethyl	$-\text{CH}_2-\text{COO}^-$	WCX
Phosphonic acid	$-\text{PO}_3-\text{H}^-$	WCX
Selenonic acid	$-\text{SeO}_3^-$	WCX
Quaternary amine	$(\text{CH}_3)_4 \text{N}^+$	SAX
Quaternary amine	$-\text{N}(\text{CH}_3)_2(\text{C}_2\text{H}_5\text{OH})^+$	SAX
Sulfonic acid	$-\text{SO}_3^-$	SCX

**WAX: weak anion-exchanger; WCX: weak cation-exchanger; SAX: strong anion-exchanger; SCX: strong cation-exchanger.**



### 1.2.3. Mobile phases in ion-exchange HPLC

In ion-exchange chromatography, the mobile phase must be compatible with the detection mode, have an appropriate pH and appropriate concentration of competing ions, provide good selectivity for the separated sample ions, effect the charge status of the analyte and the ion-exchanger, have the appropriate solvent strength to control the sample retention and elution, and provide adequate solubility for the various salts and buffers used as well as for the analyte molecules [12].

In ion-exchange chromatography the application buffer is chosen such that the analyte has a net charge which is opposite to the ion-exchanger functional group, leading to its retention [17]. There are two gradient methods for separating mixture of proteins on ion-exchange column, one is the employment of salt gradient elution- gradual elution of the immobilized proteins from the exchanger is obtained by continuous increase in competing ion concentration. Most often a gradient of salt such as NaCl (0 to 1M) is used for separation of proteins [17]. The salt ions compete with the proteins for the charged binding sites. Proteins are separated based on displacement mechanism by ions in the mobile phase competing with analyte ions for functional groups on the ion-exchange resins. This method allows bound proteins to be desorbed sequentially, according to their net charge and to be eluted from the resin consecutively.

The second gradient method is pH gradient elution. This method is used in the separation of proteins involves a change in pH for the desorption of the protein. Changing the mobile phase pH in the pH gradient results in decreasing the net charge of the proteins until at the  $\text{pH} = \text{pI}$  where the protein has a net zero charge to its theoretically leading to

its elution. Alternatively or in addition to the changed charge status of protein, changing the mobile phase pH can change the charge of the ion-exchanger to the neutral causing elution of the protein. The interaction between the analyte and exchanger is negated and, thus the protein is desorbed. This is usually used in the separation of amphoteric species, which are separated based roughly on their pI (isoelectric point) values [17, 18].

### **1.3. Ion-exchange protein HPLC**

Theoretical aspects and several factors involved in retention of protein have been studied [19-21]. Proteins are large biological molecules consisting of 20 amino acids bonded by peptide bonds between the carboxyl and amino groups of adjacent amino acid residues. The net charge of the protein depends on the summation of the charges of the acidic and basic amino acid groups in the proteins and charges of the N and C termini.

There is a characteristic pH called isoelectric point (pI) where the protein has no net charge (it is neutral). The isoelectric point depends on the number, the type, and the distribution of acidic/basic amino acids in the protein. Proteins have a net positive charge at pH more acidic than the protein's pI and have net negative charge at a pH more basic than the protein's pI.

In electrophoresis, protein separation is dependent on its net charge, with the electrophoretic mobility of a protein being directly related to the net charge of the protein. There are nuances however in ion-exchange chromatography. Studies have reported that proteins being retained by an ion-exchange column at their pI values [20].

There are several factors explaining the difference between protein's elution pH in ion-exchange chromatography and its isoelectric focusing pI. The important reason is that the protein's retention on ion-exchange column is through the interaction of only a small portion of the protein's surface interacting with the ion-exchanger. Proteins are three dimensional molecules with significant diversity in the surface functional group distribution. It is sterically impossible for all of the amino acid residues to bind to the surface of an adsorbent. Only residues that are near the external surface of the protein can make major contribution to protein retention. Proteins through the gradient eventually end up binding through their strongest interaction sites. Hence it is not the charge state of the entire protein, rather the charge state of the interacting surface regions of the protein that influence its retention and elution [20, 21]. Thus the elution pH of a protein in ion-exchange chromatography can be higher or lower than its pI. Other possible sources for deviation from pI are Donnan potential, mixed retention mechanisms of the ion-exchanger such as hydrophobic retention and anion concentration elution effects [15, 22, 23, 24]. In general most proteins elute in the pH range  $\pm 1$  from its pI [24].

The limitation of ion-exchange chromatography is that proteins will be minimally resolved that have similar net charge [25]. The resolution of proteins is high if the mobile phase pH is closer to the pI (isoelectric point) of separated proteins which can enhance the relative adsorption affinity of proteins [26]. When mixture of proteins is being separated it is not possible to maintain mobile phase pH near the pI of each individual protein because usually ion-exchange chromatography is carried out with salt gradient at constant mobile phase pH [26]. Thus pH gradient HPLC has a significant resolution advantage over conventional ion-exchange HPLC employing salt gradient elution.

## **1.4. Conventional Chromatofocusing**

pH gradient elution in ion-exchange HPLC is often used technique because analyte compounds in some cases cannot be sufficiently resolved well by salt gradient elution. The first report using a pH gradient in ion-exchange chromatography in the separation of amino acids was worked Moore et al. in 1951. This work did not draw much attention as gradients in salt concentration or ionic strength dominated in the field. However, in the late 1970s, pH gradient chromatographic technique called chromatofocusing was introduced which increased the usage of pH gradient ion-exchange chromatography in protein separation [26].

### **1.4.1. Background**

The introduction of chromatofocusing (CF) developed by Sluyterman and co-workers in 1978 overcomes the limitations of IEC and separates the proteins near their approximate pI values at low ionic strength [26]. Chromatofocusing is a chromatographic technique that combines the attributes of isoelectric focusing (IEF) and ion-exchange chromatography performing both the separation based on pI values of proteins and band narrowing through the generation of internal pH gradients [27, 28]. Due to its high resolving power and ability to retain proteins in native state, CF became a widely used analytical technique for separation of proteins [29]. This mode of chromatography confines the time of protein exposure to extreme pH values which leads to less denature of proteins [30].

There are two types of chromatofocusing methods, anion chromatofocusing and cation chromatofocusing. Cation chromatofocusing separates proteins according to their pI values on cation exchange column (negatively charged) presaturated with low pH elution buffer. In this technique the column is subjected to a high pH buffer by step change which promotes the formation of an ascending linear pH-gradient [31]. Anion chromatofocusing employs a weak anion- exchange column which has positively charged amine groups equilibrated with high pH application buffer to retain the negatively charged proteins. Proteins are eluted according to their pI values by introduction of low pH polyampholyte elution buffer through a stepwise change to the column. The elution buffer is a mixture of polymeric buffering species that buffers a wide pH range [32, 33]. As a result of the buffering capacities and mutual titration of the mobile and stationary phase functional groups, an outlet pH gradient in time and column pH gradient (internal pH gradient) in distance are produced. These two pH gradients are important parameters in protein separation.

The outlet pH gradient (pH change in time at the column outlet) affects the separation of proteins peaks, where as the internal pH gradient (pH change in distance) provides the focusing effect on peak widths [24]. The generation of these internal pH gradient and outlet pH gradients can be explained by either a buffer interaction model and or an ampholyte displacement mechanism.

In buffer interaction model, the internal pH gradient is formed inside in the column by taking the advantage of buffering action of functional groups of ion-exchanger which resist the sudden change in pH due to the step change. This method views the column as

being divided into many small sections or segments from the inlet to outlet. The shifting of the series of mobile phase aliquots through the successive column segments followed by the formation of equilibrium between each mobile phase aliquot pH passing and the pH of each column segment. The pH of exiting buffer aliquot from the column is the pH of the last segment of ion-exchanger.

For anion-exchange chromatofocusing a continuous internal pH gradient from low pH to high pH and for cation-exchange chromatofocusing an internal pH gradient from high pH to low pH in distance is produced entire the length of the column. The following will go through the process of chromatography according to the buffer interaction model for anion-exchange chromatofocusing. A protein either passes through the column with mobile phase when it and stationary phases are similarly charged or retains by the stationary phase when they are oppositely charged. The movement of the protein occurs when it is exposed to a mobile phase  $\text{pH}=\text{pI}$  zone, as this zone moves through the column. If protein would move ahead of this zone, it would become negatively charged and be retained on the column and be eluted when the  $\text{pH}=\text{pI}$  mobile phase zone passes as protein would become positively charged(or does not have a charge).The process of movement of protein by continuous relocation of its  $\text{pH}=\text{pI}$  zones is repeated until the protein exits from the column. This is the reason for focusing of proteins into narrow peak results because protein cannot get ahead of the  $\text{pH}=\text{pI}$  band [24, 34].

The displacement mechanism also explains the generation of an internal pH gradient in the column as well as an outlet pH gradient formation. This is met by the distribution of mobile phase components according to the affinities for the ion-exchanger. Different

buffer components have different affinities for the ion-exchanger. In anion chromatofocusing, the stronger acidic components bind the first available sites of ion exchanger, next available sites are bound with the next strongest acidic components down the entire column. As the elution buffer is introduced on the column, more acidic components having higher affinity displace the less acidic components having lower affinity, and these weaker acidic components further displace the even weaker components [35]. This successive displacement of lower affinity (less acidity) component by higher affinity (more acidity) component to downstream the column establishes an ascending internal pH gradient in distance from the column inlet to outlet. As the more acidic components successively elute from the column, the pH of the column is decreased in time producing a descending outlet pH gradient. This displacement method promotes the proteins to travel through the column in ampholyte bands.

The pH at the protein eluted is depending on the pH of the ampholyte band containing that particular protein. This outlet pH gradient affects the protein elution according to their pI values [24, 35, 36].

#### **1.4.2. Limitations of Chromatofocusing**

Although chromatofocusing yields considerable success in protein separation, it still exhibits several limitations [24]. There is a disadvantage in using of polyampholyte buffers in the elution buffer as these buffers are expensive and contaminate proteins. It is very difficult to eliminate these buffers from isolated proteins as they form the complexes

with isolated proteins [37-39]. Replacing polyampholytes by simple and common buffer components for elution buffer causes irregularities in linear pH gradients. These pH gradients are not smooth and are non linear, exhibiting spikes and cascade steps when compared to pH gradients produced with polymeric buffers [40].

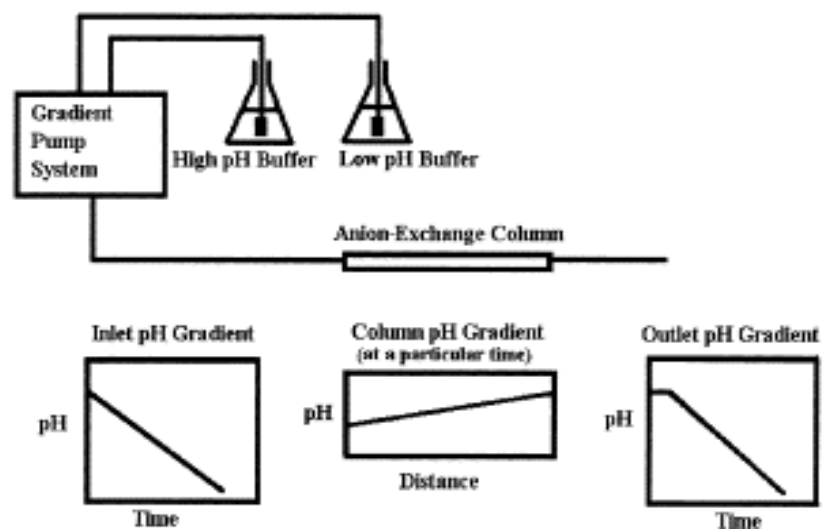
Another limitation is controlling the slope of pH gradient is difficult because low mobile phase concentrations are necessary for this technique, since higher buffer concentrations in mobile phase produce steep pH gradients because mobile phase buffer capacity exceeds the stationary phase buffering capacity. Increased column length and increased buffer capacity of stationary phase can leads to decreased pH gradient slope. Increased column length gives the better separation capacity [24]. The range of pH gradients is also often limited to small pH range as they form inconsistent gradients if the pH range is large [8, 41]. Linear pH gradients generated on low- performance resins are not good when compared to those produced on high-performance ion exchangers. In chromatofocusing technique low-performance exchangers are used usually. However the speed of analysis time is greater with high-performance chromatofocusing than low-performance chromatofocusing is used [42].

### **1.5. Gradient Chromatofocusing**

Gradient Chromatofocusing (GCF), a more flexible approach to chromatofocusing, has been developed by our laboratory to overcome the major shortcomings and to broaden the applications of conventional chromatofocusing. GCF is a chromatographic technique



that generates linear pH gradients using weak anion-exchange HPLC gradient pump system with inexpensive low molecular buffer components [29, 41]. This method shows significant advantages over conventional chromatofocusing and salt gradient ion-exchange chromatography in protein separation on DEAE column. Figure 1.1 shows the general design of gradient chromatofocusing set up. In GCF, initially a weak anion exchange column is equilibrated with a high pH application buffer (composed of multiple basic buffers with evenly spaced pKs). After injecting the sample, a low pH elution buffer (composed of multiple acidic buffers with evenly spaced pK values) is mixed in successively greater proportions with high pH application buffer to generate a linear inlet pH gradient or external pH gradient in time prior to entering the column [8, 43]. This inlet pH gradient is then introduced on high performance anion exchange column which produces the internal pH gradient (column pH gradient) in distance in the column and outlet pH gradient in time at the column outlet [44]. The formation of these two pH gradients is explained by either buffer interaction [34] of column or by the displacement of weaker acidic buffer components with the stronger acidic buffer components on the column [35, 36].



**Figure 1.1. Over view of Gradient chromatofocusing technique set up**

GCF has an advantage of producing smooth linear pH gradients using simple buffer components which is not possible in conventional chromatofocusing (although as pointed out later this generation of smooth pH gradients takes considerable trial and error effort, which is addressed as one of the specific aims of this project). The gradient mixing allows gradual change in pH instead of instantaneous change in pH that a step change causes in chromatofocusing. Gradual introduction of elution buffer onto the column gives smooth linear and reproducible pH gradients because any disturbances or irregularities in pH- gradients are also gradual. Li Shan and D.J.Anderson used different composition of gradient mixing of elution buffer (%B) in single run to produce reproducible linear outlet and inlet pH- gradients on weak anion exchange column (Protein –Pak DEAE 15HR,15 $\mu$ m,1000Å ) over a wide range of pH (7.5-3.5) in their work. They compared these results with the experiments employing conventional chromatofocusing using the same buffer components and the same packing materials. They found that the conventional technique produced pH- gradients were too steep and not smooth which led to poor resolution [43].

GCF has also the flexibility of obtaining required pH- gradient slope by varying the slope of inlet pH- gradient via changing the gradient time program [29, 41, 43]. Chromatography experiments of a standard protein mixture of conalbumin, BSA,  $\beta$ -lactoglobulin A,  $\beta$ -lactoglobulin B and ovalbumin using GCF shallow pH gradients showed better resolution when compared to steep gradients [24, 45]. A high linear and controllable outlet pH-gradients were also successfully obtained on strong anion-exchange(Mono Q 4.6/100 PE ,10mm $\times$  10mm,10 $\mu$ m) in the pH range 9.5-3.7 as well as on different strong cation-exchange columns in the pH range of 4-10 applying the same

GCF gradient mixing principle [46]. Reproducible linear pH-gradients from pH 8.5 to 4.0 produced on capillary Mono P column as well as on PL-SAX column [47].

Another significant advantage of GCF over CF is its flexibility in employing a wide range of buffer concentrations by using few common buffering species without affecting the slope of the pH-gradient for optimizing the protein separation. Shan and Anderson generated the same linear outlet pH-gradient profiles using seven different mobile phase systems which differ in their buffer concentrations on a Protein-Pak DEAE polymethacrylate column (8HR, 8 $\mu$ m dia, 1000Å pore dia). They chromatographed a sample mixture (pI given in parentheses) of conalbumin (5.9), BSA (4.7.4.9), ovalbumin (4.7),  $\beta$ -lactoglobulin A (5.23) and  $\beta$ -lactoglobulin B (5.13) using the same outlet pH gradients but generated by different buffer concentrations. Results showed an increased resolution and decreased peak width with increased mobile phase concentration. For instance, in the separation of  $\beta$ -lactoglobulin A and B, resolution is increased from 1.5 to 2.3 with the increased buffer components concentration in the elution buffer from 6.25mM to 25.0mM. The gain in the resolution of two peaks of ovalbumin in the sample from 0.7 to 2.4 is also noticed when concentration of buffer components in the elution buffer was increased from 6.25mM to 37.5mM [43]. The separation of five protein mixture on Mono P column employing the gradient chromatofocusing runs at different mobile phase concentrations (75,50,12.5mM) was also demonstrated. All five proteins well resolved at higher buffer concentration 75mM. Improved resolution was obtained for the separation of individual protein isoforms using higher buffer concentrations with same column and buffer components [41]. Thomas Anderson and co-workers also reported the influence of varying buffer concentrations (5,10,20 and 40mM) on

resolution and the peak widths of  $\beta$ -lactoglobulin A and B using the same outlet pH gradients(6.8-4.3) produced on fused silica capillaries packed with PL-SAX column( 10cm x 0.32 mm i.d,10micron,1000Å).Their results showed a better resolution obtained with 10mM buffer concentration [47].

Proteins are focused into narrow bands resulted in better peak shapes in GCF compared to NaCl gradient IEC. Superior separation of  $\beta$ -lactoglobulin A and B in GCF was demonstrated with the optimized resolution of 2.33 compared to 1.1 resolution in NaCl gradient chromatography at constant pH using the same anion-exchange material (Protein-Pak DEAE polymethacrylate 8HR,8 $\mu$ m dia,1000Å pore dia) [43]. The separation of standard protein mixture containing cytochrome C,myoglobin, conalbumin,  $\beta$ -lactoglobulin A and  $\beta$ -lactoglobulin B with pH gradient IEC (GCF) was better compared to salt gradient using the same pH gradient profile (10.5 to 3.5) [48]. Gradient chromatofocusing of  $\beta$ -lactoglobulin A and  $\beta$ -lactoglobulin B mixture showed better focusing capability obtaining one-third the peak width and demonstrated 3-fold resolution gain compared to peaks of NaCl gradient ion exchange using the same Mono P column and injecting the same amount of protein sample [41]. Results of GCF technique were compared with the result of conventional CF technique in the analysis of the standard protein mixture (conalbumin, BSA,  $\beta$ -lactoglobulin A and  $\beta$ -lactoglobulin B and ovalbumin) using the same Mono P column and the same pH gradient slope. Better resolutions and comparable peak widths achieved with GCF technique for several protein pairs [41].

Comparison of gradient chromatofocusing results with different columns was also demonstrated [41, 47]. PL-SAX material has better focusing effect almost 2-fold decrease in peak width than Mono P column in the analysis of  $\beta$ -lactoglobulin A and  $\beta$ -lactoglobulin B using the same pH gradient. It was also reported that the apparent pI values of these proteins approximately closer to their actual isoelectric points on the PL-SAX column [47]. Changing the column from DEAE to Mono P optimum resolution increased from 2.33 to 4.19 for  $\beta$ -Lac A and  $\beta$ -Lac B and optimum peak widths decreased from 0.076 to 0.044 for  $\beta$ -Lac A and 0.088 to 0.050 for  $\beta$ -Lac B. By applying the nearly same pH gradient slopes, Mono P column showed greater separation compared to DEAE column with increased resolution from 2.47 to 11.51 in the analysis of ovalbumin isoforms and decreased peak widths for oval-1 and oval-2 from 0.11 to 0.054 and from 0.20 to 0.062 respectively [41].

Two-dimensional liquid chromatography interfaced with mass spectrometry has been widely used potential technique for the separation of protein digested samples and proteomic studies by performing the ion-exchange technique as the first dimension and RP-LC as the second dimension [48, 49]. GCF has the advantage of giving an estimate of the protein pI and being able to be directly interfaced to a mass spectrometer when volatile buffers are used [50].

In order to expand the capabilities and advantages of the GCF, we worked out to optimize the different aspects described in following chapters.

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## **CHAPTER II**

### **IMPROVED LINEAR pH GRADIENT GENERATION IN GRADIENT CHROMATOFOCUSING USING NOVEL APPROACH EMPLOYING BRIDGING BUFFERS**

#### **2.1. Introduction**

Gradient chromatofocusing (GCF) is a chromatographic technique developed by the Anderson group that generates linear pH gradients using weak anion-exchange HPLC with inexpensive low molecular buffer components [1-4]. Other groups referred it as pH-gradient ion-exchange chromatography in their articles [5, 6]. In GCF an elution buffer (consisting of multiple acidic buffers with evenly spaced pKa values) is mixed in successively greater proportions with application buffer (composed of multiple basic buffers with evenly spaced pks), to generate a pH gradient in time prior to entering the column. GCF utilizing linear pH gradients provides unique capabilities in pI- based

protein separation when compared with the conventional ion-exchange and conventional chromatofocusing chromatography. It also has optimization advantages over conventional chromatofocusing through its ability to manipulate the pH gradient profile by changing the slope of the external pH gradient and the flexibility in employing a wide range of buffer concentration [3]. Increased resolution and decreased peak widths are achieved by this technique in protein separation [3,4]. Expensive polymeric ampholyte buffers used in conventional chromatofocusing are replaced by less expensive common buffer components to produce linear pH-gradients [1-4].

An objective of the pH gradient ion-exchange chromatography is to separate the complex mixture of biomolecules with high resolution using controllable and reproducible linear pH gradients. Formation of pH gradients prior to the column by proportioning pumps allows the generation of pH-gradients with slopes that are independent of the buffer concentration. The slope of the pH gradient plays a pivotal role in both the retention time of as well as band width of the protein peak.

Generation of smooth linear pH gradients requires considerable trial and error effort. Work on generating linear pH gradients on weak anion-exchange columns has been reported by our group [1-4]. Liu and Anderson attempted to produce a linear pH gradient ranging pH from pH 7.4 to 3.5, however shape of the pH-gradient is not totally linear or smooth [1]. Shan and Anderson had to use five different gradient time programs to produce reproducible linear pH gradients over the same pH range 7.5-3.5 when using seven different buffer systems differ in their concentrations [3]. They had to use complex gradient time programs when high buffer concentration mobile phases were used.

Irregularity was noted between 7.1-6.5 pH regions of the pH gradients [3]. In another study by our group a linear pH gradient from pH 9-2.6 was accomplished to separate proteins for on-line mass spectrometric analysis [7]. The gradient program used to generate the outlet pH gradient (9-2.6) was a complicated six step gradient time program. There were also irregularities at several pH regions of this gradient.

In work done by others on weak anion-exchangers a pH gradient within the pH range 8.5-4.0 was produced using low molecular buffer components [8]. Different gradient programs were used to obtain linear pH gradients in this work. However a sharp drop was observed in the pH 8.5-7.7 region of the gradients and the shape of the pH gradients was concave from pH 7.5-5 for two of the three pH gradients [8].

Several studies have employed pH gradients on strong anion-exchange columns in the analysis of proteins. A pH gradient from pH 10.5 to 3.5 separated a standard protein mixture using different buffer components with different concentrations employing multiple linear gradient steps programs [9]. However the generated pH gradient was curved and irregular. Another study reported the separation of MnP isozymes utilizing a 6.0-4.0 pH gradient in a linear 0-100%B gradient program [10]. The resulting pH gradient showed a precipitous drop and a concave shape from pH 6-5 after the plateau region. T.Ahmed and his co-workers generated various pH gradients ranging from 11.5-4.0, 10.5-4.0, 10-4 and 8.7-3.7 [5]. The shapes of these pH gradients were irregular with a sudden large drop after the plateau regions of the gradients. In another work a decreasing linear pH gradient (pH range 10-4) was produced for the anion-exchange chromatography of a monoclonal antibody from hybridoma cell culture supernatant using

the 20mM buffer components. The resulting pH gradient showed shallowness after the irregular plateaued region of the gradient. [6].

Formation of pH gradients on cation-exchange columns have also been reported in chromatography of proteins. Farnan and Moreno showed sensitive separation method utilizing a pH gradient (6-9.5) generated to separate monoclonal antibodies [11]. In their work they studied the effect of altering the relative amounts of the buffer components and also the effect of changing the concentration of the buffering species in the formation of pH gradients via 0-100% linear gradient elution program. These gradients had the shapes of a large peak increasing from pH 6 to a maximum pH 9 and then dropping back to a pH 6.

Generation of pH gradients on all types of ion-exchange columns (porous and non porous, strong and weak, anionic as well as cationic) over a pH range from 2.4-10.8 was done by employing 4mM basic buffer components in both application and elution buffers through 0-100%B linear gradient program [12]. The pH gradients produced on non porous weak anion-exchange columns were irregular and not controllable. For the porous anionic (weak and strong) and cationic (weak and strong) columns, two of the four pH gradients were fairly linear while the other two showed irregularities in several pH regions of the gradients. Review of the literature thus confirms the difficulty in generating smooth linear pH gradients in ion-exchange HPLC.

