GRADIENT CHROMATOFOCUSING AND REVERSED PHASE HPLC IN PROTEIN ANALYSIS AND LC-MS/MS DETERMINATION OF THIAZOLIDINEDIONE NL-1 IN MOUSE SERUM AND BRAIN

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Gradient chromatofocusing (GCF) is a technique developed using linear pH gradients on weak-anion exchange HPLC columns. GCF greatly extends the capabilities of chromatofocusing by overcoming the shortcomings of the conventional chromatofocusing technique. GCF has been interfaced to mass spectrometry in protein separations. Chapter 1 demonstrates the advantage of GCF technique over reversed-phase (RP) HPLC in protein analysis. All RP protein peaks were eluted in a narrower region (40 – 60% organic in a gradient) of the gradient compared to GCF, in which the proteins peaks were distributed along the entire length of the gradient due their differences in isolectric points. Regarding peak widths, some had narrower widths with RP, others with GCF. The median, average peak widths for both the techniques were similar, however, overall, GCF is advantageous when compared with RP technique in the separation of proteins.

Chapter 2 demonstrates a new procedure for characterizing weak anion-exchange (WAX) HPLC columns. Peak figures-of-merit results from injection of the 100 µM NaNO₃ standard were compared to that of injections toluene recommended by the
manufacturer (20 mM toluene) has revealed that the peak width increases by 50% for the NaNO$_3$ while the increase in toluene peak width is 30%. And, the shift in the retention time of 100 µM NaNO$_3$ from 3.17 mins to 1.52 mins beginning and after extensive use respectively has shown that 52% of function ion-exchange sites has been lost after an extensive usage.

Chapter 3, 4 and 5 demonstrates developing a quantitative LC-MS/MS method for determination of thiazolidinedione (TZD) mitoNEET ligand NL-1 in mouse serum and its pharmacokinetic applications. A sensitive LC-MS/MS assay has been developed and validated for quantification of NL-1 \{5-[(3,5-di-tert-butyl-4-hydroxyphenyl)methyl]-1,3-thiazolidine-2,4-dione\} in mouse serum. The method is suitable for pharmacokinetic (PK) studies of the parent drug NL-1 based on the preliminary serum results from dosed NL-1 mouse studies. The key pharmacokinetic parameters of NL-1 in its serum and brain concentration –were shown good penetration of drug into mice brain, More extensive metabolite studies should be performed as a part of future studies in order to investigate the fate of the analyte.
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1.1. Introduction

The Human Genome project was an international scientific research project with an ultimate goal to determine the sequence of human genome. The outstanding data obtained from this project was yielded critical information in the life sciences and medical fields. After determination of the sequence of almost 30,000 human genomes, it has become the primary tool in the identification of the proteins encoded by these genes and the correlation of the qualitative and quantitative changes of these proteins in the diagnosis and treatment of disease. Techniques that can measure and identify a multitude of proteins simultaneously are important in finding protein defects, either in structure or quantity pertinent as disease markers and/or disease etiologies [1-5].

A newly proposed method, gradient chromatofocusing (GCF) for analysis of multiple proteins in a sample has broad scope application in determining the protein content and concentrations using simple, volatile buffer components. The GCF technique uses ion-
exchange chromatography (IEC) employing linear pH gradients for protein separation. The proposed work will lead to complementary capabilities to existing chromatographic techniques.

1.1.1. IEC in protein / peptide analysis

IEC separates proteins / peptides based upon their charges. Different substances have different degrees of interactions with the ion exchanger due to differences in their charge, charge densities and distribution of charge on their surfaces. These interactions can be controlled by varying conditions such as ionic strength and pH. In the equilibrium state, the ion exchanger gets saturated with exchangeable counter-ions, usually sodium for cation-exchange or chloride for anion-exchange chromatography. Reversible binding takes place, once the charged solute molecules enter into the column. In the second step elution will occur by increasing the ionic strength using higher concentration of salts (usually 0 - 1M) or by changing the pH [6, 7].

The net charge of a protein is determined by the summation of individual charges on the protein. The pH at which net charge is zero is the *isoelectric point* (pI) of that protein. When the pH below the pI, the protein will be positively charged and at the pH above the pI it will be negatively charged. Technically pH of the mobile phase must be between the pI of the protein and pKa of the ion-exchange ligand on the stationary phase in order to achieve retention on the ion-exchanger [6, 8].