In the present study smooth linear pH-gradients were generated with simpler gradient programs by using a new approach involving bridging buffers. In this approach the



highest  $pK_a$  acidic buffer component is added in the basic application buffer and the lowest  $pK_a$  basic buffer component is included in the acidic elution buffer.

## **2.2. Experimental**

### **2.2.1. Materials**

Bis-tris methane (catalog no. A0293971) was from Fisher (New Jersey, USA). 2, 3-dimethyl pyridine (catalog no. L3501), acetic acid (catalog no.695092), lactic acid solution (catalog no.252476), 4-methylpyridine (catalog no. 239615), pyridine (catalog no. 270970), 4-chlorophenyl acetic acid (catalog no. 139262) were purchased from Sigma-Aldrich (St.Louis, MO, USA). Conalbumin (catalog no.C-0755), rat albumin serum (catalog no. A6272),  $\beta$ -lactoglobulin A (catalog no. L7880), methanol (catalog no.14262) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Bulk anion-exchange material was Protein-Pak DEAE 8HR, 8 $\mu$ m diameter, 1000 Å pore diameter, DEAE functionalized polymethacrylate) from Waters (Milford, MA, USA). HPLC grade water was generated by a Barnstead Nano system with a Nanpure Diamond Pack Organic Free DI cartridge from Thermo Scientific (West Palm Beach, FL, USA). All the solvents were filtered through 0.45 $\mu$  cellulose ester membrane filters from Millipore (Billerica, MA, USA).

In buffer system IA, mobile phase A (high pH application buffer) consisted of 10mM of each 2, 3-dimethyl pyridine (6.57), and 3-methyl pyridine (5.68). Mobile phase B (low pH elution buffer) consisted of 10mM of each acetic acid (4.76) and lactic acid (3.81), 2-

hydroxy benzoic acid (2.97). In buffer system II, application buffer A (high pH buffer) consisted of 10 mM of each 2, 3- dimethyl pyridine (6.5), 4-methyl pyridine (6.02), 3-methyl pyridine (5.68), and pyridine (5.25). Elution buffer B (low pH buffer) composed of 10 mM of each acetic acid, 4-chlorophenyl acetic acid (4.19), and lactic acid. The pKa of each buffer component is given in the parentheses.

### **2.2.2. Chromatographic conditions and pH gradient measurement**

The HPLC system consisted of a System Gold 126 solvent module gradient pump system, System Gold 508 Auto Sampler, System Gold 168 Detector from Beckman instruments (Fullerton, CA, USA). All the data collected and analyzed using 32 Karat Gold software (version 8). ). DEAE anion-exchange column (4.6 X 100 mm, 8HR, 8 $\mu$ , 1000Å). The pH gradient was monitored online after the detector using a FC45C flow cell equipped with a flow-through pH electrode (from Sensorex, S450CD). The volume of the pump mixing chamber to the column was 0.8 mL and the void volume of the column was 0.33 mL. The flow rate of the mobile phase was 1.0 mL/min. The sample injection volume was 100 $\mu$ L. The column was equilibrated for 50 min with the application buffer prior to the start of each run till the column pH reaches the pH of the application buffer.

The pH gradients were monitored on-line after the detector by collecting pH measurements using a FC45C flow cell (200 $\mu$ L) equipped with a flow-thru pH electrode from Sensorex (S450CD) that connect directly to the tubing used by HPLC system. A Denver Instrument pH meter (Model 250) with data logging capabilities was interfaced

by the micro flow-thru electrode for data acquisition every two minute intervals. The micro flow-thru electrodes allow the pH measurements at the low flow rates (100 uL/min). These electrodes are advantageous because they measure pH on-line in no time as opposed by taking the pH measurements over time by collecting fractions. This process eases the laborious efforts that were once required to measure pH of the fractions manually in development of linear gradients.

### **2.3. Results and discussion**

Generation of pH-gradients is crucial to the separation of proteins because it largely determines the quality and usefulness of the separation. A pH-gradient that is appropriate to the separation can be generated when the slope, shape and range of the pH-gradient should be well controllable. The pH-gradient should be reproducible, linear and smooth as this largely determines the resolution of the separation. Experiments were done to get smooth linear descending pH-gradients without a column in place by delivering different proportions of application and elution mobile phases by HPLC gradient pumps. In these experiments trial and error gradient programming was done using the buffer system IA. The buffer components of buffer system IA is given in Table 2.1. But the resulted pH gradients were irregular in some pH regions and showed poor linearity with pH range from 8.5 to 2.5.

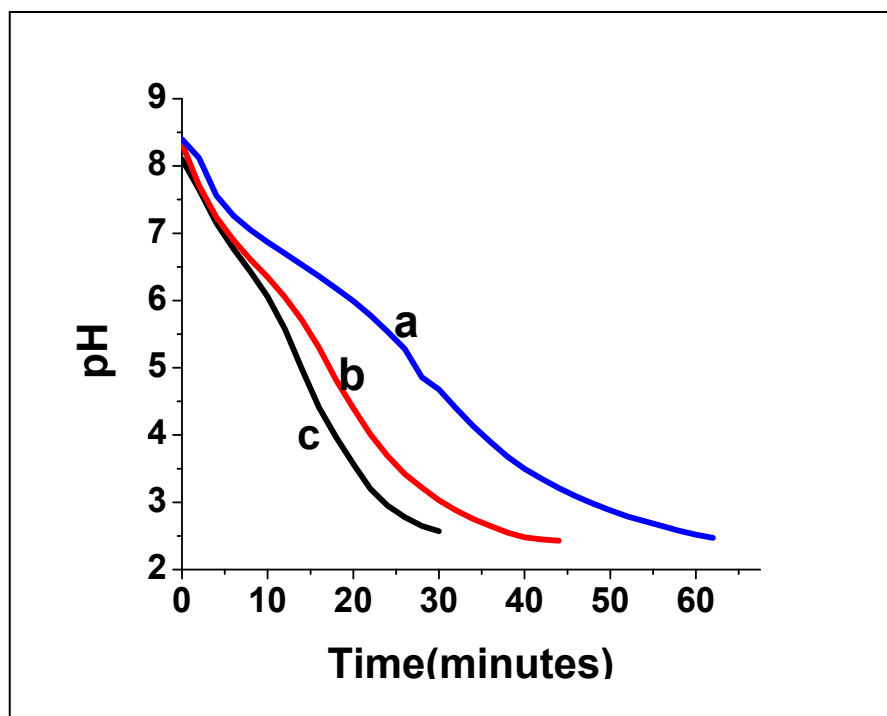
**Table 2.1. Buffer components used for buffer system IA**

<b>Basic application buffer(10mM)</b>	<b>Acidic elution buffer(10mM)</b>
2,3-dimethyl pyridine (6.57)	Acetic acid (4.76)
3-Methyl Pyridine (5.68)	Lactic acid (3.81)
	2,5-dihydroxybenzoic acid (2.97)

### 2.3.1. Development of linear pH gradients by manipulation of gradient programs

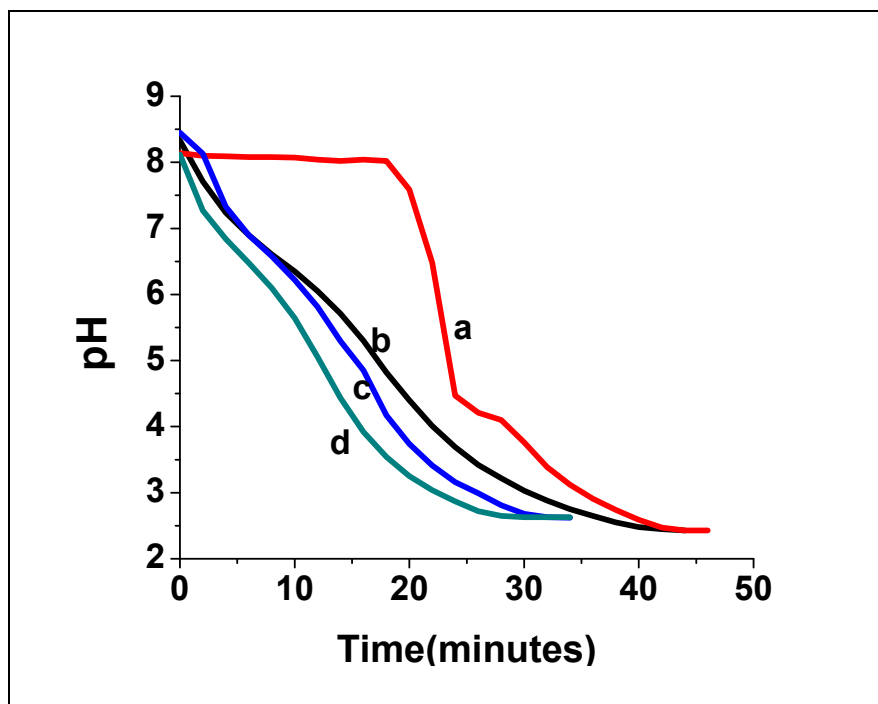
Achieving good linearity with a smooth slope in the pH-gradients is a labor intensive and time consuming process. Figures 2.1, 2.2 and 2.3 show the formation of poor pH gradient using dead volume union in place of a column generating a pH gradient employing different pump-proportioned external mixing of increasing elution buffer mixing with decreasing amounts of application buffer, both containing 10 mM of 2,3-dimethyl pyridine ( $pK_a$  6.57), 10 mM of 3-Methyl pyridine (5.68), 10 mM acetic acid (4.76), 10 mM of lactic acid and 10 mM of 2,5-dihydroxy benzoic acid (2.97). The  $pK_a$  values of these buffering species are evenly spaced (approximately 1) throughout the pH range 8.5-2.5.

In Figure 2.1, pH gradients a, b and c were produced with a 0%-100%B linear gradient program for different length of gradient times by delivering a different percentage of mobile phase per minute. The resulting pH gradients were irregular. The pH gradient plot a in Figure 2.1 showed a drop in pH at the beginning first three minutes, shallowness in the mid pH region followed by a short steep portion and ending with a more shallow pH gradient. To improve the linearity, the rate of gradient change in the problematic region was varied as given in plots b and c in Figure 2.1. Although the increased gradient rate did eliminate some of the first plateau region in the gradient (pH range 7.2-6.7), a significant plateau region still remained.



**Figure 2.1. Non-linear pH-gradients formed by buffer system IA without column. Gradient time programs used are 0-100%B in 60min, 40 min and 28 min for a, b, and c pH-gradients respectively**

To achieve better linear pH gradients without plateau regions, multiple slope time gradient steps were incorporated into the program as opposed to previously used one slope 0-100% B linear gradient time program. The established pH gradient profiles with these multi step programs are shown in Figure 2.2. The first attempt (plot a) utilized program that generated a precipitous drop in the pH from 8 to 4 after a long plateau region followed by irregularity at the end portion of the gradient. The mobile phase mixing rate was 2.5% / min (plot a in Figure 2.2). In order to correct slope of the pH gradient, higher gradient was attempted to deal with beginning plateau and mid regions. The modified mobile phase rates throughout the pH gradients were 3.33% / min (plot b in Figure 2.3), 3.4% / min - 3.57% / min – 3.7% / min (plot c in Figure 2.3); 3.58% / min – 3.8% / min – 3.57% / min (plot d in Figure 2.3).



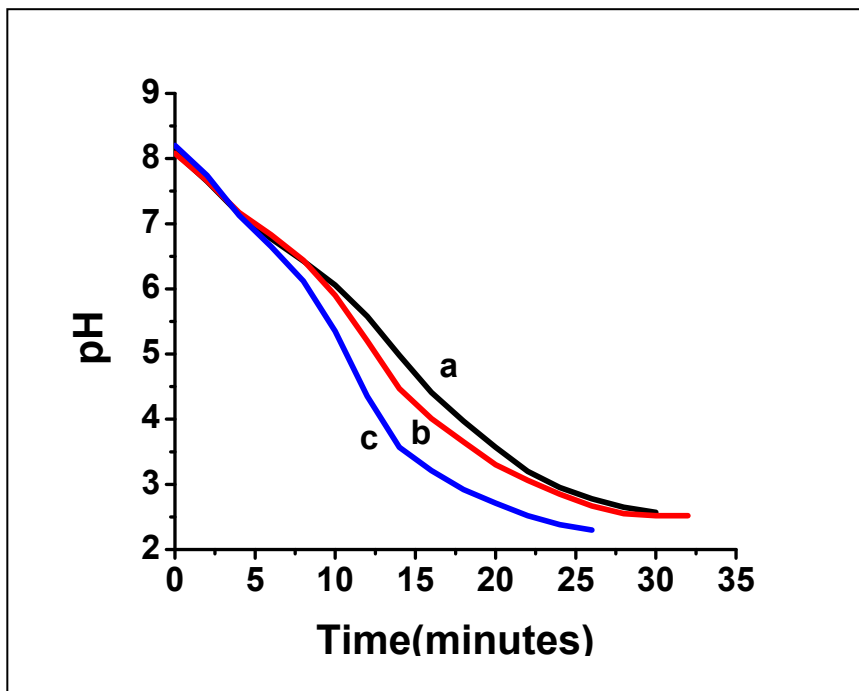
**Figure 2.2. Non-linear pH gradients formed by without column with buffer system**

**IA. Gradient time programs used for (a) 0%-100% B in 15 min, 40-70%B in 10 min, 70-100%B in 15 min; (b) 0%-2% B in 1 min, 2-22%B in 6 min, 22-32%B in 3min, 32-53%B in 14 min, 53%-100%B in13.5 min (c) 0-20.4% B in 6 min, 20.4-46.4% B in 7 min, 46.4-100% B in 15.7 min; and d) 0%-17.9% B in 5 min, 17.9-52.1% B in 9 min, 52.1% B-100% B in 13.4 min**



These modified time programs did not yield a completely linear pH gradient. In general these pH gradient profiles were steeper in the first three minutes, shallow from 3 to 10 min followed by a concave shape.

Other multiple linear step gradient programs were then attempted to eliminate the irregularity in the pH gradients, however the slope at the beginning pH region of the gradients could not be controlled. The resulting pH gradients (plots a, b and c) are given in Figure 2.3. In these experiments attempts were made to decrease the shallowness of the mid pH regions as well as decrease the steepness of the pH gradients (plots a and b in Figure 2.3) by giving the mobile phase at faster rates. The gradient program resulting in plot c (Figure 2.3) pH gradient yielded the best resulting however the pH gradient still had a steeper drop in mid region.



**Figure 2.3. Nonlinear pH-gradient formation with buffer system IA without column. Gradient time program were (a) 0-100% B in 28 min (b) 0-20% B in 6 min, 20% B-50% B in 6 min, 50% B-100% B in 15 min (c) 0-10% B in 3 min, 10-30% B in 4min, 30-66% B in 6 min, 66% B-100% B in 10 min**

These multiple step gradient trials by varying the gradient rate continuously at different time periods of the pH gradient were not able to yield a smooth linear gradient.

### **2.3.2. Improved linear pH-gradient generation by *bridging* buffer components between basic application buffer and acidic elution buffer**

A new approach to generate linear pH- gradients has been worked out. As discussed above attempt to achieve smooth linear pH gradient by varying gradient rate is a very laborious process that does not produce completely linear pH gradients. This approach does not address the inherent problem that lack of buffering capacity in mid pH gradient regions due to there being progressively less application buffer and successively more elution buffer in the mid region. This leads to a sudden drop in pH even though the  $pK_a$  of buffer components are evenly spaced. This reduced buffering capacity leads to the noted irregularities in the pH gradients.

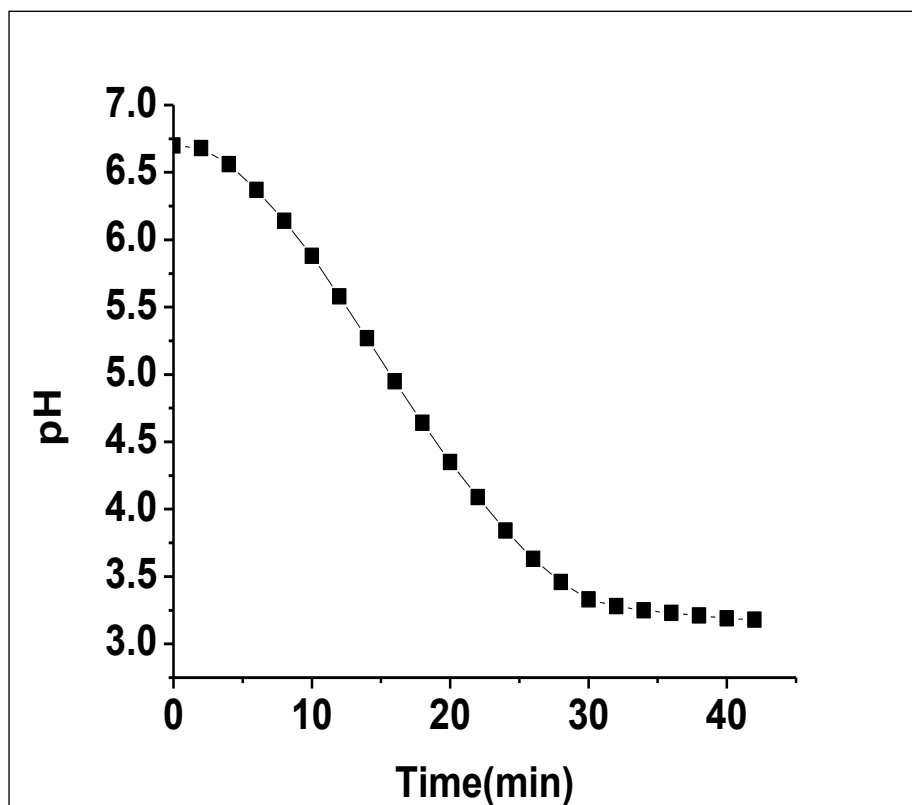
The problem was solved by introducing “bridging” buffers components between application buffer and elution buffers. The composition of buffer systems IA was modified by adding the lower  $pK_a$  application buffer component to the elution buffer and high  $pK_a$  elution buffer component to the application buffer. This bridging of the application and elution buffer solutions leads to sufficient buffer capacity throughout the desired pH range which results in smooth linear pH-gradients. The buffer system in Table 2.1 was modified by adding the 5mM of acetic acid ( $pK_a=4.76$ ) to the application buffer and 5mM of 3-methyl pyridine ( $pK_a=5.68$ ) to the elution buffer. This modified buffer

system IA (Table 2.2) produced an excellent linear pH gradient with a simple 0%B-100%B linear gradient program as given in Figure 2.4.

Adding the high  $pK_a$  elution buffer component to the application buffer lower the pH of the application buffer while at the same time adding the low  $pK_a$  elution buffer component increased the pH of the elution buffer. The pH range of the gradient decreased from 8.5-2.5 to 6.7- 3.0 by adding the 5mM acetic acid to application buffer and 5mM of 3-methyl pyridine to the elution buffer that can be observed in Figure 2.4.

**Table 2.2. The modified buffer system IA with bridging**

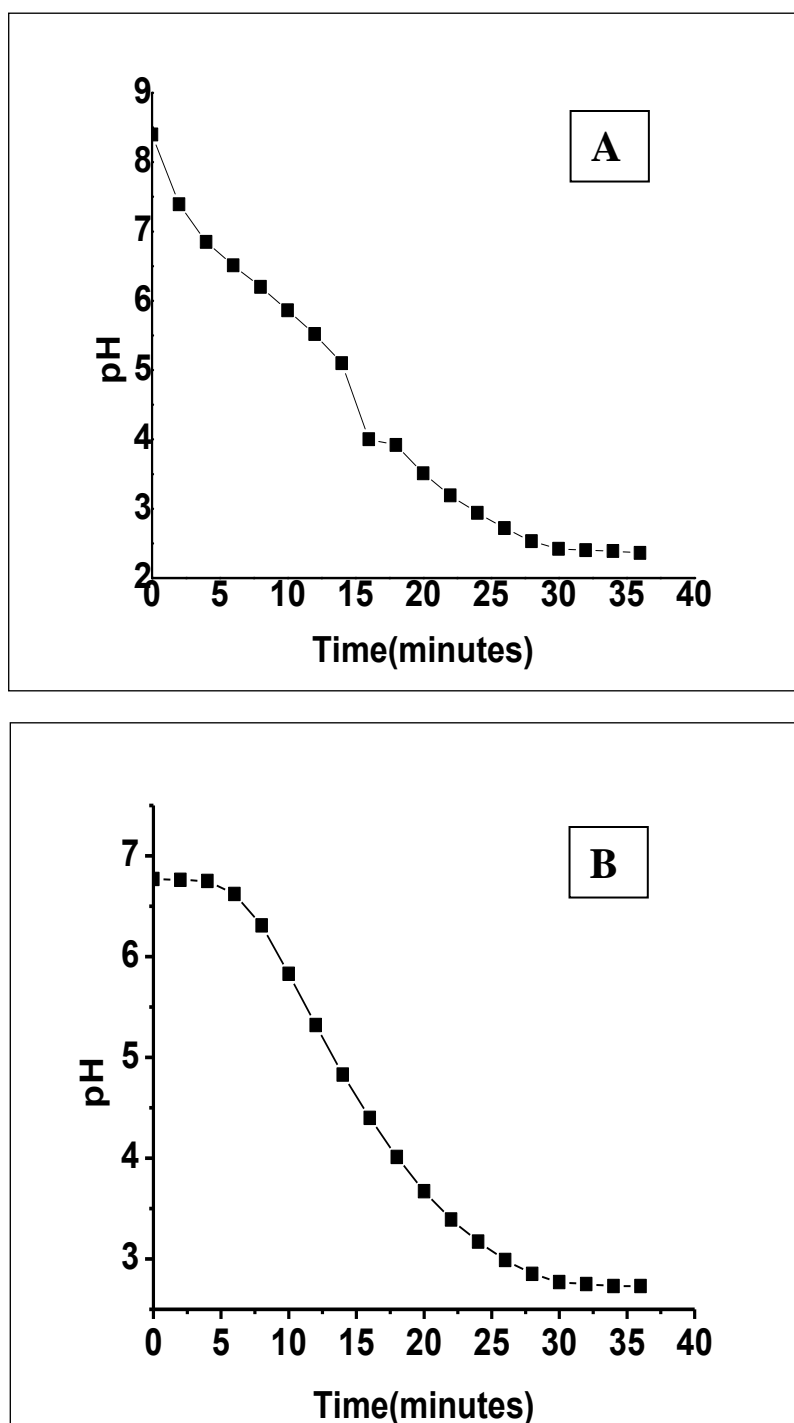
<b>Basic application buffer  (each component10mM)</b>	<b>Acidic elution buffer  ( each component10mM)</b>
2,3-dimethyl pyridine  (6.57)	Acetic acid (4.76)
3-Methyl Pyridine  (5.68)	Lactic acid (3.81)
	2,5-dihydroxybenzoc acid  (2.97)
<b><i>Bridging (5mM</i></b>  <b><i>Acetic acid (4.76)</i></b>	<b><i>Bridging (5mM)</i></b>  <b><i>3-methyl pyridine (5.68)</i></b>



**Figure 2.4. The pH gradient profile produced with bridging buffer system IA with 0-100% B in 30 minutes (3.33%/ min) without column in place**

### **2.3.3. Generating smooth linear pH gradient with buffer system II A**

This bridging technique has also been utilized to generate smooth linear pH-gradient without the column with the buffer system IIA which has more buffer components compared to buffer system I. Buffer system IIA is given in Table 2.3.  $pK_a$  values of these buffering species are evenly spaced (approximately 0.5) over the entire pH range from 6.7 to 2.5. Figure 2.5 shows the linear pH-gradient profiles with buffer system IIA with bridging and without bridging. The buffer component 4-chlorophenyl acetic acid is only partially soluble in water. Thus 5% methanol was used in the application and in the elution buffer of buffer system IIA with and without bridging.



**Figure 2.5. pH gradients produced by buffer system IIA with no column in place (A) without bridging and (B) with bridging. Gradient programs were used for (A) and (B) 0% -100% B in 30 min**



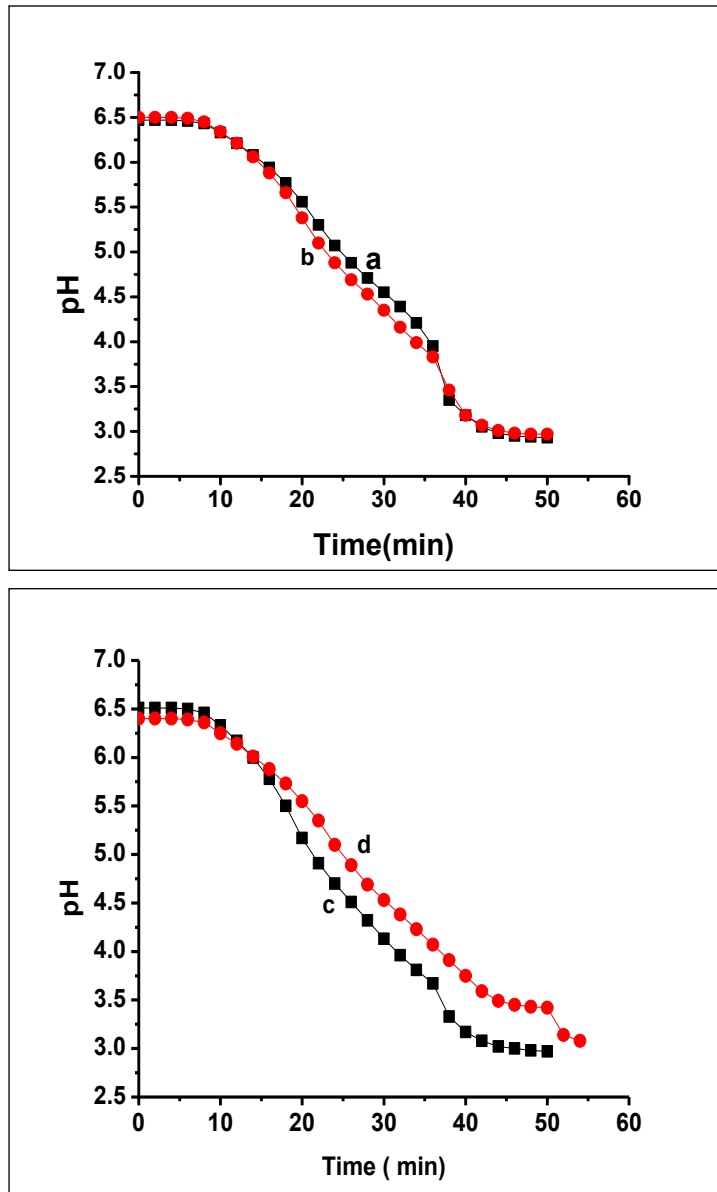
**Table 2.3. Buffer components used for Buffer system IIA without bridging and with bridging.**

Without bridging (each component 10 mm)		With bridging (each component 10 mm)	
Applica tion buffer	Elution buffer	Application buffer	Elution buffer
2,3- dimethy l pyridin e(6.57)	Acetic acid (4.76)	2,3-dimethyl pyridine(6.57 )	Acetic acid (4.76)
4- methyl pyridin e (5.68)	4- chlorophen yl acetic acid (4.19)	4-methyl pyridine (6.02)	4- chlorophenyl acetic acid (4.19)
3- methyl pyridin e (5.68)	Lactic acid (3.81)	3-methyl pyridine (5.68)	Lactic acid (3.81)
Pyridin e (5.25)	2, 5- dihydroxy benzoic acid (2.97)	Pyridine (5.25)	2, 5-dihydroxy benzoic acid (2.97)
		Bridging (5mM)  Acetic acid	Bridging (5mM)  Pyridine

#### **2.3.4. Producing linear pH-gradient profiles on weak anion-exchange column (DEAE column)**

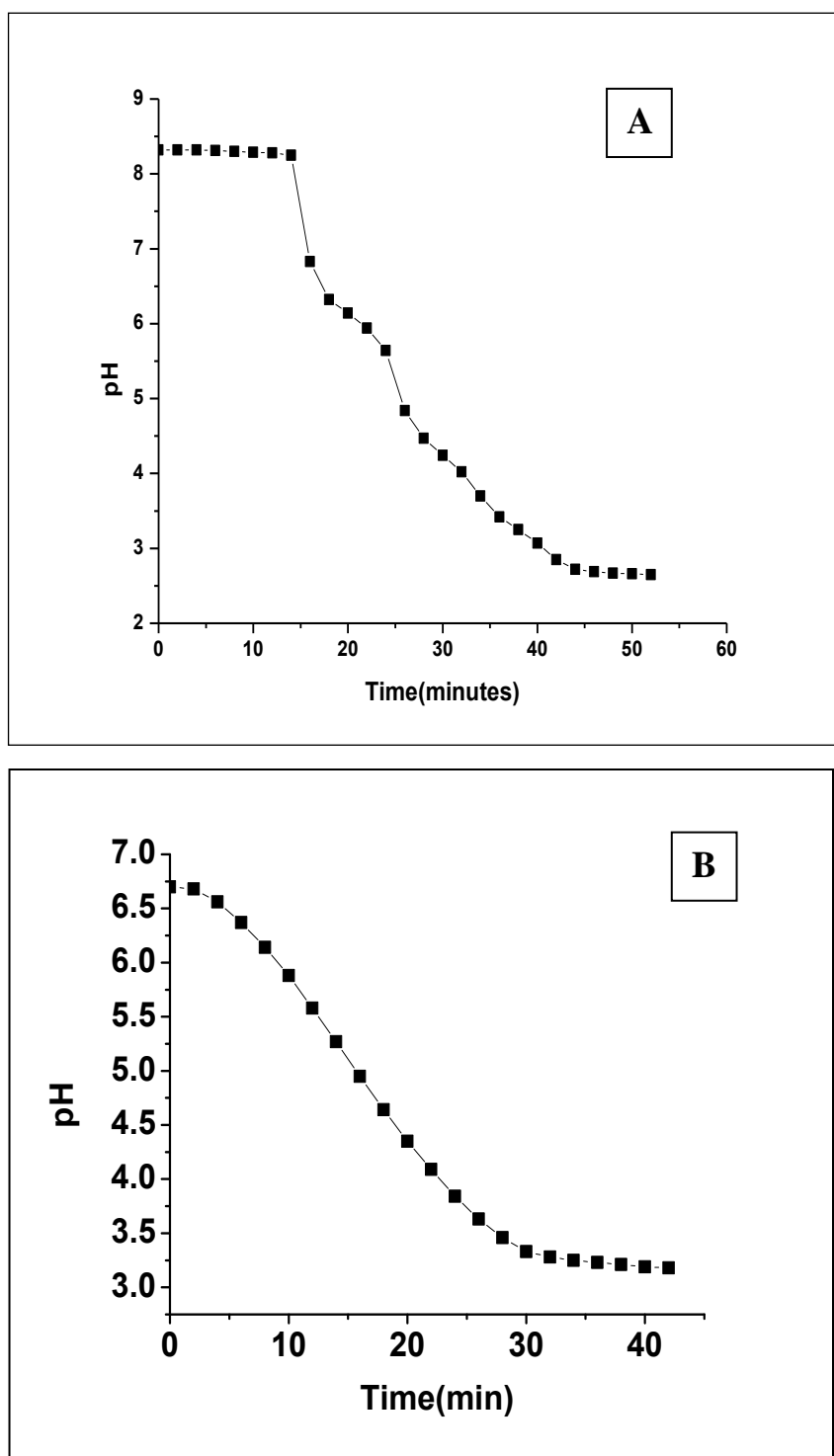
After attaining the linearity of the pH gradients for the two buffer systems (IA & IIA) without column in place, experiments were done for generating linear pH-gradients on the DEAE.

Non-linearity in the pH-gradient was noted in column studies employing the bridging buffer system given in Table 2.2. The same application and elution buffers were used as previously used without column to produce linear pH gradients (Figure 2.4). But there were drops in pH from 36 min to 38 min at the end region of pH-gradients which can be observed in Figure 2.6 (plot a). Since the drop in pH was in the 3.9 to 3.35 pH range, the region at which lactic acid buffer ( $pK_a$  3.81), lactic acid concentration was successfully increased from 10 mM to 15 mM, to 20 mM. The resulting pH gradients, however, did not improve as shown in Figure 2.6. The possible reason for not achieving linearity in the end region of the pH gradient is the strong retention of the most acidic component 2, 5-dihydroxy benzoic acid in its anionic form on the column. The strong retention of the anion of 2, 5-dihydroxy benzoic acid displaces the anionic form of lactic acid on the column where it combines with  $H^+$  in the mobile phase leading to decrease in pH.

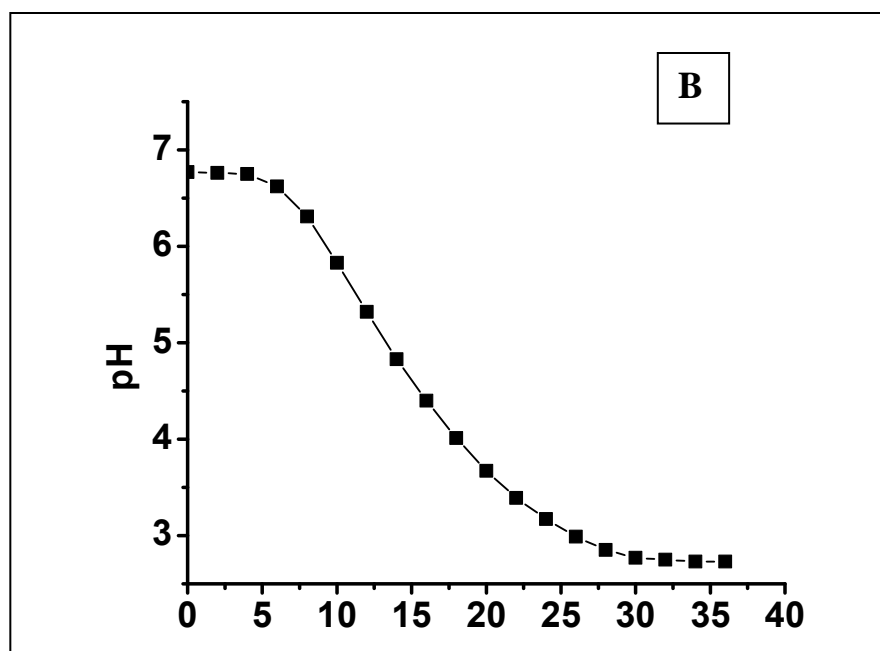
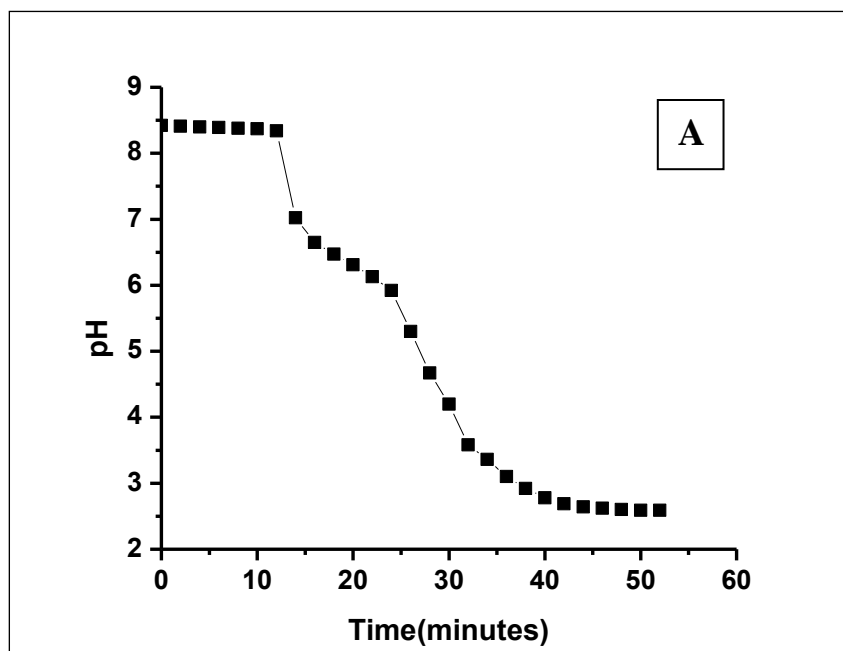


**Figure 2.6. Lactic acid concentration increased from 10mM (a) to 15 mM (b) and 20mM (c), 2, 5-dihydroxy benzoic acid concentration decreased from 10mM to 5mM (d). Gradient program used was 0-100%B in 40 min.**

Eventually the anionic form of the lactic acid are displaced and there is limited binding of anionic form of 2, 5-dihydroxy benzoic acid (anion-exchanger sites filled up), so then the pH drops rapidly to the elution buffer pH. The 2, 5-dihydroxy benzoic acid was removed from the elution buffer to eliminate this non-linear pH drop at the end region of the pH-gradient. Gradients of the resulting modified buffer system IA of Table 2.2 and the buffer system IIA (Table 2.3) without 2, 5-dihydroxy benzoic acid, pH gradients were then run on DEAE ion-exchanger with and without bridging. The resulting pH gradients are shown in Figures 2.7 and 2.8. The outlet pH gradients produced without bridging had a precipitous drop after the beginning plateau region and showed irregularity throughout the pH gradient. The bridging buffer system however yielded a smooth linear pH gradient. The same results were obtained for Buffer System IIA without 2, 5-dihydroxy benzoic acid are given in Figure 2.8.



**Figure 2.7. Outlet pH gradient on DEAE column generated from 0-100% B gradient of Table 2.2 buffer system IA without 2, 5-dihydroxy benzoic acid without bridging (A) and with bridging (B)**



**Figure 2.8. Outlet pH gradient on DEAE column generated from 0-100% B gradient of Table 2.3 buffer system IIA without 2, 5-dihydroxy benzoic acid without bridging (A) and with bridging (B)**

Finally of separation standard proteins were run employing the gradient of bridging buffer system IA (Table 2.2) and IIA (Table 2.3) both of which had the buffer component 2, 3-dimethyl pyridine. It was found that several of protein peaks were missing (either from irreversible adsorption or precipitation of the proteins). It was found that removal of the 2, 3-dimethyl pyridine replaced with bis-tris methane in the application buffers solved the problem. The final buffer systems (buffer system I B and buffer system II B) that yielded excellent linear pH gradients which were compatible with the protein chromatography are given in Table 2.4, with the resulting linear pH gradients plotted in Figure 2.9.

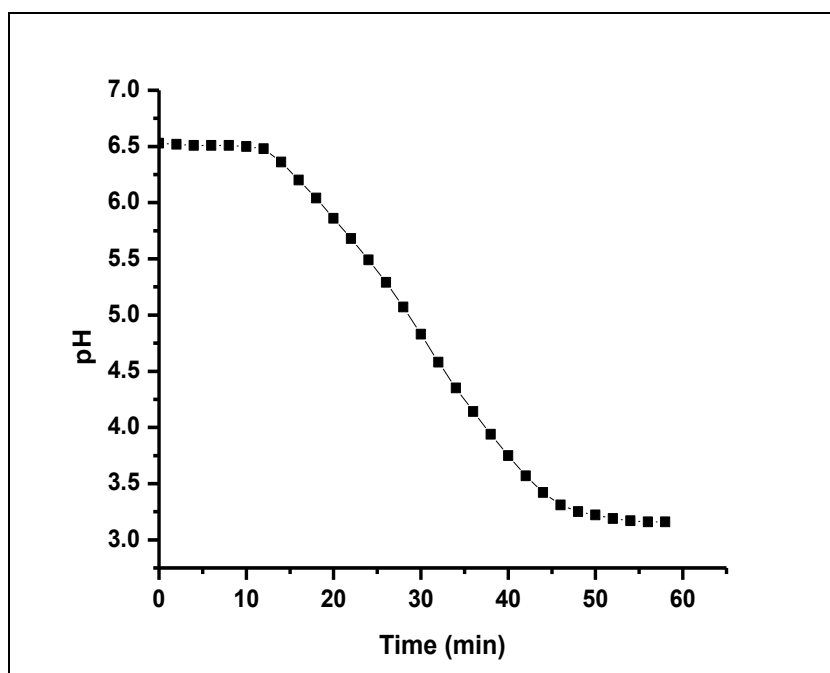
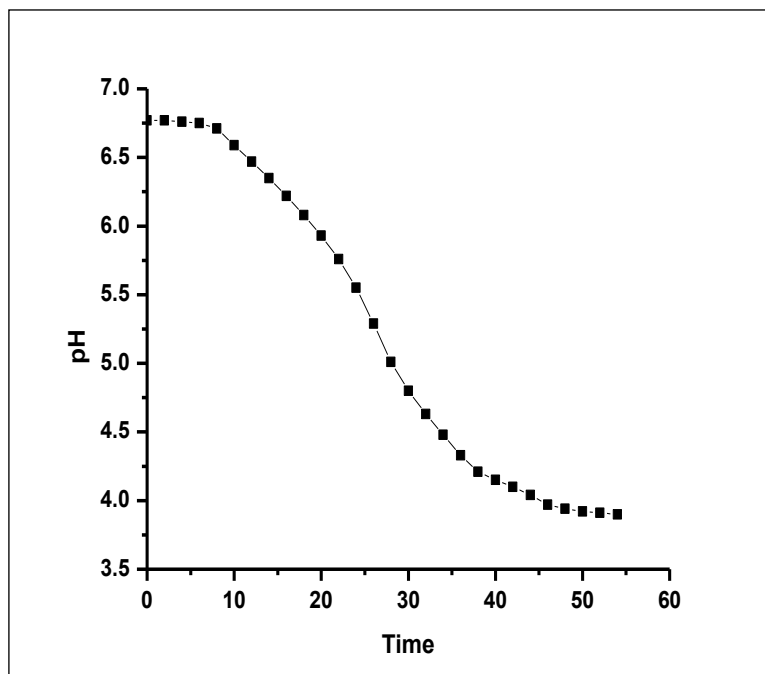
## **2.4. Conclusion**

This new way of generating linear pH-gradients by incorporation of bridging buffer components in the application and elution buffers eliminates non-linear drops in pH that are problematic in current gradient HPLC techniques, producing well controlled and reproducible linear pH-gradients.

**Table 2.4. Composition of optimized bridging buffer systems I B and II B**

Buffer System I B		Buffer system II B	
(each component 10 mM)		(each component 10 mM)	
Application buffer (pK <sub>a</sub> )	Elution buffer (pK <sub>a</sub> )	Application buffer (pK <sub>a</sub> )	Elution buffer (pK <sub>a</sub> )
Bis-tris methane (6.5)	Acetic acid (4.76)	Bis-tris methane (6.5)	Acetic acid (4.76)
3-methyl pyridine (5.68)	Lactic acid (3.81)	4-methyl pyridine (6.02)	4- chlorophenyl acetic acid (4.19)
		3-methyl pyridine (5.68)	Lactic acid (3.81)
		Pyridine (5.25)	
<b><i>Bridging (5mM)</i></b>	<b><i>Bridging (5mM)</i></b>	<b><i>Bridging (5mM)</i></b>	<b><i>Bridging (5mM)</i></b>
<b><i>Acetic acid</i></b>	<b><i>3-methyl pyridine</i></b>	<b><i>Acetic acid</i></b>	<b><i>Pyridine</i></b>





**Figure 2.9. Outlet pH gradients on DEAE column generated from 0-100% B gradient of Table 2.4 buffer systems IB (A) and IIB (B) with bridging**

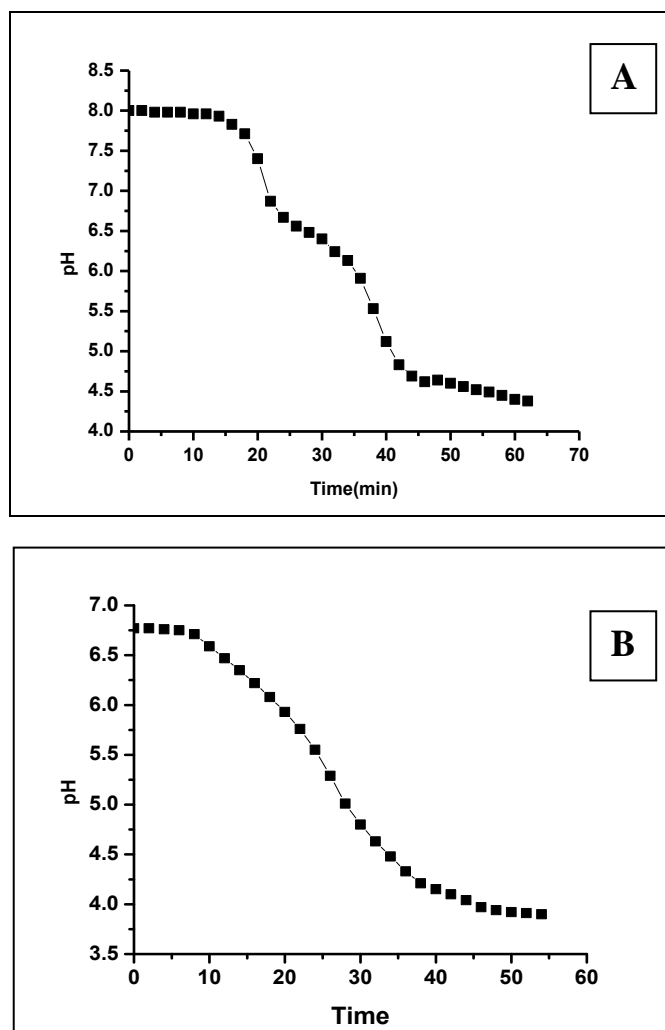
## **2.5. Separation of protein mixture on pH gradients produced with bridging and without bridging with buffer system IB**

The results of pH gradient generated without bridging are compared with the results of pH gradient produced with bridging with buffer system IB in the chromatographic separation of three protein mixture of conalbumin ( $pI = 5.9$ ), rat albumin ( $pI = 5.7$ ), and  $\beta$ -lactoglobulin A ( $pI = 5.13$ ) on DEAE weak anion-exchange column(same column used for generating pH gradients). Results showed pH gradients generated with bridging buffer system IB gave significantly higher resolutions of the proteins compared to non-bridging buffer system IB. Gradients for each are shown in Figure 2.10. Resolutions increased with the bridging buffer system significantly from 3.5 to 8.26 for conalbumin and rat albumin and 1.6 to 3.02 for rat albumin and  $\beta$ -lactoglobulin A pairs, as summarized in Table 2.5. A two-fold gain in the average resolution from 2.5 to 5.6 was obtained for the bridging buffer system compared to without bridging buffer system.

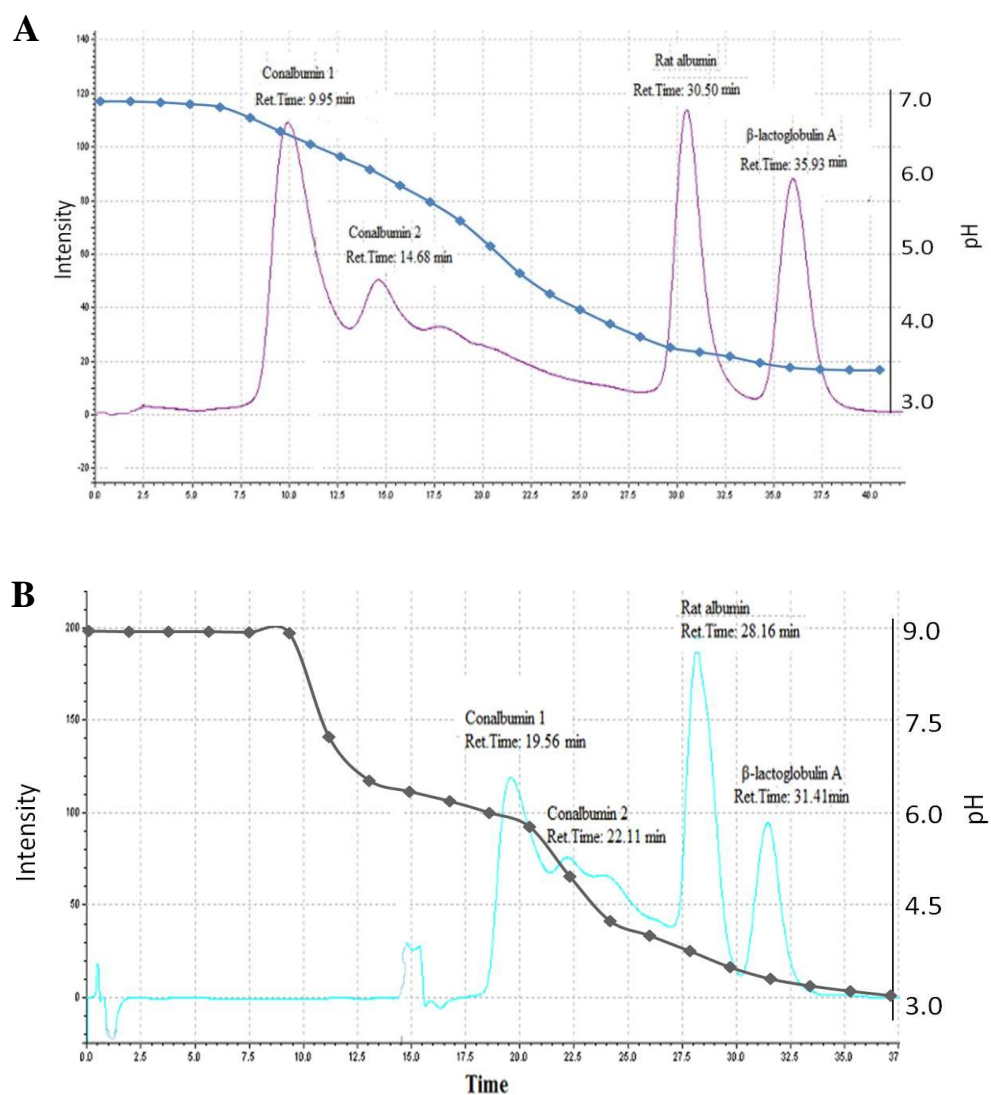
**Table 2.5. Resolution and peak widths of proteins with and without bridging for gradients generated with buffer system IB**

Proteins	Resolution( $R_s$ )	
	Buffer System IB	
	Without bridging	With bridging
Conalbumin and Rat albumin	3.5	8.26
Rat albumin and $\beta$ -lactoglobulin A	1.6	3.02
<i>Average</i>	<i>2.55</i>	<i>5.64</i>

Proteins	Peak widths at half height (min)		Peak widths in pH units	
	Buffer system IB		Buffer system IB	
	Without bridging	With bridging	Without bridging	With bridging
Conalbumin	2.19	2.08	0.38	0.15
Rat albumin	1.37	1.48	0.32	0.13
$\beta$ -lactoglobulin A	1.21	1.58	0.12	0.08



**Figure 2.10. Outlet pH gradients on DEAE column generated from 0-100% B gradient of buffer systems IB without bridging (A) and with bridging (B)**



**Figure 2.11. Chromatogram of a sample containing conalbumin, rat albumin, and  $\beta$ -lactoglobulin A with bridging (A) and without bridging (B) with buffer system IB**

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## **CHAPTER III**

### **The effect of the number of buffer components on separation of proteins in high-performance gradient chromatofocusing ion-exchange using linear pH-gradients**

#### **3.1. Introduction**

Background discussion on gradient chromatofocusing (GCF) is covered in section 1.6 of Chapter 1. Studies of the effect of gradient slope and buffer concentration [1-3] have been done, showing that these experimental factors are important in the optimization of the chromatography. This gives GCF significant advantage over conventional chromatography which cannot control these factors. In the present study the effect of number of buffer components resulting in more closely spaced  $pK_a$ s in the gradient is studied.



## **3.2. Experimental**

### **3.2.1. Materials and injected samples**

Conalbumin (catalog no.C-0755),  $\beta$ -lactoglobulin A (catalog no. L7880), methanol (catalog no.14262),  $\beta$ -lactoglobulin B (catalog no. L8005), ovalbumin (catalog no. A-2512), bovine albumin serum (catalog no. A5503), were purchased from Sigma-Aldrich (St. Louis, MO, USA). All proteins samples were prepared in deionized water and 100  $\mu$ M of each protein was injected.

Bulk anion-exchange material was Protein-Pak DEAE 8HR, 8  $\mu$ m diameter, 1000 Å pore diameter, DEAE functionalized polymethacrylate) from Waters (Milford, MA, USA). HPLC grade water was generated by a Barnstead Nano system with a Nanpure Diamond Pack Organic Free DI cartridge from Thermo Scientific (West Palm Beach, FL, USA). All the solvents were filtered through 0.45  $\mu$  cellulose ester membrane filters from Millipore (Billerica, MA, USA).

### **3.2.2. Chromatographic conditions on DEAE column**

The HPLC system consisted of a System Gold 126 solvent module gradient pump system, System Gold 508 Auto Sampler, System Gold 168 Detector from Beckman instruments (Fullerton, CA, USA). All the data collected and analyzed using 32 Karat Gold software (version 8). DEAE anion-exchange column (4.6 X 100 mm, 8HR, 8  $\mu$ , 1000Å). The pH gradient was monitored online after the detector using a FC45C flow cell equipped with a flow-through pH electrode (from Sensorex, S450CD). The volume of

the pump mixing chamber to the column was 0.8 mL and the void volume of the column was 0.33 mL. The flow rate of the mobile phase was 1.0 mL/min. The sample injection volume was 100uL. The pH gradients and proteins were monitored at 280nm. The column was equilibrated for 50 min with the application buffer prior to the start of each run till the column pH reaches the pH of the application buffer.

The pH gradients were monitored on-line after the detector by collecting pH measurements using a FC45C flow cell (200uL) equipped with a flow-thru pH electrode from Sensorex (S450CD) that connect directly to the tubing used by HPLC system. A Denver Instrument pH meter (Model 250) with data logging capabilities was interfaced by the micro flow-thru electrode for data acquisition every two minute intervals. The micro flow-thru electrodes allow the pH measurements at the low flow rates (100uL/min). These electrodes are advantageous because they measure pH on-line in no time as opposed by taking the pH measurements over time by collecting fractions. This process eases the laborious efforts that were once required to measure pH of the fractions manually in development of linear gradients.

Buffer system IB consisted of an application buffer mobile phase A composed of 10mM of bis-tris methane (6.5), 10mM of 3-methyl pyridine (5.68) and 5% methanol, adjusted to pH 6.5 with concentrated ammonium hydroxide and an elution buffer mobile phase B, consisting of 10mM of acetic acid (4.76), 10mM of lactic acid (3.81) and 5% methanol, without any pH adjustment. Buffer system IIB consisted of an application buffer mobile phase A composed of 10mM each of bis-tris methane (6.5), 4-methyl pyridine (6.02), 3-

methyl pyridine (5.68), pyridine (5.25) solution and 5% methanol with the pH adjusted to 6.5 using concentrated  $\text{NH}_4\text{OH}$ .

An elution buffer mobile phase B, consisting of 10mM each of acetic acid (4.76), 4-chlorophenyl acetic acid (4.19), lactic acid (3.81) and 5% methanol, without any pH adjustment. The  $\text{pK}_a$  values for the buffer components are given in parentheses.

### **3.2.3. Chromatographic conditions on commercial PEI weak-anion exchange column**

PEI weak anion- exchange column (50 X 2.1 mm, 5 $\mu$ , 300Å) was purchased from The Nest group (Columbia, MD 21045, USA). The flow rate was 0.3 mL/ min. Chromatographic conditions are the same as indicated in section 3.2.2.

## **3.3. Results and Discussion**

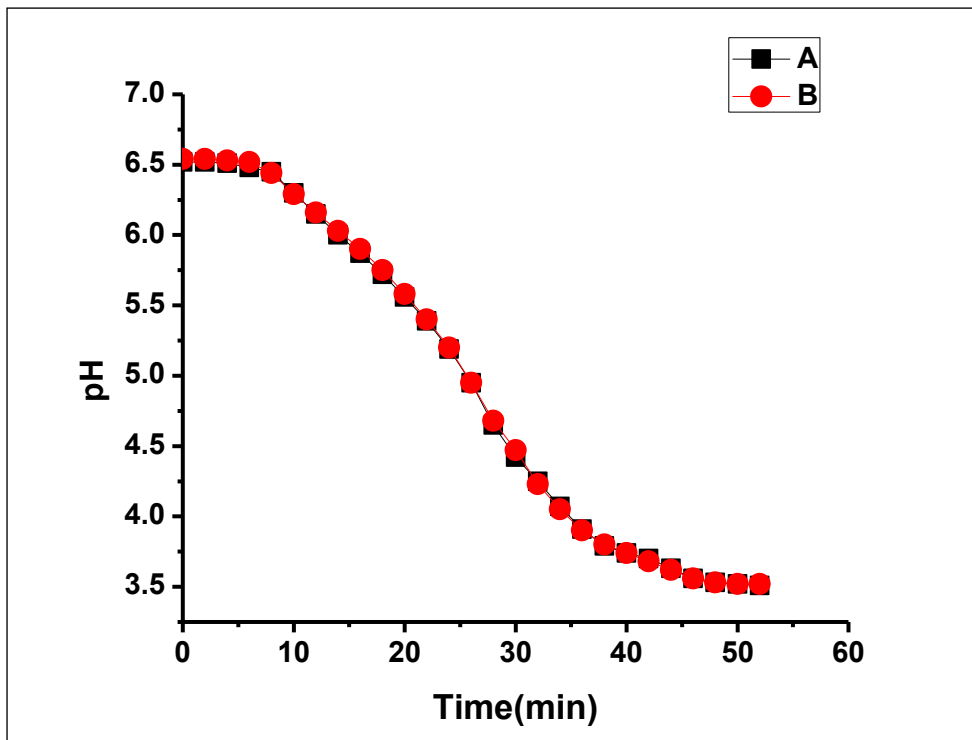
### **3.3.1. Superimposition of pH-gradients generated with two buffer systems**

Reproducible, excellent linear pH-gradients using bridged buffer system IB and buffer system IIB was achieved, as presented in Chapter 2. These buffer systems differ in their number of buffer components. Since buffer system II has more number of buffer components, different gradient time programs were used for buffer system II to achieve

the superimposition with buffer system I by trial and error gradient mixing of buffer components.

The effect of the number of buffer components in the mobile phase buffer was studied on resolution and peak width of protein separation. Buffer system with more buffer components had buffer species added to the buffers such that there was smaller  $pK_a$  difference between buffer components. In order to study this effect, pH-gradients needed to be matched exactly such that difference in chromatographic separation characteristics could be attributed solely to difference in number of buffer components.

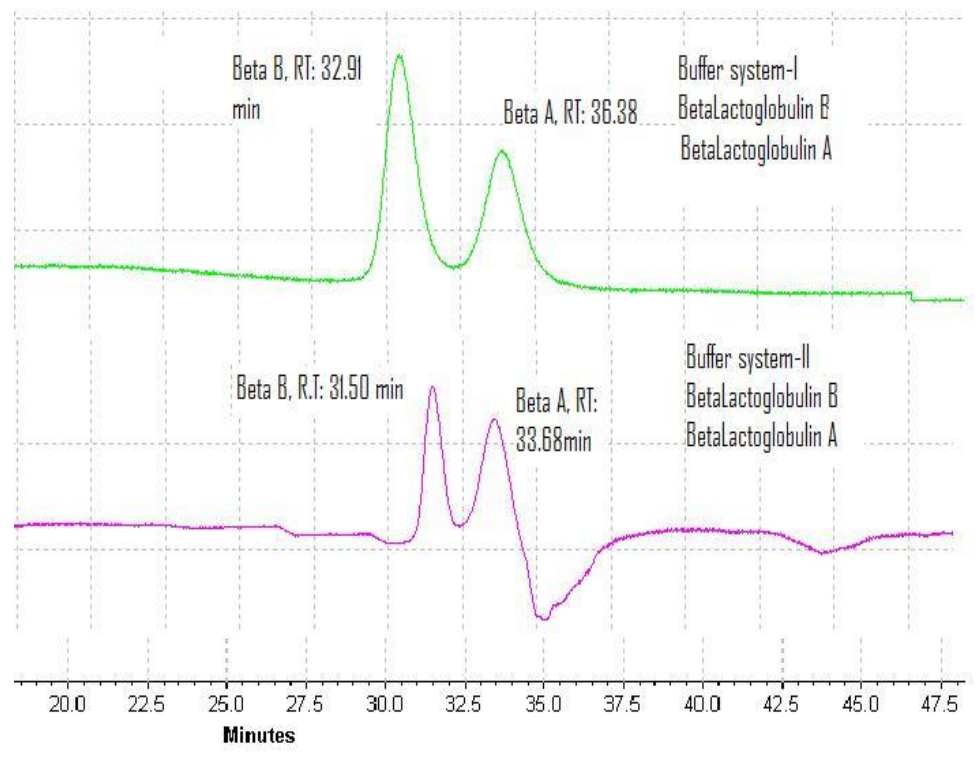
Numerous trials were done to exactly match the pH gradients of the two buffer systems. The optimized gradient programs and the resulting pH gradients for the two buffer systems are shown in Figure 3.1, in which shows that exact superimposability of the gradients was achieved.



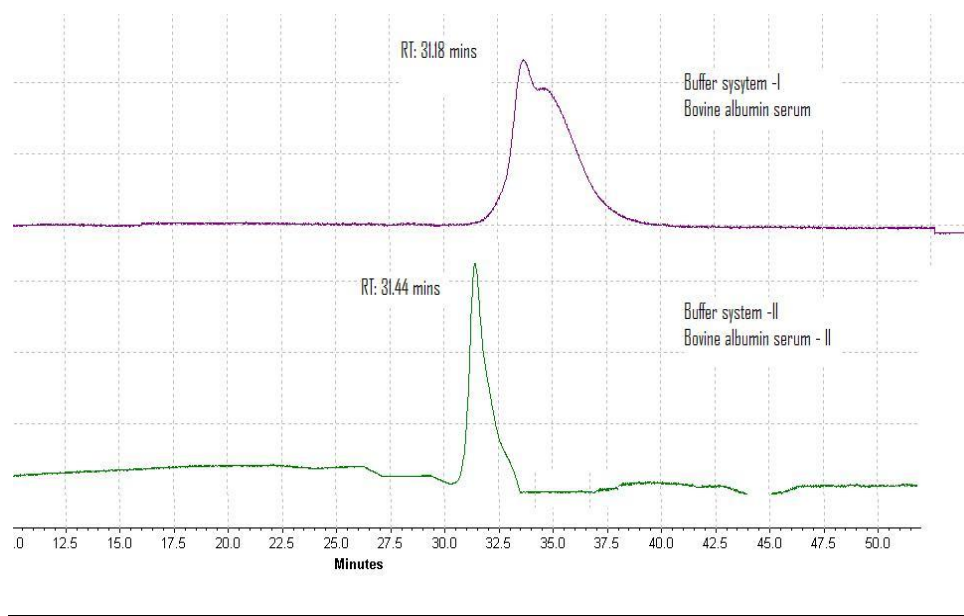
**Figure 3.1. Superimposable pH-gradients of buffer system I (A) and buffer system II (B). The gradient time programs used for buffer system I (A): 0 -62.7% B in 22 min, 62.7 -78% B in 6 min, 78-100% B in 7.09 min and for buffer system II (B) 0 - 45.19% B in 17 min, 45.19 -60.03% B in 6 min, 60.03-81.03% B in 6 min, 81.03-93% B in 14.59 min.**

### **3.3.2. Comparing the buffer systems IB and IIB in the GFC separation of proteins on the DEAE column**

Chromatographic separation experiments of five standard proteins  $\beta$ -lactoglobulin A (5.13),  $\beta$ -lactoglobulin B (5.34), bovine albumin serum (4.9), conalbumin (5.9) and ovalbumin (4.6) were done on DEAE weak anion-exchange column employing the superimposed linear pH-gradients given in Figure 3.1 for the two buffer systems (protein pIs are given in the parentheses). Comparison of the GCF chromatograms for the two buffer systems are given in Figures 3.2, 3.3 and 3.4.

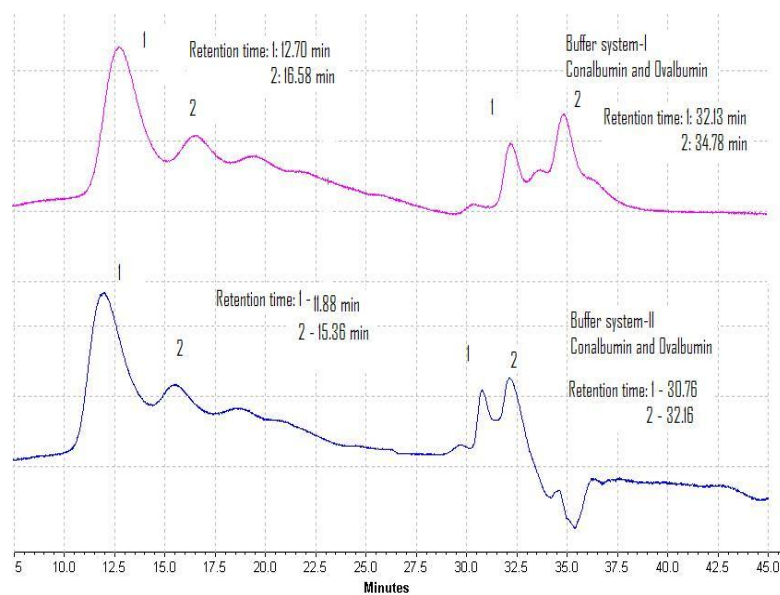


**Figure 3.2. GCF chromatography of  $\beta$ -lactoglobulin A and B with two buffer systems IB (upper plot) and IIB (lower plot) on DEAE column.**



**Figure 3.3 . GCF chromatography of bovine serum albumin with two buffer systems IB (upper plot) and IIB (lower plot) on DEAE column.**





**Figure 3.4. GCF chromatography of conalbumin and ovalbumin with two buffer systems IB (upper plot) and IIB (lower plot) on DEAE column.**

### **3.3.2.1. Peak widths results**

Results showed that all the proteins were focused into narrower bands with the more component buffer system II compared to the less component buffer system I as summarized in Table 3.1. The greatest decrease in peak width was seen for bovine albumin serum which decreased in peak width at half height from 2.69 min to 0.7 min and for  $\beta$ -lactoglobulin B which decreased from 1.11 min to 0.5 min.

**Table 3.1. Comparison of the peak widths at half height of proteins separated with linear pH gradients (Figure 3.1) of buffer system IB and buffer system IIB on DEAE column**

<b>Protein</b>	<b>Peak widths at half height(min)</b>		<b>Peak widths in pH units</b>	
	<b>Buffers system I</b>	<b>Buffer system II</b>	<b>Buffers system I</b>	<b>Buffer system II</b>
Conalbumin	1.78	1.4	0.3	0.18
Ovalbumin	0.71	0.46	0.4	0.3
Bovine albumin serum	2.69	0.7	0.32	0.09
$\beta$ -lactoglobulin B	1.11	0.5	0.18	0.18
$\beta$ -lactoglobulin A	1.24	0.94	0.73	0.19
<i>Average</i>	<i>1.50</i>	<i>0.78</i>	<i>0.39</i>	<i>0.18</i>
<i>Median</i>	<i>2.69</i>	<i>0.7</i>	<i>0.32</i>	<i>0.09</i>

### 3.3.2.2. Resolution results

Resolutions were calculated for different protein pairs using the data of individually chromatographed proteins. The resolution for each protein pair is calculated using following equation:

$$R_s = \frac{2.354(t_{R2} - t_{R1})}{2(W_{h2} + W_{h1})}$$

where  $R_s$  is the resolution;  $t_{R2}$  and  $t_{R1}$  are the retention times of peak 2 (late eluting peak) and peak 1 (early eluting peak) respectively, and  $W_{h2}$  and  $W_{h1}$  are the width of peak 2 and peak 1 at half height respectively.

Resolutions were found to be higher for four of the five proteins in their GCF separation using buffer system IIB (more buffer components) compared to buffer system IB (less buffer components). For example the resolution increased from 4.7 to 10.3 and 0.3 to 0.6 for BSA and conalbumin and BSA and ovalbumin pairs, respectively. The resolution of the various adjacent eluting protein pairs are given in Table 3.2.

**Table 3.2. Comparison of resolution of proteins separated with linear pH gradients**

**(Figure 3.1) of buffer system IB and buffer system IIB on DEAE column**

<b>Proteins</b>	<b>Resolution (<math>R_s</math>)</b>	
	<b>Buffer system I</b>	<b>Buffer system II</b>
Ovalbumin&Conalbumin	8.8	10.5
BSA & Ovalbumin	0.3	0.6
$\beta$ -lactoglobulin B& BSA	0.3	0.5
$\beta$ -lactoglobulin B& $\beta$ -lactoglobulin A	1.74	1.6
BSA & Conalbumin	4.708	10.3
<i>Average</i>	<i>3.16</i>	<i>4.7</i>
<i>Median</i>	<i>1.74</i>	<i>1.6</i>

### **3.4. Chromatographic separation of three standard proteins on commercial PEI weak anion-exchange column**

Chromatographic separation of conalbumin,  $\beta$ -lactoglobulin A, and  $\beta$ -lactoglobulin B was carried out on commercially purchased PEI weak anion-exchange column (50 X 2.1 mm, 5 $\mu$ , 300Å) employing the same superimposed pH gradients generated with buffer system IB and buffer system IIB.

#### **3.4.1. Peak width and resolution results**

The results of protein peak widths at half height and resolutions resulting from the exact same pH gradient runs employing buffer system IB and buffer system IIB on the PEI commercial column are given in Table 3.3 and Table 3.4. Results show a significant effect of number buffer components on peak widths and resolution. Proteins were focused into narrow bands with buffer system II. Peak widths decreased between 15 – 31% in comparing buffer system IB and system IIB results (Table 3.3). The resolution increased from 11.2 to 18.0 and from 1.05 to 5.8 in the separation of  $\beta$ -lactoglobulin B & conalbumin and  $\beta$ -lactoglobulin B and  $\beta$ -lactoglobulin A, respectively, in comparing buffer system IB with buffer system IIB, respectively (resolution increases with more buffer components).

Resolutions and peak widths at half height are also compared between packing column to commercially purchased column for conalbumin,  $\beta$ -lactoglobulin A, and  $\beta$ -lactoglobulin B for both buffer systems IB and IIB. The average peak widths at half height significantly decreased as shown in Table 3.3, more than 50% from 1.37 to 0.67 for buffer system I and from 0.94 to 0.54 for buffer system II for the PEI commercial column compared with the DEAE column. The average resolutions for the PEI commercial column compared with the DEAE column were increased from 4.96 to 6.10 for buffer system IB and from 10 to 12 buffer system II in the chromatography of conalbumin,  $\beta$ -lactoglobulin A, and  $\beta$ -lactoglobulin B with buffer system IIB as shown in Table 3.4.

**Table 3.3. Comparison of the peak widths at half height of proteins separated with linear pH gradients of buffer system IB and buffer system IIB on commercial PEI and DEAE columns**

Proteins	Peak widths at half height(min)			
	Buffer System IB		Buffer System IIB	
	Commercial column	Packed column	Commercial column	Packed column
Conalbumin	1.16	1.78	0.99	1.4
$\beta$ –lactoglobulin B	0.40	1.11	0.33	0.5
$\beta$ –lactoglobulin A	0.45	1.24	0.31	0.94
<i>Average</i>	<i>0.67</i>	<i>1.37</i>	<i>0.54</i>	<i>0.94</i>



**Table 3.4. Comparison of resolution of proteins separated with linear pH gradients of buffer system IB and buffer system IIB on commercial PEI and DEAE columns**

Proteins	Resolutions ( $R_s$ )			
	Buffer System IB		Buffer System IIB	
	Commercial column	Packed column	Commercial column	Packed column
$\beta$ -lactoglobulin B & Conalbumin	11.16	8.18	18.04	18.4
$\beta$ -lactoglobulin A & $\beta$ - lactoglobulin B	1.05	1.74	5.8	1.6
<i>Average</i>	<i>6.10</i>	<i>4.96</i>	<i>12</i>	<i>10</i>

The reasons for obtaining improved resolution and narrower peak widths for commercially purchased PEI anion exchange column (2.1 x 50 mm, 5 $\mu$ , 300Å) when compared to self packing DEAE column (4.6 x 100 mm, 8 $\mu$ , 1000Å) are the different particle diameters used and difference in the packing of two columns. Packing material particle size ( $d_p$ ) is a physical dimension that has significant influence on the performance of an HPLC column. Smaller particle sizes give higher peak efficiencies. Factors affecting resolution include selectivity, retention capacity, and efficiency. A column's particle size particularly affects the efficiency term of the resolution equation. Efficiency is quantified by the number of theoretical plates (N) in a given column. A theoretical plate refers to one complete equilibrated transfer (or partition) of a solute between the mobile and stationary phases. Efficiency, as measured by the number of theoretical plates (N), inversely related to the particle size ( $d_p$ ) as given by the well known van Deemter equation:

$$H = L/N = A*d_p + B/v + C*d_p^2*v$$

where H is the plate height, L is the length of the column,  $d_p$  is the diameter of the packing material, v is the linear velocity of the mobile phase, and A, B, C are constants. Since values of A, B, and C are dependent on column/packing material/analyte characteristics it is not possible to establish the exact quantitative relationship between a change in  $d_p$  and N. However, it can be reasonably assumed that the B term is negligible, because it is only at impractically slow mobile phase linear velocities that this term is significant. Thus the relationship between  $d_p$  and N is inversely proportional ranging

from  $d_p$  and  $d_p^2$ . As particle size is decreased, efficiency increases, and greater resolution is achieved.

### **3.4.2 Discussion of effect of number of buffer components on peak width**

The displacement mechanism of ampholyte displacement chromatography [4,5] proposes the formation of a pH gradient within the column through a gradient distribution of different acidic ampholytes within the anion exchange column, with the proportion of stronger to weaker acidic components gradually decreasing down the length of the column. In this mechanism more acidic components successively move down the column displacing less acidic components continuously lowering the pH of the column. This displacement process displaces the proteins down the column through successive pH bands of ampholytes that have the same affinity for the ion-exchanger sites as the protein. These pH bands push one another down the column at the same rate.

If the elution buffer has a greater number of buffer components, protein passes through stronger/more acidic pH zones which push and force them to be displaced completely from the anionic sites of the ion-exchanger. As a result, the pH bands of ampholyte become narrower (spreading of these pH bands decreases) thus the proteins which are in that narrower pH bands are focused into narrow bands and will have smaller peak widths. As more elution buffer enters the column, additional stronger acid components displace the weaker acid components, and these weaker components displace even weaker acid substances downstream to it.

One can propose similar mechanism for movement of buffer component in gradient chromatofocusing, with the stronger buffer components displacing weaker buffer components. Thus with more buffer components in the mobile phase having closer  $pK_a$ s the proteins will travel in the bands that match their  $pI$  and since the  $pH$  bands are narrower the proteins themselves will elute in narrower bands.

### **3.5. Development of multi buffer component system for generating linear $pH$ gradients to separate multiple forms of prolactin having closely related $pI$ values**

#### **3.5.1. Background**

Prolactin (PRL) is a protein hormone and regulates the mammary gland and reproductive system. Besides these its best known physiological actions, more than 300 different and distinct actions of PRL have been reported which include effects on growth and development, osmoregulation and behavior, endocrinology and metabolism, brain and behavior, immune regulation and protection [6]. Studies have been reported that this broad versatility in biological activities of prolactin is due to its structural polymorphism which exists in several molecular sizes. The analysis of prolactin in animals was analyzed by different methods like electrophoresis, RIAs, bioassay, chromatography of plasma and pituitary extracts revealed that the hormone is existed in more than one form [7]. Fractionation by size exclusion chromatography of PRL from male rat pituitaries and plasma showed the size heterogeneity of this protein hormone ranging from 30.4, 27.1, 25.6, 24.3, 23.6, 21.5KD and the chromatofocusing of pituitary PRL predominant size

variant 24.3KD exhibited the charge variants of pI 5.34, 5.31, 5.26, 5.20 and 5.14[8]. Three differently charge isoforms of rat prolactin was isolated by molecular sieve chromatography on Sephadex G-100 and column electrophoresis in agarose suspension as well as the electrophoresis resolved prolactin activity into three to four immune active components have been reported[9]. Studies have been reported that the arising heterogeneity in molecular size variants related to genetic and posttranslational events in pituitary cells as well as metabolic process like glycosylation and proteolysis [8, 9]. A size-exclusion HPLC method was developed to separate the three different prolactin forms: monomeric prolactin (~23 KDa), big prolactin (~60 KDa), and macro prolactin (~150 KDa) [10].

The present work utilizes anion-exchange gradient chromatofocusing method, optimizing the separation of the multiple forms of the enzymes and proteins by focusing into narrow bands.

### **3.5.2. Experimental**

#### **3.5.2.1. Materials and injected samples**

HEPES (catlog no. H3375), BES (catlog no.B9879) , PIPES (catlog no.P6757 ) , bis-tris methane (catalog no. A0293971) from Fisher (New Jersey, USA), 2-methyl benzimidazole (catlog no.65840), 4-methyl pyridine(catlog no. 239615, 3-methyl pyridine (catlog no. 42053), isoquinoline (catlog no. I28208), pyridine (catlog no. 270970), trimethyl aceticacid (catlog no. T71803), acetic acid (catlog no. H3375), 4-

chlorobutanoic acid (catlog no.C29835), 4-chloropenyl acetic acid (catalog no. 695092), lactic acid (catalog no. 252476), ammonium bicarbonate (catalog no. A6141), prolactin from sheep pituitary (L6520) were purchased from Sigma-Aldrich (St.Louis, MO). A poly weak anion exchange column (50 x 2.1 mm, 3 $\mu$ , 300 Å) was purchased from The Nest Group (Columbia, MD, USA). Protein sample prepared in DI water. 50  $\mu$ L of 100 uM prolactin was injected

Mass spectrometric materials for infusion experiments of eluted prolactin isoform peaks were acetonitrile (catalog no.34967), formic acid (catalog no. F0507), methanol (catalog no. 14262) from Sigma (St.Louis, MO). The ultra mcicro C4 spin column for desalting (p/n: SUM SS04V) was purchased from The Nest group (Columbia, MD)

### **3.5.2.2. Chromatographic conditions**

PEI weak anion- exchange column (50 X 2.1 mm, 3 $\mu$ , 300Å) was purchased from The Nest group (Columbia, MD 21045, USA). The flow rate was 0.2 mL/ min. Chromatographic conditions are the same as indicated in section 3.2.2.

#### **3.5.2.2.1 Thirteen component buffer system**

A thirteen component buffer system was developed to generate a linear shallow pH gradient that consisted of multiple buffer components with evenly spaced pK<sub>a</sub> values approximately 0.25. In this buffer system, application mobile phase A (high pH buffer)

consisted of 10 mM of each HEPES (7.4), BES (7.09), PIPES (6.76), bis-tris methane (6.5), 2-methyl benzimidazole (6.19), 4-methyl pyridine(6.02), 2-ethyl pyridine(5.89),3-methyl pyridine(5.68), isoquinoline (5.42), and pyridine (5.25). Elution buffer B (low pH buffer) composed of 10 mM of each trimethyl acetic acid (5.03), acetic acid (4.76), 4-chlorobutanoic acid (4.52), 4-chlorophenyl acetic acid (4.19), and lactic acid (3.81). The  $pK_a$  values for the buffer components are given in parentheses.

#### **3.5.2.2.2. Six component buffer system with NaCl**

Application buffer composed of 10mM of each ammonium bicarbonate, bis-tris methane, 3-methyl pyridine, and 5mM of acetic acid. Elution buffer consisting of 10 mM of each acetic acid, lactic acid, chloroacetic acid, 5 mM of 3-Methyl pyridine, and 25mM of NaCl.

#### **3.5.2.3. Mass spectrometric infusion experiments of eluted prolactin isoform peaks**

After injecting the prolactin (50uL of 100uM) on PEI weak anion-exchange column, the fractions were collected for each separated isoform of prolactin immediately after the detector at their retention times when it eluted. Five fractions were collected for five forms and were infused on mass spectrometer for molecular weight determination. Since each form of prolactin fraction eluted with buffer components, the fractions were desalted to remove all buffers before infusing on mass spectrometer. The ultra micro spin

C4 column was used for desalting of all collected fractions of prolactin forms. These columns will retain non-polar solutes such as peptides, proteins, and detergents. Salts, buffers and polar solutes will not be retained.

Initially the column was conditioned by pipetting 200uL of 100% methanol into the column to soak the column bed in it. Then the column was equilibrated two times with 200uL of 0.1% formic acid in deionized water. 100uL of prolactin fraction was injected onto the column and washed the column twice with the 200uL of 0.1% formic acid in DI water. This will enable the protein to bind the column and flush out/ remove all the salts from the samples as well as from the column. The sample was then eluted using 200uL of 0.1% formic acid in 60% acetonitrile. The eluted protein isoform samples were speed vac dried and reconstituted in 0.1% formic acid in 60% acetonitrile for mass spectrometry infusion analysis.

Infusion experiments were carried out for desalted fraction samples using a Bruker's Esquire HCT ion trap mass spectrometer (Billerica, Massachusetts, USA) equipped with electro spray ionization (ESI) in positive mode to determine the molecular mass of each form of prolactin. The mass spectrometer parameters were as follows: The scan range is 100-2800 m/z; nebulizer gas at 15 psi; drying gas flow is 10L/min; drying temperature is 320 °C. The maximum accumulation time was 2 ms and the infusion rate was 10uL/min.

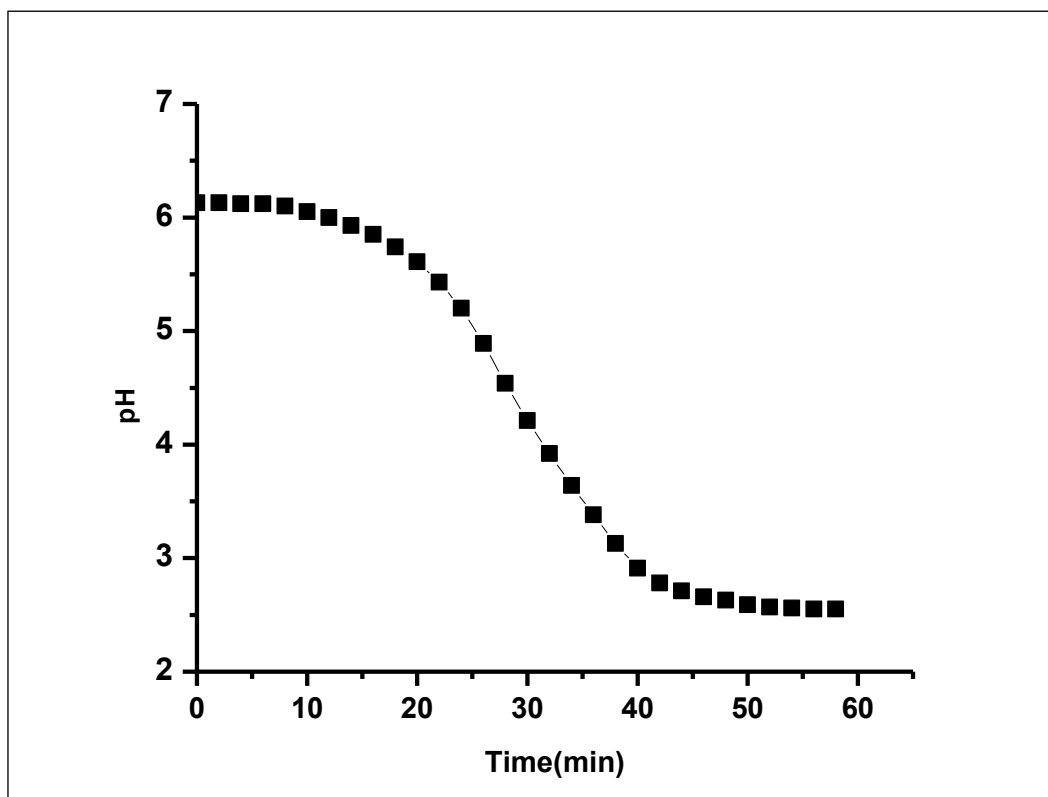


### **3.5.3. Results**

#### **3.5.3.1 GCF of prolactin isoforms with thirteen buffer component system**

A linear pH gradient ranging pH from 6 to 2.5 was generated on the PEI weak anion exchange column with a simple 0%B-100% B in 35 minutes gradient program utilizing the application and elution mobile phases described in section 3.5.2.2 with the resulting gradient given in Figure 3.5.

The bridging design discussed in chapter 2 was not required for generating a smooth linear pH gradient with the thirteen component buffer system. The reason behind this was the buffer components were chosen with pKa values evenly spaced apart by approximately 0.25 pH units over the range (6 to 2.5) of the pH gradient. The difference in spacing was much less for this new buffer system compared to buffer system IB and buffer system IIB. This smaller spacing of pKa values between buffer components provided high even buffering capacity necessary for generating smooth pH gradients throughout the desired pH range. The resulted linear pH gradient can be seen in Figure 3.5.



**Figure 3.5. Linear pH gradient generation with thirteen buffer component buffer system on PEI weak anion-exchange column with a simple 0-100%B in 35 min linear gradient program.**

After producing the desired slope pH-gradient using this new buffer system, the generated gradient was employed to resolve the different variants having closely related pI values of commercial prolactin (from sheep pituitary) isoforms. Prolactin was injected on the PEI weak anion exchange column, however no prolactin peaks eluted from the column. Attempts were made to elute the prolactin by increasing the concentration of elution buffer from 10mM to 25mM and adding 25mM of NaCl in elution buffer did not solve the problem. The reason might be the protein was denatured or precipitated as it was exposed to more organic environment.

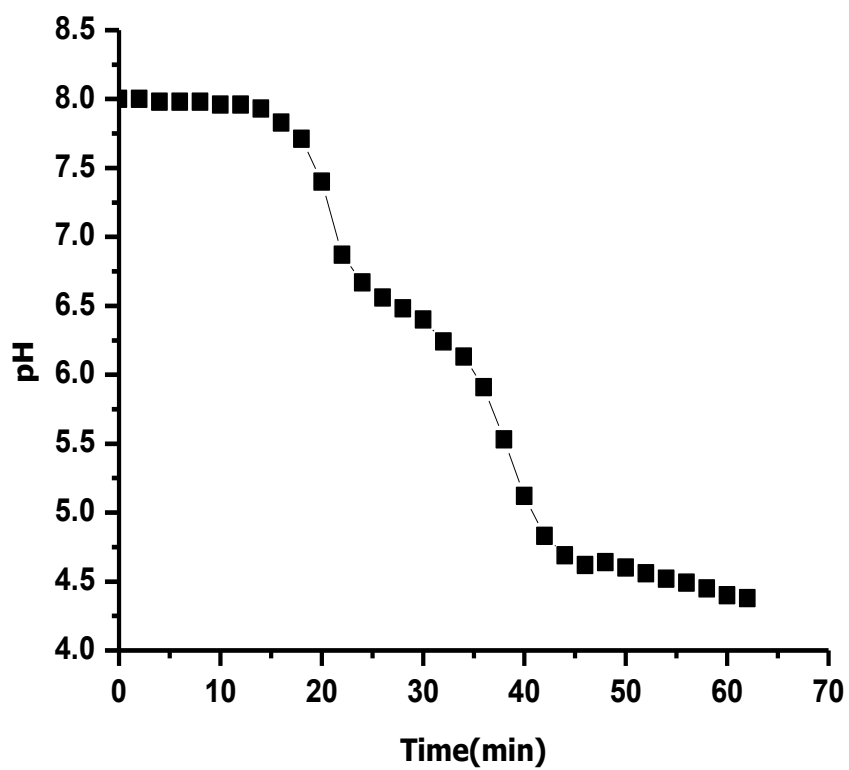
#### **3.5.3.2 GCF of prolactin isoforms with six buffer component buffer system**

The irreversible adsorption problem was solved by using six component buffer system with NaCl which resolved the prolactin variant isoforms. The buffer components are given in Table 3.3. One buffer component was introduced in application buffer and 25mM of sodium chloride was added to elution buffer in order to increase the ionic strength. The bridging technique was utilized for this buffer system to generate the linear pH gradient for the separation of prolactin isoforms.

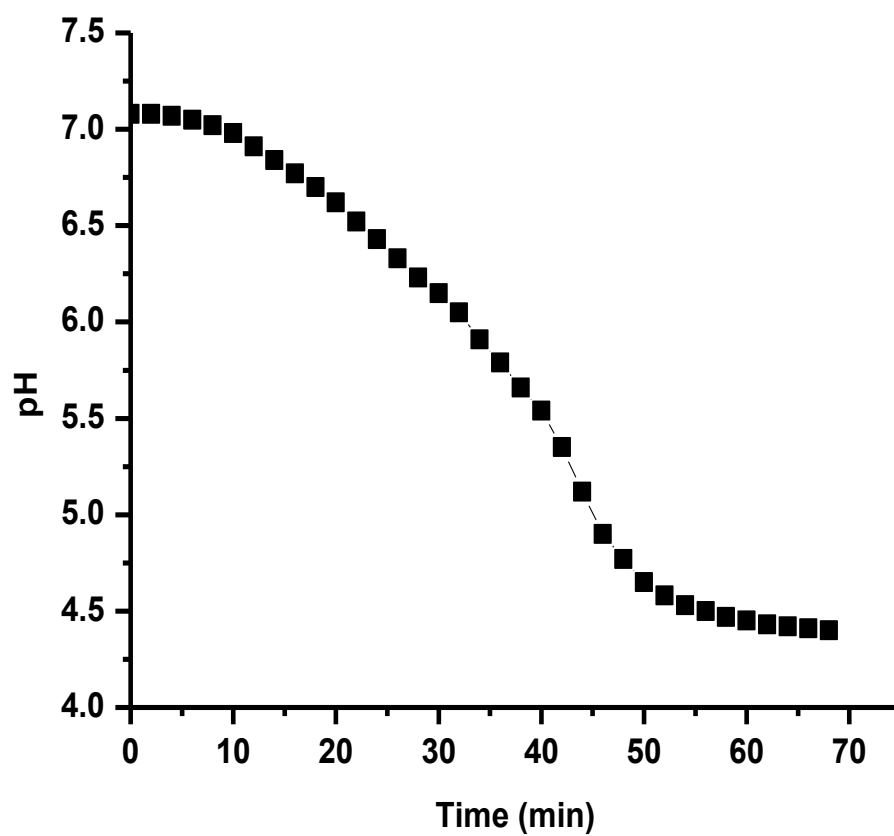
The linear pH gradients without and with the bridging buffer components are plotted in Figures 3.6 and 3.7, respectively. The resulting GCF chromatogram of the prolactin isoforms using the six component buffer system with NaCl and with bridging buffer components is given in Figure 3.8, showing excellent resolution of the five prolactin isoforms.

**Table 3.5. Six component buffer with bridging components and NaCl**

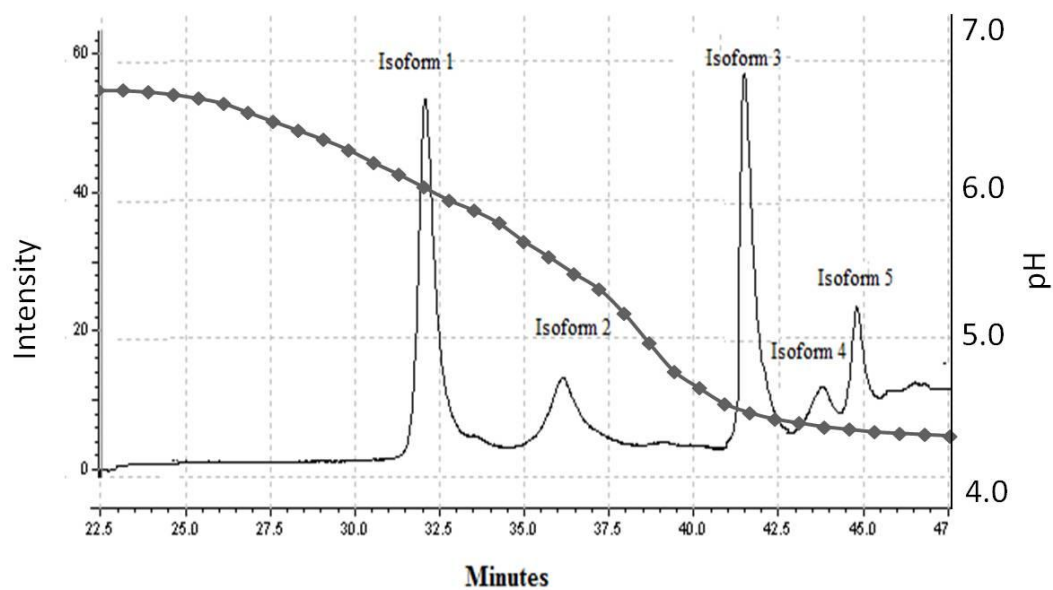
<b>Basic application buffer (each component 10mM)</b>	<b>Acidic elution buffer (each component 10mM)</b>
Ammonium bicarbonate  (9.26)	Acetic acid (4.76)
Bis-Tris      methane (6.5)	Lactic acid (3.81)
3-Methyl      pyridine (5.68)	Chloroacetic acid (2.)
<i>5mM of acetic acid</i>	<i>5mM of 3-methyl pyridine</i>
	<i>25 mM Sodium chloride</i>



**Figure 3.6. The pH gradient generated with six component buffer system with NaCl without bridging with 0 -100%B in 35 mins linear time gradient program**



**Figure 3.7. The pH gradient generated with six component buffer system with NaCl with bridging with 0 -100%B in 35 mins linear time gradient program**



**Figure 3.8.** GCF separation of different isoforms of prolactin on PEI weak anion-exchange column using pH linear gradient of Figure 3.7.

Peak widths at half height, pI values of prolactin isoforms according to literature, and the elution pH are given in the Table 3.4. Resolution of adjacent eluting peaks of the prolactin isoforms was calculated from retention times and peak widths at half heights and given in Table 3.5.



**Table 3.6. Peak widths at half height, literature pI and elution pH of isoforms of prolactin**

<b>Prolactin isoforms</b>	<b>Peak widths at half height (min)</b>	<b>Literature pI values of prolactin isoforms</b>	<b>Elution pH of prolactin isoforms</b>
Isoform 1	0.52	5.34	5.81
Isoform 2	0.68	5.31	5.75
Isoform 3	0.47	5.26	5.35
Isoform 4	0.73	5.20	5.12
Isoform 5	0.28	5.14	4.94

**Table 3.7. Resolution of adjacent eluting prolactin isoforms**

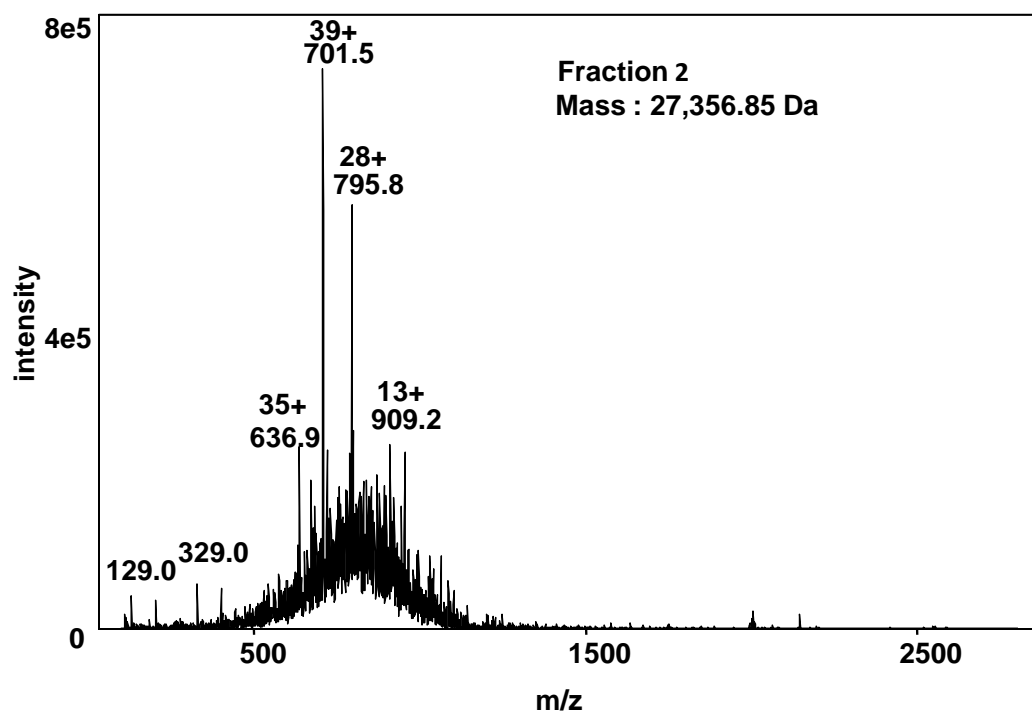
<b>Prolactin isoform pair</b>	<b>Resolution (<math>R_s</math>)</b>
Isoforms 1& 2	4.16
Isoforms 2& 3	9.69
Isoforms 3 & 4	1.24
Isoforms 4& 5	1.37

### **3.6. Molecular mass determination of prolactin variants**

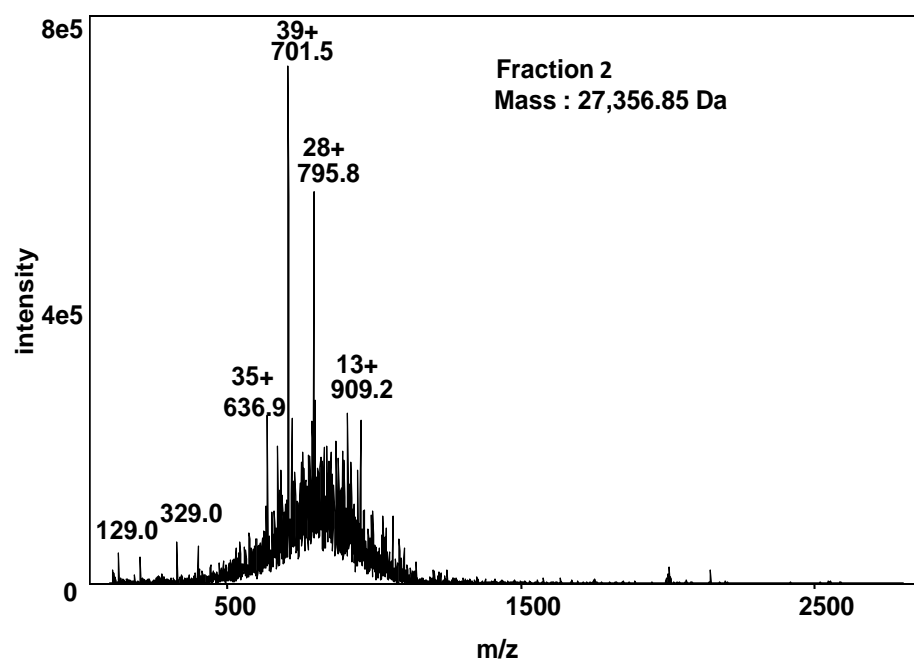
Studies reported that the prolactin shows the size heterogeneity and exists in several molecular sizes ranging from 30.4 KD to 21 KD [3]. So infusion experiments were carried out to determine the molecular masses of pI based isolated multiple forms prolactin.

#### **3.6.1. Results**

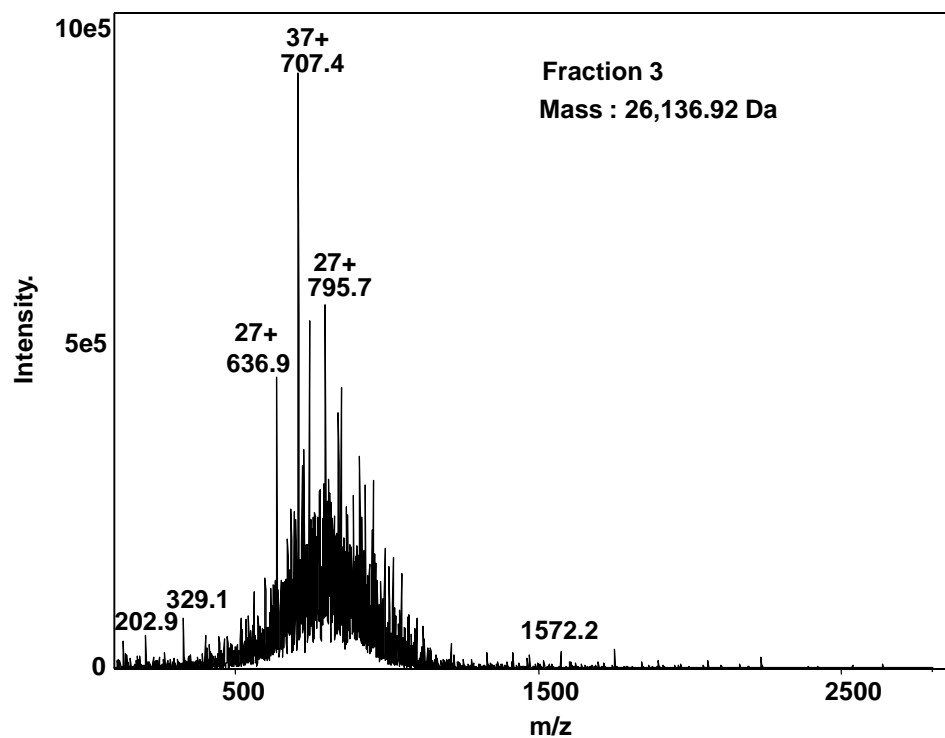
Prolactin hormone exhibits size heterogeneity ranging from 30 kDa to 21.5 kDa (30.4, 27.1, 25.6, 24.3, 23.6, 21.5 kDa) according to published literature. The deconvoluted mass spectrums of five fractions of prolactin forms gave the molecular masses of 21.5, 27.3, 26.1, 21.5, 26.9 kDa respectively. The molecular masses of the five peaks are within the molecular mass range for prolactin, giving supporting evidence that the peaks are likely prolactin peaks. The infusion mass spectrums of five fractions of prolactin isoforms and corresponding molar masses of each fraction are shown in Figures 3.9 – 3.13.



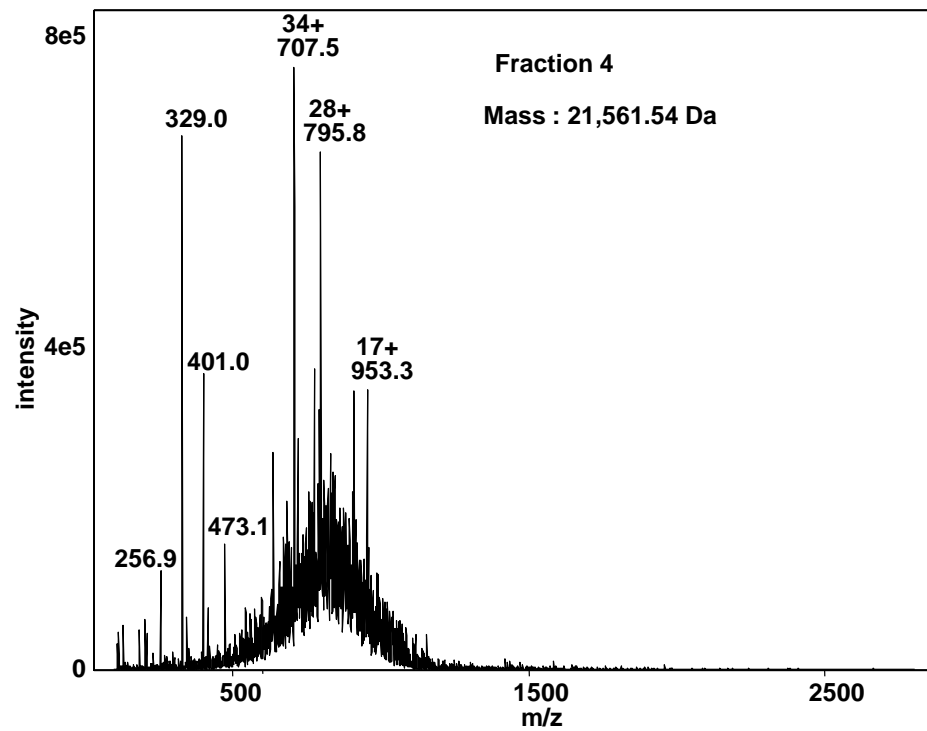
**Figure 3.9. The infusion mass spectrum of isoform 1**



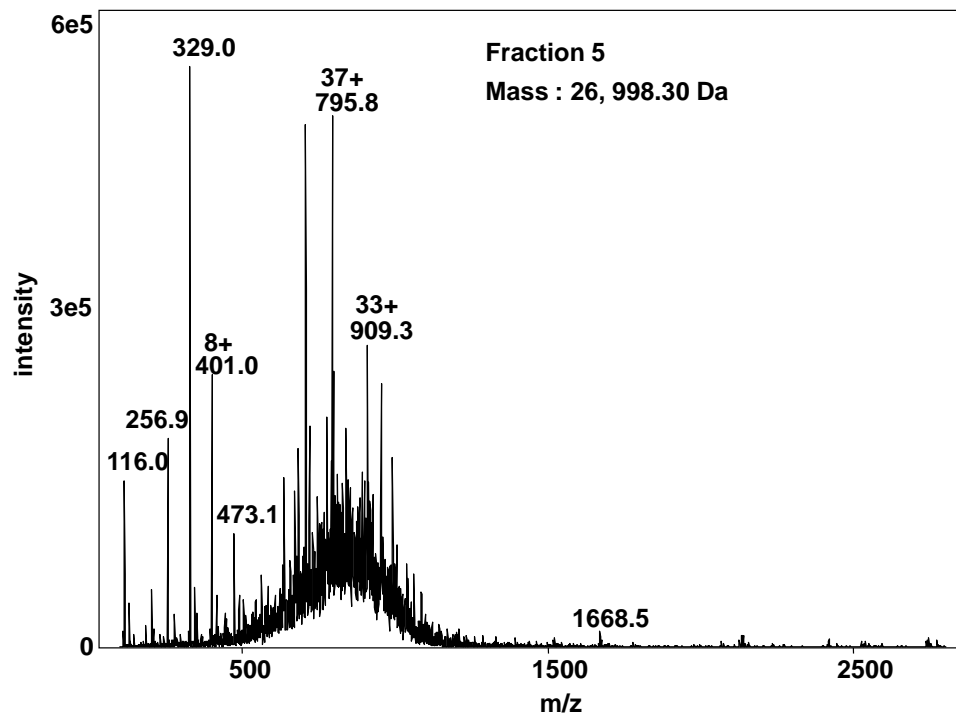
**Figure 3.10.** The infusion mass spectrum of isoform 2



**Figure 3.11. The infusion mass spectrum of isoform 3**



**Figure 3.12. The infusion mass spectrum of isoform 4**



**Figure 3.13. The infusion mass spectrum of isoform 5**



### 3.7. REFERENCES

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## **CHAPTER IV**

### **Bioanalytical Methods and Applications**

#### **4.1. Introduction**

Bioanalytical methods developed for the accurate quantification of drugs and their metabolites in biological samples are the key parameters in producing accurate and reproducible data which in turn are utilized in the assessment and interpretation of pharmacokinetics, bioequivalence, bioavailability, and toxicokinetics findings. This plays a vital role in understanding diseases, clinical diagnosis, and drug discovery and development [1]. It is known that bioanalytical methods and technologies are continually changing and improving significantly to yield reliable results which can be analyzed accurately. This facilitates to develop well characterized and fully validated analytical methods. In the last decade there have been remarkable advancements in bioanalysis technologies, which fulfill the requirements of clinical and pharmaceutical fields, especially in the field of mass spectrometry with the development of new interfaces, ionization, and detection techniques[2]. Due to its more rapid throughput and increased

sensitivity, hyphenated mass spectrometry (LC-MS-MS) based assays significantly replaced the conventional HPLC-UV, GC and other bioanalytical techniques[3, 4].

Major advances in the field of drug discovery and development aided increase in the number of new drug targets. However, the number of new drug approvals has not kept pace with the increased cost of their development. Increasingly, innovative uses of biomarkers are employed in an attempt to speed new drugs to market [5, 6]. Biomarkers are increasingly used in drug development to improve the understanding of disease as well as therapeutic effects of drugs contributing to improving the patient care. The biomarkers are used in monitoring the different biological entities including proteins, nucleic acids and metabolites to reflect the disease progression and therapeutic progression [Biomarker (medicine) wiki]. Presence of biomarkers often in low amounts in biological samples, diverse nature of biomarker analysis and its wide range applications in drug development bringing great challenges in obtaining reliable detection and validation. So there is a need to develop a standardized method and validation guidelines to measure the biomarkers [7].

Apart from wide range of bioanalytical applications in biomarker detection, the development of quantitative bioanalysis has significantly been increased in pharmaceutical drug discovery and development[3]. Quantitative bioanalysis provides the information to understand the pharmacological properties including absorption, distribution, metabolism and elimination (ADME), as well as toxicity to guide the drug screening for lead compounds.

#### **4.1.1. Bioanalysis applications in drug discovery and development**

In the process of drug discovery evolution, pharmacokinetics, toxicity and preclinical drug metabolism studies play crucial role in identification and optimization of lead compounds. An ideal drug candidate should exhibit the ability be absorbed in blood stream, reach desirable concentration for effective activity, and be excreted without producing toxic metabolites. Knowledge and understanding of pharmacokinetic and metabolism characteristics of the lead compounds is required in drug discovery [8]. Different stages of drug development process are shown in Figure 4.1.

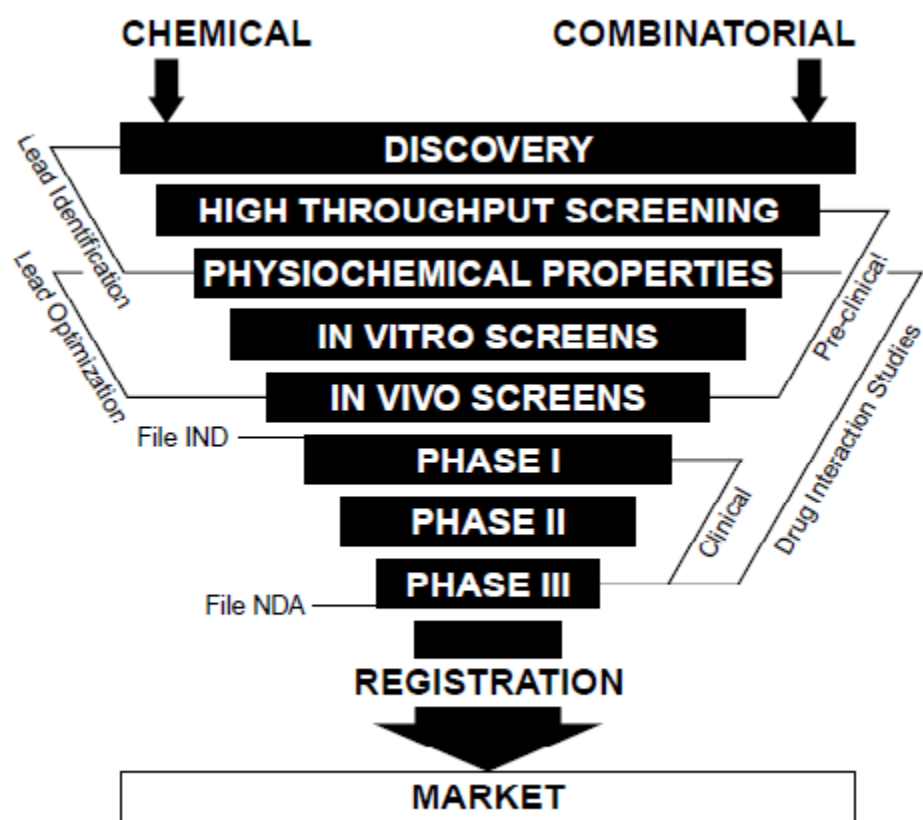


Figure 4.1. Various stages of drug discovery

Pharmacokinetic (PK) studies provide a mathematical basis to evaluate the time course of drugs and their effects in the body. PK describes how the body affects a specific drug after administration through the absorption, distribution, metabolism, and excretion processes. These pharmacokinetic processes usually referred to as ADME, determine the drug concentration in the body when the drug is administered. PK enables the ADME mechanisms to be quantified [9] .

Absorption is the process that the drug entering the blood stream circulation after administration of it by any route. The process of dispersion of a drug rapidly from blood to throughout the fluids and various tissues of the body is called distribution. A drug is excreted directly through an excretory route like urine, bile etc. from the body which is known as elimination. The drug eliminated indirectly via enzymatic and biochemical transformation by the liver as is called metabolism. Metabolism is the elimination of foreign and undesirable compounds from the body. It is also known as detoxication . The drug path from the blood is represented in Figure 4.2. Pharmacokinetic data is very useful in optimization of the dosage form design and establish the dosage interval [10, 11].

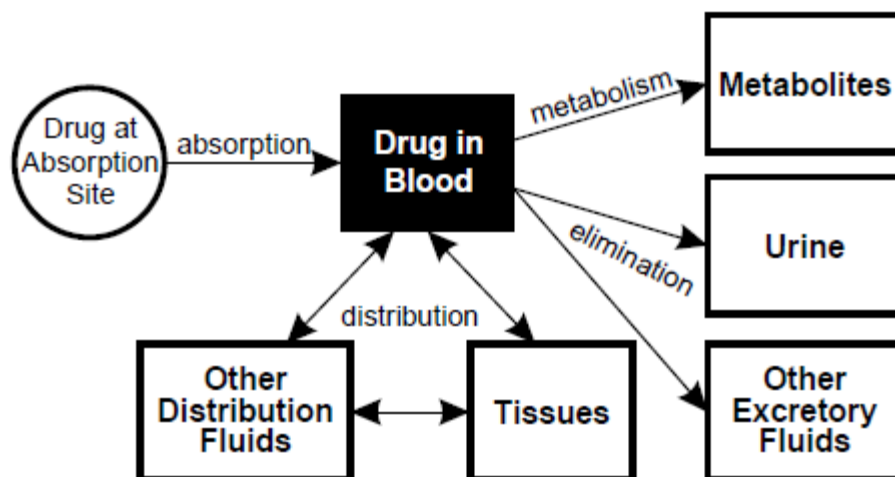


Figure 4.2. Schematic diagram of ADME process of a drug



The rates of ADME process can be characterized by zero-order reaction and first order reaction. In zero-order reaction, considering if the amount of drug A is decreased at a constant rate and then the rate of elimination of A is:

$$dA/dt = -k^* \quad (k^* = \text{zero-order rate constant})$$

The rate of elimination of the drug is independent of the concentration of the drug present in the body. In the first-order reaction, the elimination rate of drug A decreases as the drug A concentration decreases in the body[12]. This relationship is

$$dA/dt = -kA \quad (k = \text{first-order rate constant})$$

Elimination of the most of the drugs at therapeutic dosages generally follows the first-order kinetics. Common measurements and important parameters used in PK (Figure 4.3) analysis are  $C_{\max}$  (the maximum concentration recorded),  $T_{\max}$  (the time taken to reach  $C_{\max}$ ), AUC (area under the curve – a measure of the exposure to the drug), and  $t_{1/2}$  (elimination half-life – the time taken for the plasma concentration to fall by half its original value) [11-13].

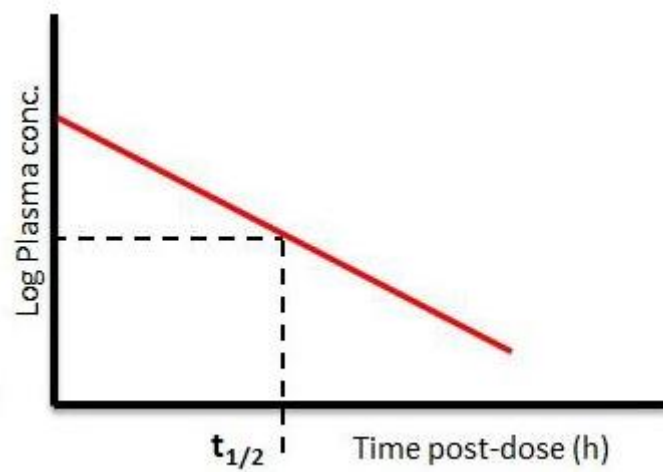
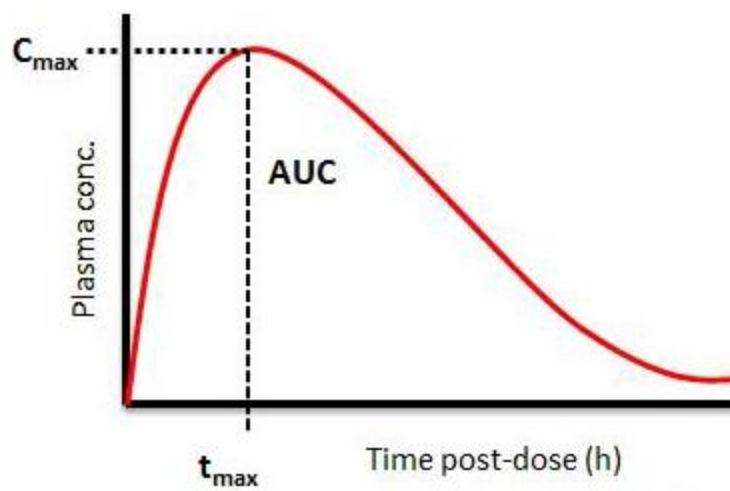


Figure 4.3. Common measurements used in PK analysis

High-throughput screening *in-vitro* ADME assays have been implemented in early drug discovery to study the drug-drug interaction and to identify and eliminate the compounds with poor drug properties as well as to promote potential drug candidates for *in-vivo* pharmacokinetic profiling [13-15]. However, *in-vitro* ADME assay results cannot truly represent the real physiological environment and always cannot assess the *in-vivo* PK parameters properly. In spite of many advances in *in-vitro* technologies, these approaches are not always reliable and accurate which may lead to mistaken conclusion about drug metabolism [16, 17]. The PK profile of a compound is controlled by many physicochemical and chemical properties of the molecule such as lipophilicity, solubility, permeability and metabolic stability. The *in-vivo* ADME processes through an intact animal or human are frequently far more complex than in isolated *in-vitro* systems. Therefore it is necessary to have *in-vivo* testing and confirmations of *in-vitro* ADME results in early drug discovery to improve the candidate selection through animal models [18, 19].

In order to predict the drug behaviors *in-vivo* and *in-vitro* accurately, there is always a continuous demand for bioanalytical method's support in pharmaceutical industries. These bioanalytical methods are essential to construct a concentration-time profile. The techniques are used to measure the concentration of drug in biological fluids or matrix, most often plasma, serum, and urine. So proper bioanalytical methods employed for the quantification should be selective and

sensitive. These requirements can be accomplished by advanced chromatographic and mass spectrometric techniques and accurate sampling procedures. LC-MS based methods have become widely accepted as an integral part of the drug discovery and development[3].

#### **4.2. Quantitative LC-MS/MS analysis for small molecules and pharmaceutical analysis**

Recent bioanalytical technologies have been considerably expanded over the past decade for quantitative determination of small molecules and large molecules in biological samples accurately. The combination of high-performance liquid chromatography (HPLC) with tandem mass spectrometry (MS/MS) detection is considered as the preferred method for the quantitative determination of drugs and metabolites in biological fluids due to its high sensitivity, selectivity, and short analysis time [2, 20, 21]. In pharmaceutical analysis, the hyphenated LC-MS is the most powerful technique because of the complex nature of the matrix (often plasma or urine) and the need for high sensitivity to observe concentrations after a low dose and a long time period [2, 3].

The most common instrumentation used in this application is LC-MS with a triple quadrupole mass spectrometer with ESI or APCI interface. Tandem mass spectrometry is usually employed for added specificity[2].

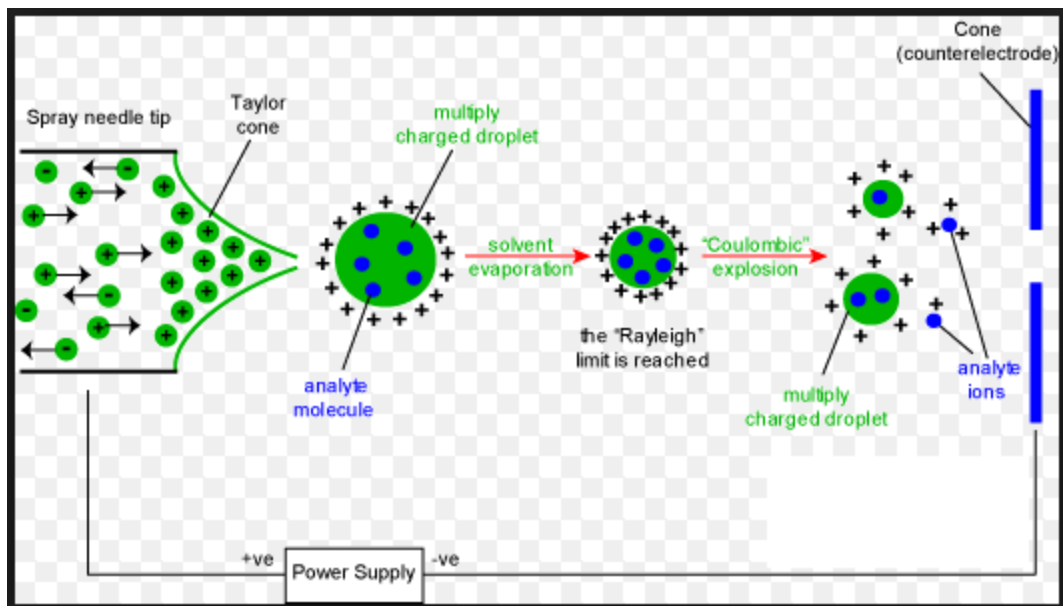
#### 4.2.1. MS detection

General mechanism of MS detection is the analyte in liquid flow from the HPLC goes into three components of mass spectrometer of ionization source in which the sample is ionized, mass analyzer in which the ions are separated and sorted according to their mass and charge ratio, and the detector which measures and detects the separated ions. The mass spectrometry is capable of not only indicating the presence of impurities but also able to give the molecular weights of underlying components and to distinguish between different molecules.

HPLC/MS became a success with the introduction of atmospheric pressure ionization (API) ten years back which enables the MS analysis by generating ions in a stream of liquid after HPLC separation. The most widespread method for quantifying small molecules is quadrupole MS interfaced with a number of API sources such as electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI). These ionization sources convert the sample from liquid phase to gaseous state [2].

In electrospray ionization, the analyte is introduced to the ion source in solution either from a syringe pump or as the eluent flow from liquid chromatography. The analyte solution flow passes through the electrospray needle that has a high potential difference (with respect to the counter electrode) applied to it (typically in the range from 2.5 to 4 kV). This forces the spraying of highly charged fine droplets from the needle with a surface charge of the same polarity to the charge on the needle. After solvent is further evaporated from these droplets, the droplet shrinks until it reaches the point that the surface tension can no longer sustain the charge at which point a coulombic explosion

occurs and the droplet is diffused apart leaving the ions to enter to the mass analyzer. The process of ESI is shown in Figure 4.4.



**Figure 4.4. The schematic diagram of ESI source**

Unlike ESI, APCI evaporates the solvent by passing it through a heated tube where the high voltage is applied to needle to generate a corona discharge and forming the plasma ions in the solvent. These solvent ions ionize the analyte molecules by means of gas phase reactions[2].

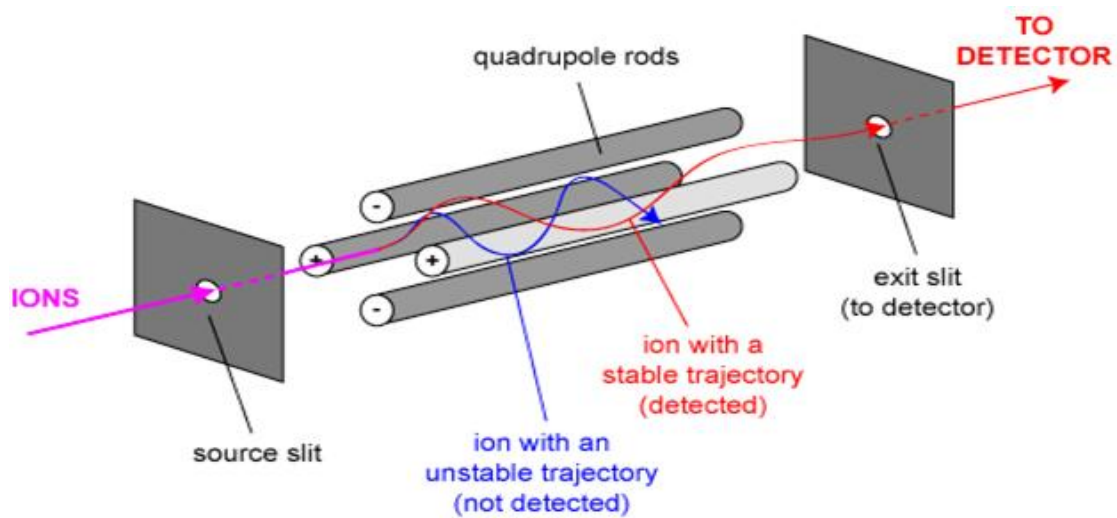
Mass analyzers separate the ions according to their charge to mass ratio ( $m/z$ ) by accelerating the ions with applying electric or magnetic field. Based on mass range limit, analysis speed, mass accuracy, and resolution, there are different types of mass analyzers are used. These include quadrupole MS, ion-trap MS, and time of flight (TOF) MS.

The quadrupole mass analyzer (Figure 4.5) uses an electric field to select and separate the ions with a particular  $m/z$ . It consists of four parallel metal rods where adjacent rods have opposite voltage polarity applied to them. The voltage applied to each rod is the summation of a constant DC voltage and a varying radio frequency.

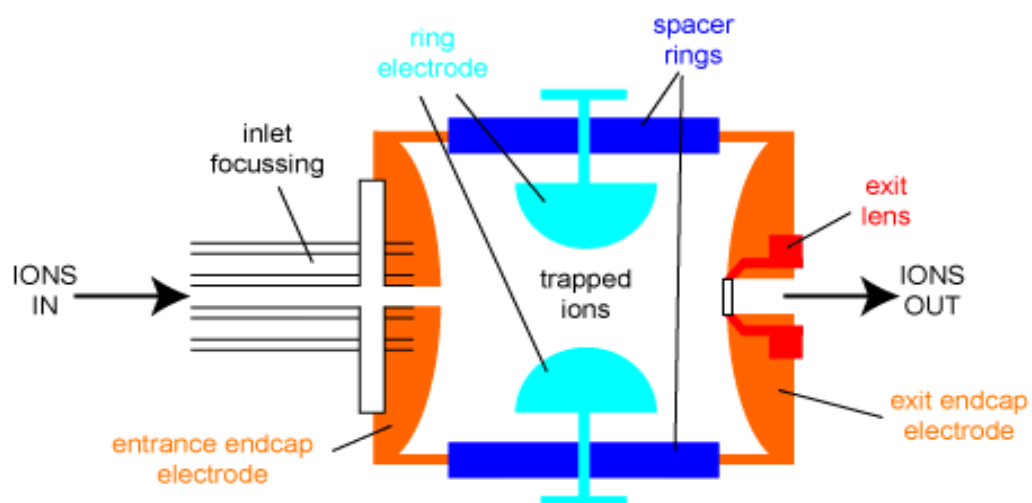


The electric force on the ions causes the ions to travel down the quadrupole between the rods. Only ions of a certain mass-to-charge ratio will reach the detector for a given ratio of voltages. Quadrupole analyzers have a limited  $m/z$  range, high sensitivity and mass accuracy, but low percentage of ion transmission. Triple quadrupole (QqQ) mass analyzer consists of three quadrupoles arranged in a linear series. Precursor ions filtered in the first quadrupole (Q1) are dissociated in the collision cell ( $q_2$ ) in the presence of inert gas such as Ar, He or N<sub>2</sub> gas, and resulting selected fragments are filtered or scanned by third quadrupole (Q3) achieving high specificity.

The quadrupole ion-trap mass analyzer employs similar principles as the quadrupole analyzer mentioned above, it uses an electric and magnetic fields for the separation of the ions by mass to charge ratios. The ion-trap MS (Figure 4.6) has the advantage of its high sensitivity and resolution. Time-of-flight (TOF) mass spectrometers use an electric field to accelerate gas phase ions toward a detector. The  $m/z$  of an ion will determine how long it takes for it to travel from the source to the detector, with low  $m/z$  ions traveling faster relative to high  $m/z$  ions. Several designs of TOF analyzers exist, some using a linear flight tube and others using 1 or more reflectrons that change the direction of ion flight and improve resolution or the ability to distinguish two  $m/z$  ratios from one another. TOF analyzers have an essentially unlimited  $m/z$  range and very high sensitivity, mass accuracy, and percentage of ion transmission, but a limited dynamic range. The modern MS instrument hybridizes different types of mass analyzers on one instrument to broaden the tandem mass spectrometry applications.



**Figure 4.5. The operation of quadrupole mass analyzer**



**Figure 4.6. A schematic way of a ion-trap mass analyzer**

The mass spectrometric detection of target analyte ions employ selected ion monitoring (SIM) and multiple reaction monitoring (MRM) scanning modes for LC-MS and LC-MS/MS methods respectively. The SIM mode detection allows to select the desired  $m/z$  value for the target analyte by the instrument. This mode of analysis requires a single quadrupole and only the  $m/z$  of a precursor ion because no fragmentation is induced. Because only a limited mass-to-charge ratio range is transmitted or detected by the instrument, this operation typically results in significantly increased sensitivity.

Comparing with SIM, MRM mode detection provides the unparalleled specificity because this analysis utilizes triple quadrupole mass analyzer to select and analyze a specific analyte. In MRM mode, two stages of mass filtering are employed on a triple quadrupole mass spectrometer. In the first stage, an ion of interest (the precursor) is preselected in Q1 and induced to fragment by collisional excitation with a neutral gas in a pressurized collision cell (q2). In the second stage, instead of obtaining full scan  $ms/ms$  where all the possible fragment ions derived from the precursor are mass analyzed in Q3, only a small number of sequence-specific fragment ions (transition ions) are mass analyzed in Q3. This targeted MS analysis using MRM enhances the lower detection limit as compared to full scan  $ms/ms$  analysis by allowing rapid and continuous monitoring of the specific ions of interest. Particular precursor ions and product ions are selected for detection based on their unique pathways, MRM provides a great improvement in signal to noise ratio.

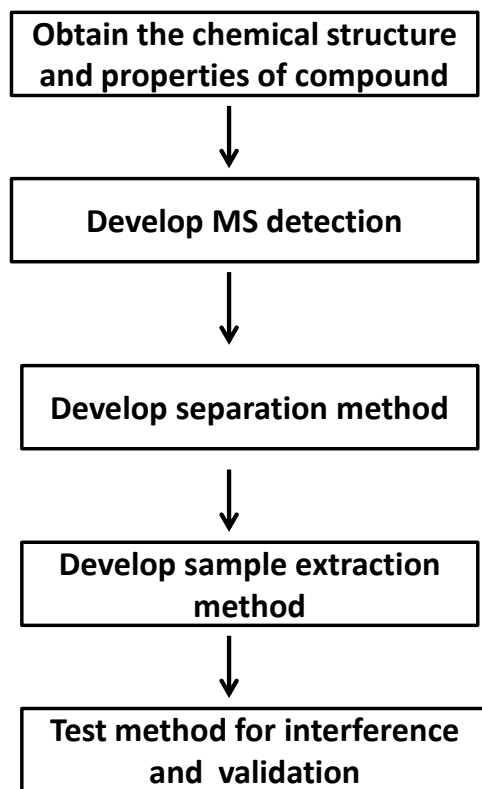
#### **4.2.2. HPLC separation**

Liquid chromatography is the basic separation technique for the separation of analyte of interest from the components of mixture. High-performance liquid chromatography (HPLC) is the most widely used separation method in bioanalysis. The mechanisms of liquid chromatography are based on distribution and partition of analyte between liquid mobile phase and stationary phase. There are different types of chromatographic methods based on different types of stationary phases applied. These include ion-exchange chromatography (IEC), reversed-phase chromatography (RPLC), normal phase chromatography (NPLC), and size-exclusion chromatography (SEC) etc.

Besides traditional HPLC, there are some modern approaches such as ultra-performance chromatography and hydrophilic interaction chromatography (HILIC) to improve the chromatographic separation efficiency and resolution. By using smaller particles, speed and peak capacity can be extended to new limits, termed Ultra Performance Liquid Chromatography (UPLC). This technique takes full advantage of chromatographic principles for separations using columns packed with smaller particles and higher flow rates for increased speed, with superior resolution and sensitivity over conventional HPLC by handling elevated pressure.

All small molecule compounds and peptides are separated by RP-LC with a C18 column and mass spectrometric detection. A guard column is used to prevent the contamination from the injected biological samples and damage from the mobile phase additives. A successful quantitative LC-MS/MS method development requires three important interlinked methodologies: MS detection, chromatographic separation and sample preparation. The steps involve in general method development process shown in Figure 4.6.

Several types of sample preparation methods are existed to extract the analyte from the biological matrices such as solid phase extraction (SPE), liquid-liquid extraction (LLE), and protein precipitation (PP).



**Figure 4.7. Method development workflow for small molecules**

### **4.3. Development and validation a LC-MS/MS method for determination of voltage-gated calcium channel and NMDA receptor antagonist NGP1-01 in mouse serum**

#### **4.3.1. Abstract**

NGP1-01 (8-benzylamino-8, 11-oxapentacyclo [5.4.0.0<sup>2</sup>, 6.0<sup>3</sup>, 10.0<sup>5</sup>, 9] undecane) is a heterocyclic cage compound with multifunctional calcium channel blocking activity that has been demonstrated to be neuroprotective in several neurodegenerative models. The primary mechanism of neuroprotection is through the blocking of the N-methyl-D-aspartate receptor (NMDA) and the L-type voltage-gated calcium channel to modulate excessive calcium influx into neurons. A sensitive internal standard LC-MS/MS method was developed and validated to quantify NGP1-01 in mouse serum. The internal standard was 8-phenylethyl-8, 11-oxapentacyclo [5.4.0.0<sup>2</sup>(2, 6).0<sup>3</sup>(3, 10).0<sup>5</sup>(5, 9)] undecane. Sample preparation involved a protein precipitation procedure by addition of acetonitrile. Chromatographic separation was carried out on a Phenomenex Kinetex phenyl-hexyl column (100 x 2.1 mm, 2.6  $\mu$ m) employing a linear gradient (45% to 95% in 6 min) of 5mM ammonium acetate in 2% acetonitrile mixing with increasing proportions of 5mm ammonium acetate in 100% acetonitrile. Detection was achieved by QTrap 5500 mass spectrometer (AB Sciex) employing electrospray ionization in the positive mode. Multiple-reaction-monitoring (MRM) was employed for NGP1-01 ( $m/z$  266  $\rightarrow$  91) and for the internal standard ( $m/z$  280  $\rightarrow$  105). The method validation was carried out in accordance with Food and Drug Administration (FDA) guidelines. The method had a linear range of at least 0.5–50 ng/mL with a correlation coefficient 0.999. The intra-assay and inter-assay precisions (%CV) ranged from 1.0 to



4.3% and accuracies (% relative error) ranged from -2.5% to 3.4%. The analyte was stable for at least 2 months at -20° C, for at least 10 hours at room temperature and for at least three freeze thaw cycles. The absolute recovery was 99 to 102% with a %CV  $\leq$  4.5%. The optimized method was applied in the measure of NGP1-01 in serum, brain and retinal samples from dosed mice.

#### **4.3.2. Introduction**

An increasingly important focus in drug discovery research in recent years is development of multifunctional drugs, agents with more than one therapeutic mechanism. Various terminologies besides multifunctional has been used in the literature for this category of drugs that is based on multiple therapeutic effects, including: designed multiple ligand, dual mechanism, dual ligand, bifunctional, multimechanistic, multimodal, pan agonist, and hybrid drugs. Two extensive reviews of the literature on the development and use of multifunctional drugs applied to various diseases have been published [22, 23]. Significant advantages of multifunctional drugs over conventional drugs that target one protein include: 1) many diseases have multi-factorial pathology and 2) there is a reduced likelihood of multifunctional drugs developing resistance [24]. Use of multifunctional drugs is also considered a better therapeutic strategy compared to use of multiple drugs (polypharmacy) to treat multi-factorial pathology due to the inherent complexities in polypharmacy approaches resulting from different degrees of bioavailability, pharmacokinetics and metabolism of the different drugs [25, 26]. In addition, the drug-drug interactions that often occur in the polypharmacy regimen, leads

to a combined, or even multiplied, toxicity that is not inherent in a multifunctional drug [27].

One area in which multifunctional drugs can potentially make a significant impact is neurodegenerative diseases, many of which have been shown to have multi-factorial pathoetiological effects and origins [28, 29]. Multiple etiologies in neurological diseases are indicated from multifunctional drug studies [29-31] including: anti-amyloid, anti-cholinesterase and anti-oxidant activity in Alzheimer's disease [32, 33]; anti-cholinesterase and monoamine oxidase-B (MAO-B) inhibitor activity in Alzheimer's disease, Parkinson's disease and Lewy body disease [34, 35]; iron chelator and MAO-B inhibitor activity in Alzheimer's disease [24] ; adenosine A<sub>2A</sub> receptor and MAO-inhibitory activity in Parkinson's disease [36] ; and pertinent to the present work, dual mechanism of modulating calcium entry in neuronal cells via L-type calcium channels and *N-methyl-D-aspartate* (NMDA) receptors important for its neuroprotective action in Alzheimer's, stroke and other neurodegenerative diseases [37]. In addition to the above, there are multiple etiologies in Huntington disease, amyotrophic lateral sclerosis, schizophrenia, depressive illness and stroke, Glaucoma and other eye diseases [29].

A deficiency in current therapies in neurodegenerative diseases is neuroprotection activity to prevent further loss of neurons. Thus, recent investigations have sought to develop neuroprotective agents to treat chronic neurodegenerative disorders such as Parkinson's and Huntington's diseases [19]. Excessive influx of calcium into neuronal cells resulting in cell death (a pathological process known as excitotoxicity) has been implicated in continuous and irreversible loss of neurons from the central nervous system

and certain regions of the brain leading to neurodegeneration [38-41]. Thus proteins controlling calcium influxes into neuronal cells are potential drug targets for developing the neuroprotective agents.

A promising multifunctional agent which has shown neuroprotection in neurodegenerative disease systems is NGP1-01, the pentacycloundecylamine 8-benzylamino-8, 11-oxapentacyclo [5.4.0.02, 6.03, 10.05, 9] undecane. NGP1-01 (Figure 4.8A) is a heterocyclic cage compound first characterized by Van der Schyf group in the mid 1980s [42] . NGP1-01 has been shown to produce neuroprotective effects by inhibiting calcium uptake by acting as an uncompetitive antagonist of both the ligand-operated calcium channel (NMDA receptors) and the voltage-gated calcium channels (VGCC) in neuronal cells [42-45], thus preventing the increased intracellular calcium excitotoxicity effect leading to neuronal cell death by necrotic or apoptotic mechanisms [46-48] .

NGP1-01 and derivatives of NGP1-01 have also been shown to have other functional effects pertinent to neurodegenerative diseases, in addition to the aforementioned inhibition effect on calcium uptake by neuronal cells. First, it has also been shown that VGCCs provide an alternate route for iron uptake by neuronal cells which contributes to neuronal cell toxicity and death [49]. NGP1-01 was found to reduce iron uptake in brain endothelial cells via the VGCC route [50]. Second, NGP1-01 and its derivative afforded neuroprotection by a presumed blocking of the dopamine transporter in the 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) Parkinsonian mouse model

[41, 51]. Third, NGP1-01 was found to exhibit considerable neuroprotection in a mouse middle cerebral artery occlusion model after transient focal brain ischemia [52].

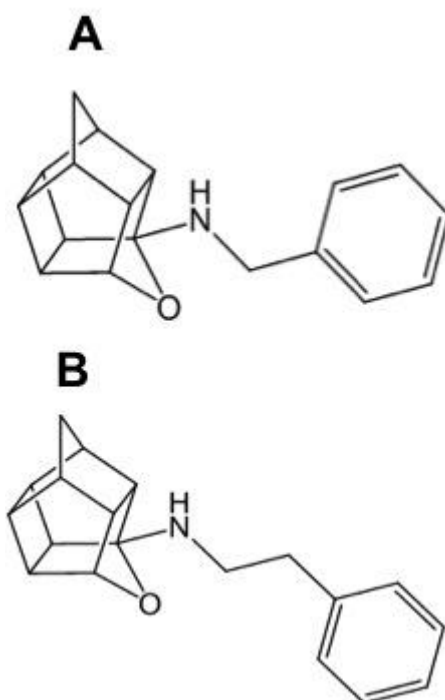
Thus NGP1-01 is a promising therapeutic candidate for treatment of neurodegenerative disorders through its multimodal effects. Development of sensitive analytical methodology for its determination in biological samples is warranted. The only reported analytical method for the determination of NGP1-01 is a HPLC technique employing UV absorbance detection at 210 nm, which was applied to the compound in aqueous solutions studying compound stability [53, 54] This technique is not applicable to biological samples because of a poor limit of detection and low analytical specificity inherent in absorbance detection. Although these studies also developed a LC-MS technique for NGP1-01, it employed out-of date particle beam ionization technology and it was not used analytically, being used solely for mass spectral identification of the HPLC peaks.

The present work reports the development and validation of a sensitive and specific LC-MS/MS technique for NGP1-01, applied to the determination of NGP1-01 in mouse serum, brain and retinal tissues.

### **4.3.3. Experimental**

#### **4.3.3.1. Chemicals and Materials**

NGP1-01 (Figure 4.8A) and the internal standard (IS), 8-phenylethyl-8, 11-oxapentacyclo [5.4.0.0(2, 6).0(3, 10).0(5, 9)] undecane (phenyl-ethyl-NGP1-01) (Figure 4.8B), were synthesized and purified [42, 51]. HPLC grade dimethyl sulfoxide (DMSO) and Optima LC/MS grade acetonitrile (ACN) were from Fisher Scientific (Fair Lawn, NJ, USA). ACS reagent grade ammonium acetate was from Sigma-Aldrich (St. Louis, MO, USA). HPLC grade water was from a Barnstead NANO pure water purification system with a Nanopure Diamond Pack Organic Free cartridge from Thermo Scientific (West Palm Beach, FL, USA). Mobile phases were filtered through 0.45 $\mu$ m membrane filters from Millipore (Billerica, MA, USA). Homogenizing apparatus was from Kontes Glass (Vineland, NJ, USA). Six individual lots of mouse serum (Non Swiss Albino) were from Innovative Research (Novi, MI, USA). Dosed serum, brain, and retina samples were from four different mice. Animal procedures were carried out according to the procedure approved by the Animal Care and Use Committee (IACUC) at Northeast Ohio Medical University.



**Figure 4.8.** The chemical structures of NGP1-01 (A) and the IS, phenyl-ethyl-NGP1-01(B)

#### **4.3.3.2. Stock and working DMSO solutions**

Stock solutions of NGP1-01 and phenyl-ethyl-NGP1-01 (IS) were prepared at a concentration of 1mg/mL in DMSO. Serial dilutions of NGP1-01 stock solution with DMSO gave the corresponding working DMSO solutions at concentrations of 10, 20, 40, 100, 200, 400, and 1000 ng/mL. In the same manner, NGP1-01 quality control (QC) working DMSO solutions were prepared with DMSO at concentrations of 24, 140, and 800 ng/mL. The internal standard (IS) working solution at 100 ng/mL was prepared by dilution of IS stock solution with DMSO. All stock and working solutions were stored at -20<sup>0</sup> C in amber glass vials.

#### **4.3.3.3. Preparation of calibration and quality control working serum solutions**

Calibrator and QC working serum solutions were prepared using six lots of pooled commercial mouse serum as diluent. The calibrator working serum solutions at 0.5, 1, 2, 5, 10, 20, and 50 ng/mL were prepared by spiking 10µL of the corresponding NGP1-01 working DMSO solutions into 200 µL of pooled mouse serum. QC working serum solutions at 1.2 (low), 7.0 (mid) and 40 (high) ng/mL were prepared by addition of 10µL of the corresponding NGP1-01 working DMSO solutions to 200 µL of pooled serum. The calibrator and QC working serum solutions were vortexed for 20 sec, and then stored at -20<sup>0</sup> C before sample preparation and the LC-MS/MS analysis described below.

#### **4.3.3.4. Preparation and storage of dosed samples**

Dosed serum, brain and retinal samples were stored at  $-20^{\circ}\text{C}$  until sample preparation steps were done as described below. A portion of the dosed mice brain was weighed and homogenized with twofold amount of phosphate buffered saline (PBS) (e.g. 100 mg brain / 200  $\mu\text{L}$  PBS) for two minutes using the homogenizing apparatus. In the same manner, each dosed mouse retina was weighed and twofold amount of PBS was added and homogenized. These prepared brain and retinal samples were processed as described in section 2.5.

#### **4.3.3.5. Preparation of calibrator standards, QC standards, blanks and dosed samples for analysis**

The calibrator and QC working serum solutions and the dosed serum samples were thawed to room temperature and taken through the preparation steps (as were the prepared brain and retinal samples), as given below. A volume of 10  $\mu\text{L}$  of IS work solution was added to 200  $\mu\text{L}$  of each sample to give a final IS concentration of 5 ng/mL. Double blanks were prepared by adding 20  $\mu\text{L}$  of ACN to 200  $\mu\text{L}$  of pooled commercial serum without adding the analyte and IS, where as single blanks were prepared by spiking 10  $\mu\text{L}$  of IS work solution and 10  $\mu\text{L}$  of ACN into 200  $\mu\text{L}$  pooled serum. Next, a protein precipitation step was done to all samples as follows. After a 20 seconds vortex, 800  $\mu\text{L}$  of ACN was added to each of the sample which were then sonicated for 10



minutes and centrifuged at 13,000 g for 20 minutes. The supernatants were then pipetted into HPLC autosampler vials for LC-MS/MS analysis. QC standards (1.2, 7 and 40 ng/mL) were run with the dosed samples to confirm acceptable performance of the method.

A series of dilutions were done (10-fold or 100-fold) with single blanks in order to adjust the concentration of NGP1-01 in the mouse dosed serum, brain and retinal samples to be within the linear range of the calibration standards.

#### **4.4. LC-MS/MS analysis**

The analysis was performed using an LC-MS-MS system in which a Shimadzu UPLC system (Columbia, MD, USA) was interfaced to an AB Sciex QTrap 5500 mass spectrometer equipped with an electrospray ionization (ESI) source (Framingham, MA, USA). The UPLC system consisted of a Prominence DGU-20A<sub>3R</sub> inline degasser, two LC-30AD pumps, a SIL-30AC autosampler, and a CBM-20A controller. Following the sample preparation described above, 10 µL of each prepared sample (section 2.5) was injected on to a Kinetex phenyl-hexyl column (100 x 2.1 mm, 2.6 µm) from Phenomenex (Torrance, CA, USA). An optimized linear gradient of mobile phase of A, 5mM of ammonium acetate in 2% ACN, and mobile phase B, 5mM of ammonium acetate in 100% ACN at 0.2 ml/min, was developed. The gradient program is given in Table 4.1. The column was equilibrated for 10 minutes before each sample injection. The run time for each injection was 12 min.

**Table 4.1. HPLC gradient program**

<b>Minutes</b>	<b>B%</b>
0-3	45 (isocratic)
3-9 (linear)	45-95
9-12	95 (isocratic)

Mass spectrometric analysis was positive electrospray ionization employing multiple reaction monitoring (MRM) at the transitions of  $m/z$  266  $\rightarrow$  91 for NGP1-01 and 280  $\rightarrow$  105 for IS. The optimized ionization MS parameters were as follows: ion spray voltage (5000 V), declustering potential (60 V), entrance potential (8 V), collision energy (40 V), collision exit potential (9 V), and nebulizer temperature (450 °C). Data acquisition and processing were done using the Analyst software (version 1.6.1) from AB Sciex.

#### **4.5. Method validation**

A complete bioanalytical method validation of NGP1-01 in mouse serum was done following the FDA guidelines for industry ([www.fda.gov/downloads/Drugs/Guidances/ucm070107.pdf](http://www.fda.gov/downloads/Drugs/Guidances/ucm070107.pdf)) and other reference [55]. The analytical method was validated for linearity, selectivity, precision and accuracy, recovery, matrix effect and stability.

Calibration curves were established by plotting the peak area ratios of NGP1-01 to IS (y) versus the spiked NGP1-01 concentrations (x) of the calibration standards (n=2 for each calibrator). The slope and correlation coefficient of the calibration curve were calculated using a weighted (1/x) linear regression.

Six double blanks were matched to six 0.5 ng/mL calibrator standards prepared from six individual lots of mouse serum to determine any extent of interfering chromatographic peaks and the signal response of the lowest calibrator.

Precision and accuracy were assessed by analyzing the of 1.2, 7.0 and 40 ng/mL QC standards. Intra-assay [within a day, n=5 for each QC standard (5 samples, each individually prepared and analyzed according to sections 2.5 and 2.6, respectively)] and inter-assay (five days, one of each of the QC standards prepared and analyzed each day, for n=5 for each QC standard) precision was determined as % CV. Accuracy was determined by comparing experimentally measured concentrations of NGP1-01 in QC standards versus theoretical values.

The absolute extraction recovery was determined by comparing the experimentally determined NGP1-01 peak areas of QC standards (1.2, 7.0 and 40 ng/mL), prepared and analyzed normally (NGP1-01 added prior to sample preparation), with the NGP1-01 peak areas of QC standards prepared with NGP1-01 added after sample preparation (n=3 for each pre- and post-addition QC standard).

The matrix effect study for mouse serum was assessed by comparing the peak areas of NGP1-01 for QC standards (1.2, 7.0 and 40 ng/mL) with the peak areas of NGP1-01 for similar concentrations of QC standards prepared in 80% ACN in water (n=3 for each QC and matched standard).

The stability of NGP1-01 in pooled serum was evaluated by preparing QC standards at two different concentrations (1.2 and 40 ng/mL). The stability of NGP1-01 was tested for the following conditions: (1) short term stability at room temperature for 8 h (pre-preparation), (2) stability at room temperature for 10 h (post-preparation), (3) long-term stability stored at -20<sup>0</sup> C for 60 days (pre-preparation), (4) freeze-thaw stability through three freeze-thaw cycles for three consecutive days (pre-preparation). All

experiments were run in triplicates for each QC concentration. These QC standards were compared with the theoretical values.

The stock solution stabilities of NGP1-01 and IS were evaluated by comparing the experimentally determined NGP1-01 concentrations of QC standards at 1.2 and 40 ng/mL (n=3) prepared from stored NGP1-01 stock solution (at -20 °C) for nine months and freshly prepared NGP1-01 stock solutions.

#### **4.5.1. Precision calculations for matrix factor and absolute recovery studies**

The matrix factor and absolute recoveries were determined as a ratio of average peak areas (see Table 4.3 and 4.4), in which the numerator and the denominator peak areas are experimentally determined average peak areas, each having a standard deviation.

### **4.6. Results and discussion**

#### **4.6.1. Mass spectra and liquid chromatography**

It was observed that NGP1-01 and the IS produced protonated molecular ions with strong intensity at m/z 266 and 280 respectively. As can be seen in their product ion spectra (Figure 4.9), predominant daughter ions of m/z 90 and 105 were generated by fragmentation of m/z 266 and 280, respectively. The MRM transitions of m/z 266 → 91

for NGP1-01 and 280 → 105 for IS were chosen for quantification in this study (Figure 4.10).

Various isocratic schemes employing different concentrations of ACN or methanol were investigated, but the peak shapes were irregular and the NGP1-01 and IS peaks were not as well separated. Optimized resolution and good peak shapes for NGP1-01 and IS were achieved using mobile phases of 5 mM ammonium acetate in 2% ACN (A) and 5 mM ammonium acetate in 100% ACN (B) employing a linear gradient (Table 4.1). Addition of ammonium acetate in the mobile phase not only enhanced the signals but also improved the peak shapes for both the analyte and IS. Also, no carryover was noted after a high concentration NGP1-01 run followed by a blank run.

Various gradient programs of methanol in 5 mM ammonium acetate were also evaluated. However the chromatographic performance was not as good as that obtained with the ACN gradient as given in Table 4.1.

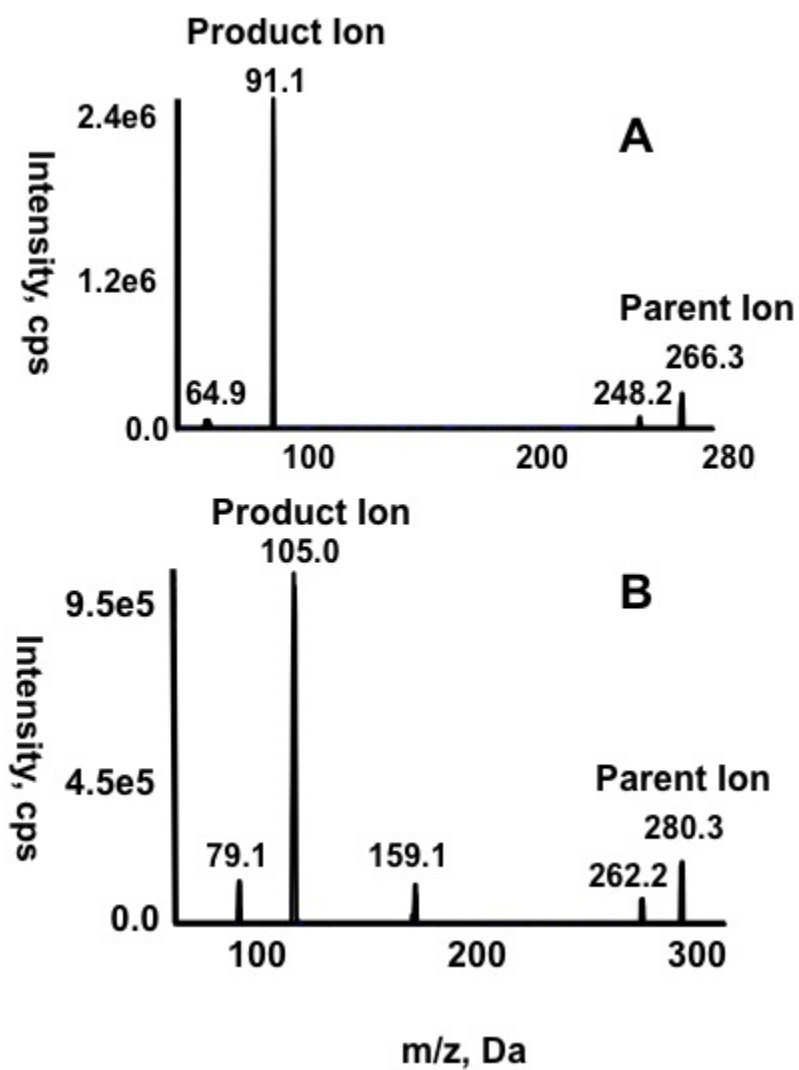
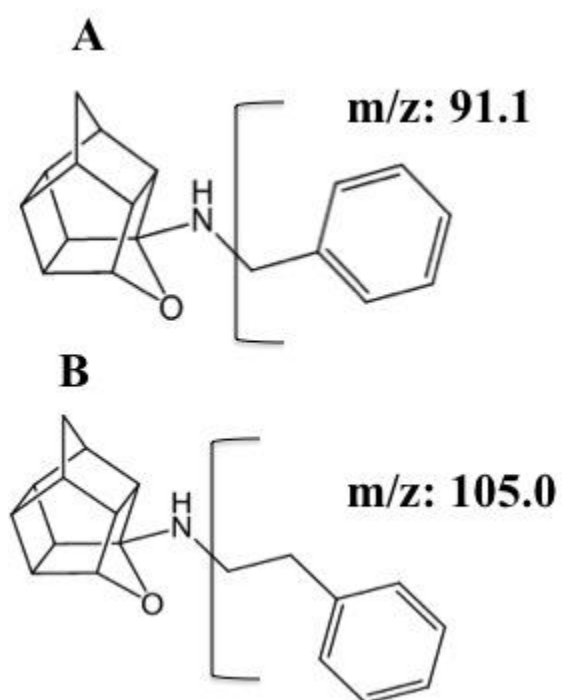


Figure 4.9. The production ion spectra of NGP1-01(A) and the internal standard (B)



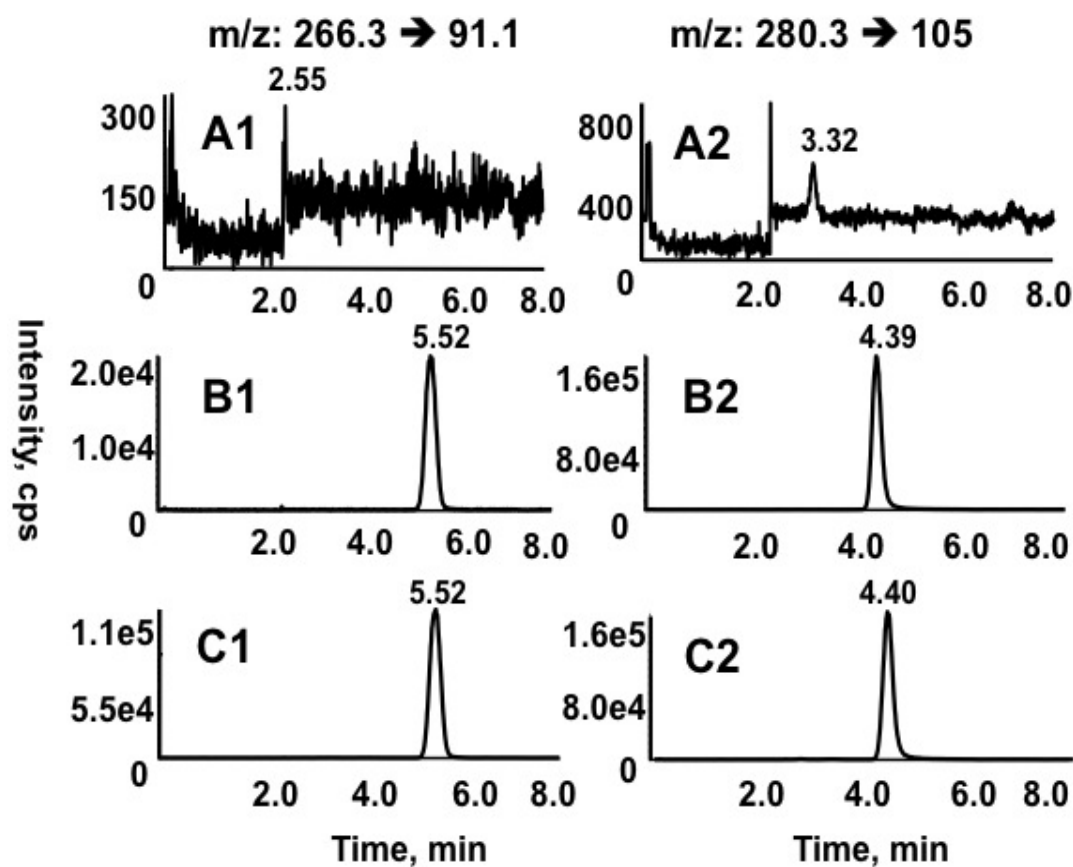
**Figure 4.10.** The proposed fragmentation patterns of NGP1-01 (A) and the IS (B)



#### 4.6.2. Calibration plot and chromatographic interference assessment

A calibration plot of the peak area ratios of NGP1-01 to the IS (y) versus NGP1-01 concentration (x) employing linear regression using a weighting factor of  $1/x$  yielded  $y = 0.239x + 0.0185$  with  $r = 0.9996$ , confirming linearity in the range of 0.5-50 ng/mL. The lowest calibration standard of 0.5 ng/mL in the present work (accounting for a 5-fold dilution resulting from the sample preparation in the present work) is three orders of magnitude better than the limit of detection of the only published HPLC technique for NGP1-01 [33]. Given that the signal/noise detector response for this 0.5 ng/mL calibrator is high [see Figure 4.11B (1 and 2)] means that the actual detection limit and limit of quantification is much lower for the present technique.

Chromatographic interfering peaks of this method was evaluated by comparative analysis of the lowest calibration standard (0.5 ng/mL) matched with double blanks prepared from six individual lots of mouse serum. As shown in Figure 4.11A (1 and 2), there were no interfering peaks at the retention time windows of the analyte and the IS at 5.52 and 4.39 min, respectively. The precision (%CV) and accuracy (%RE) for the lowest calibration standard were 5% and 1% respectively. A representative NGP1-01 dosed serum sample diluted to 2.8 ng/mL is shown in Figure 4.11C (1 and 2).



**Figure 4.11.** Representative MRM chromatograms of NGP1-01 (A1, B1, C1) and phenyl-ethyl-NGP1-01(A2, B2, C2) for double blank (A1 and A2), 0.5ng/mL NGP1-01 calibration standard and 5ng/mL IS (B1 and B2), and a dosed mouse serum

**sample at 2.8ng/mL NGP1-01 and 5ng/mL IS (actual concentration after dilution)  
mouse serum (C1 and C2).**

#### **4.6.3. Precision and accuracy**

Intra- and inter-assay precisions and accuracies were evaluated by analyzing low, mid and high QC standards for five replicates. As summarized in Table 4.2, the %RE for intra- and inter-assays were in a range of -2.5% to 3.4%. The coefficients of variation (%CV) for intra- and inter-assay were  $\leq 4.3\%$ . These values are within the acceptable limits according to FDA guidelines.

**Table 4.2. Precision and accuracy of intra-assay and inter-assay for the quantification of NGP1-01 in mouse serum (n=5)**

**Intra-Assay**

	<b>Spiked NGP1-01 conc.(ng/mL)</b>	<b>Measured NGP1-01 conc.(ng/mL) Mean±SD</b>	<b>Precision %CV<sup>a</sup></b>	<b>Accuracy %RE<sup>b</sup></b>
Low	1.2	1.17±0.02	1.7	-2.4
Mid	7.0	7.24±0.07	0.9	3.4
High	40	39.2±1.7	4.4	-2.1

**Inter-Assay**

	<b>Spiked NGP1-01 conc.(ng/mL)</b>	<b>Measured NGP1-01 conc.(ng/mL) Mean±SD</b>	<b>Precision %CV<sup>a</sup></b>	<b>Accuracy %RE<sup>b</sup></b>
Low	1.2	1.172±0.013	1.11	-2.5
Mid	7.0	7.054±0.094	1.33	1.14
High	40	40.32±0.697	1.73	0.5

$$^a \%CV = (\text{Standard Deviation}/\text{Mean}) \times 100\%$$

$$^b \%RE = [(\text{Measured} - \text{Spiked})/\text{Spiked}] \times 100\%$$

#### **4.6.4. Matrix effect and absolute recovery**

The matrix effect was determined as a matrix factor (MF) by calculating the ratio of peak areas from serum blanks (after processing) spiked with NGP1-01 at three QC concentrations to the peak areas of NGP1-01 added to 80% ACN at the same concentrations. The matrix factor (MF) for each concentration was calculated and given in Table 4.3. Results yielded MF ratios of 1.01 – 1.02, indicating that there is no matrix interference.

The absolute recoveries were determined for QC standards (1.2, 7.0, and 40 ng/mL) comparing peak areas for pre and post-preparation addition of NGP1-01 in triplicate. As indicated in Table 4.4, excellent recoveries were obtained over three concentrations for NGP1-01 in mouse serum matrix. The absolute recoveries were 99%-102%, with a %CV  $\leq$  4.5%.

**Table 4.3. Absolute Matrix factor of NGP1-01 in mouse serum (n=3)**

<b>NGP1-01 conc. (ng/mL)</b>	<b>Mean matrix factor</b>	<b>CV%<sup>a</sup></b>
	<b>± SD</b>	
1.2	1.01±0.048	4.7
7.0	1.01±0.028	2.8
40	1.01±0.036	3.6

$$^a\text{CV\%} = (\text{Standard Deviation}/\text{Mean}) \times 100\%$$

$$\text{MF} = \frac{\text{Peak area of NGP1-01 in serum}}{\text{Peak area of NGP1-01 in 80\% ACN}} \times 100 \%$$

**Table 4.4. Absolute recovery of NGP1-01 in mouse serum (n=3)**

<b>NGP1- 01 conc. (ng/mL)</b>	<b>Mean recovery  ± SD</b>	<b>CV%<sup>a</sup></b>
1.2	101±4.4	4.3
7.0	99±0.50	0.5
40	101±0.92	0.9

<sup>a</sup> CV% = (Standard Deviation/Mean) x 100%

$$\text{Recovery} = \frac{\text{Peak area of NGP1-01 spiked before extraction}}{\text{Peak area of NGP1-01 spiked after extraction}} \times 100 \%$$

#### **4.6.5. Stability**

The stability of NGP1-01 in mouse serum was determined under different storage conditions by measuring the QC standards at two concentrations using the calibration curves constructed from freshly prepared calibrators and comparing with theoretical values. As summarized in Table 4.5, pre-preparation samples were found to be stable (with a recoveries ranging from 97-101%): (1) stored at room temperature for 8 hours; (2) stored at -20° C for 2 months; and (3) after 3 freeze-thaw cycles. Post-preparation NGP1-01 samples were also found to be stable after storing at room temperature for 10 hours (97-102%). The stock solution stability of NGP1-01 after 9 months stored at -20°C was determined to be 98% with a %CV of 5%. These results indicate that NGP1-01 was stable for the experiments undertaken in the present work.



**Table 4.5. Stability studies of NGP1-01 in mouse serum pre- and post-preparation**  
(QC standards) (n=3)

<b>Stored sample</b>		<b>Spiked NGP1-01 conc. (ng/mL)</b>	<b>Measured NGP1-01 conc. (ng/mL) Mean ± SD</b>	<b>Stability (%Recovery<sup>a</sup>)</b>
2 months at -20 <sup>0</sup> C (Pre-preparation)	Low	1.2	1.17±0.04	98
	High	40	40.9±0.70	102
3 Freeze thaw cycles (Pre-preparation)	Low	1.2	1.16±0.01	97
	High	40	40.3±0.37	101
10 hr at RT (Post-preparation)	Low	1.2	1.16±0.05	97
	High	40	40.6±0.20	102
8 hr at RT (Pre preparation)	Low	1.2	1.17±0.02	98
	High	40	40.2±0.20	101

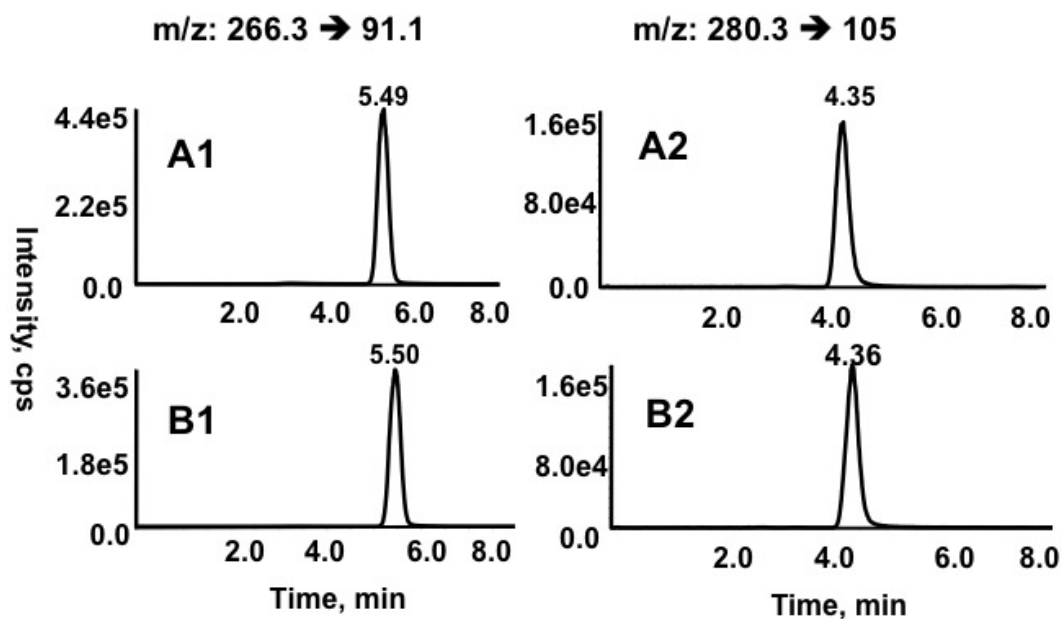
$$^a\% \text{Recovery} = \text{Measured} / \text{Spiked} \times 100\%$$

#### **4.6.6. Method applicability to pharmacokinetic studies**

This validated LC-MS/MS assay has been successfully applied for the determination of NGP1-01 in dosed serum, brain and retinal samples at a 1-hour time point from four different mice. The mice were given a single dose of NGP1-01 at 20mg / kg by intraperitoneal administration. The results are given in Table 4.6. The results show that NGP1-01 distributed into the blood as well as into the brain and retinal tissues. The high concentrations of NGP1-01 in brain and retinal tissues at 1 hour time point indicate a rapid distribution to these tissues from blood, readily passing the blood brain barrier in mice. Representative chromatograms of NGP1-01 in dosed serum, dosed brain, and dosed retina can be seen in Figure 4.11C (1 and 2) and Figure 4.12.

**Table 4.6. Measured concentration of NGP1-01 in dosed serum, brain and retina samples at 1 hr time point**

Amount injected	Administration	Measured conc. (ng/mL)		
		Mean $\pm$ SD (4mice, n=1)		
20mg/kG	Intraperitoneal	Serum	Brain	Retinal
		19.5 $\pm$ 9.2	44.0 $\pm$ 7.5	74.0 $\pm$ 41.7



**Figure 4.12. Representative MRM chromatograms of NGP1-01 (A1 and B1) and phenyl-ethyl-NGP1-01 (A2 and B2) for a dosed mouse brain sample at 11.3 ng/mL NGP1-01 (actual concentration after dilution) (A1 and A2) and for a dosed mouse retina sample at 9.2 ng/mL NGP1-01 (actual concentration after dilution) (B1 and B2).**

#### **4.7. Conclusion**

A rapid and sensitive internal standard LC-MS/MS method has been developed and validated for the quantitative measurement of NGP1-01 in mouse serum. A simple protein precipitation sample preparation method was used. The method employed a phenyl-hexyl reversed phase HPLC column for separation of the analyte and the internal standard from matrix interference and a MRM mode detection for sensitive and selective detection of the compounds. An ACN gradient with addition of ammonium acetate to the mobile phase was found to give good chromatographic performance in terms of resolution and peak shape. The assay gave a minimum linear calibration range of 0.5 - 50 ng/mL. The intra and inter-assay precision (%CV) and accuracy (%RE) of this method are well within the FDA guideline limits. NGP1-01 in serum was found to be stable under different storage conditions. This method has been applied to quantify NGP1-01 in limited number of dosed mouse serum samples to show the suitability for undertaking full NGP1-01 pharmacokinetic studies.

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## **CHAPTER 5**

### **FUTURE RESEARCH PLAN**

Gradient chromatofocusing has shown a great promise in the separation of protein and multiple forms of protein isoforms in terms of resolution and peak widths by employing generated smooth linear pH gradients on weak anion-exchange columns. But more studies to be done to make gradient chromatofocusing more competent separation technique in the field of protein characterization. The future research plans for GCF are described as follows:

Generating smooth linear pH gradients by introducing bridging buffers between application and elution buffers on different ion-exchange columns including strong anion-exchange column, weak cation-exchange column as well as strong cation-exchange columns. So far the pH range of generated pH gradients were 6.5 to 2.5 for weak anion-exchange columns as discussed in previous chapters. This pH range can be extended from 12 to 2 and 2 to 12 for anion-exchange and cation-exchange columns respectively by adding buffer components in application and elution buffers. This broad pH range of pH gradient generation will enable to isolate more number of proteins. Then study the effect

of number of buffer components on the separation of protein mixture employing generated smooth linear pH gradients on different kind of ion-exchange columns. This study will help us to find out what kind of columns are the best for gradient chromatofocusing separation of protein samples.

Computer modeling of the generation of pH gradients in gradient chromatofocusing: In gradient chromatofocusing, there are three different pH gradients include inlet, column, and outlet pH gradients. If a computer model is developed for the gradient chromatofocusing process, the above three pH gradients can be calculated by using this model without doing any experiment. This will save a lot of time to generate these pH gradients and enable to better understanding of the GCF process.

Integrating the gradient chromatofocusing with quadrupole-time-of flight mass spectrometry to detect and molecular mass determination of multiple forms of prolactin and lipoxygenase by carefully selecting some volatile buffer components which are compatible with mass spectrometry. The few of the buffer components of buffer systems discussed in previous chapters can be replaced with new buffer components which are volatile to generate smooth linear pH gradients. The generated pH gradients can be employed in GCF-MS pI based separation and detection of multiple forms of different proteins. If this can be achieved, GCF will become more powerful technique for protein analysis.

Applying GCF-MS technique to analyze the biological samples: GCF-ESI-MS technique has shown great promise in characterization of the standard protein samples. Since no biological samples have been analyzed so far by this technique, more studies need to be done to use this technique for separating and analyzing biological samples.