Ion-exchange packing material can be classified into two types based on the charge, cation and anion exchangers. In the cation-exchange chromatography, positively charged molecules are attracted to a negatively charged stationary phase matrix. Conversely, in
the anion-exchange chromatography, negatively charged molecules are attracted to a positively charged stationary phase matrix [9]. The cation and anion stationary phases are further sub-divided as weak or strong. The terms weak and strong indicate the susceptibility of the charge of the ion-exchange ligand to changes in pH. Strong ion-exchange groups maintain their charge by having a high pKa for anion-exchange groups and a low pKa for cation-exchange groups. Sulfonic acids, quaternary amines are as strong groups because of their ionization do not vary appreciably from pH range 1-14. On the other hand, weak-ion-exchange groups have a narrower pH range in which the ligands are charged because the pKa of the ion-exchange groups have intermediate values. Carboxyl groups and tertiary amines are in this category [7].

Besides the type of ion exchanger there are mobile phase factors affecting the chromatography such as pH, ionic strength, choice of the buffering substance and gradient profile. The starting pH and concentration of buffer species in the application or elution buffers is important [10, 11]. Most of the separations use 10-25 mM buffer concentrations but can be as high as 50 mM [12]. David et al. showed that the introduction of 5% organic modifier to the mobile phase buffers helped in betterment of the proteomics analyses [13]. It was also found that high resolution with long gradient times at low flow rates. A step gradient can cause a band broadening where as continuous shallow gradient slopes exhibit excellent chromatographic peak shape [14-16].

The general plate theory is not applicable to protein separation in IEC. For example column length does not significantly affect resolution, while in the chromatography of small columns increasing column length increases the number of plates and thus increases resolution. It has been found that very short columns can be effective in protein
separation. In this order Yoshio et al. reported that a strong cation-exchange column with the length of 75mm and 150 mm provided almost identical resolution [17].

There are several advantages of IEC in protein analysis. For one, the native conformation and biological activity during chromatography occurs in IEC, whereas in reversed-phase (RP) chromatography, protein denaturation occurs as organic concentration increases. Le Bihan et al. reported that a tryptic digest of a protein extract derived from human K562 cells observed that large numbers of molecular peptides are identified using online strong cation-exchange separation compared to the more conventional reverse phase RP- LC/MS approach [16].

1.1.2. Chromatofocusing in protein / peptide analysis

The continuous development of IEC lead to the development of a technique called chromatofocusing (CF) by Sluyterman and co-workers [18, 19]. This technique employs IEC using a pH gradient to separate biomolecules with acid / base functionalities, principally used in the purification of proteins and peptides [20-22]. The CF technique is popular as the separation as the based on pIs of proteins in mixtures. The buffers used in CF are polyampholytes which contain range pKa functionalities to achieve an even buffering capacity over the chosen pH range. The capabilities of iso-electric focusing (IEF) in terms of determination of pI values approximately or in achieving the more resolution is notable. Chromatofocusing on the other hand only approximates a protein pI and does not achieve the same resolution as IEF [23]. Development of pH gradients in CF is not a steady-state process as in IEF.
Conventional CF has several limitations. It is difficult to remove the polyampholyte substances from eluted proteins because of complex formation which is problematic in protein purification procedures. This technique is limited to the low concentration buffers employing step gradients (typically 2.5 – 5mM), since higher concentrations usually give unacceptably steep pH gradients and consequently poor resolution of the proteins [24-27].

In an effort to get away from using high molecular weight polyampholytes several investigators attempted to use multiple low molecular weight buffers. Hearn et al. and Hutchen’s group employed small molecular weight buffer components in order to generate a linear pH gradient using a step change but were not successful in generating a smooth pH gradients [28]. Frey et al. have been able to generate smooth linear and concave pH gradients using step changes between buffers containing positively or neutrally charged low molecular mass amine buffer components on anion-exchange columns. However the range of pH was small and technique was limited to lower concentration buffers (2-5mM) in order to generate reasonable pH gradient slopes [29-31]. Anderson’s group developed a technique called gradient chromatofocusing (GCF), which had been applied successfully in the separation of proteins using linear pH gradients employing low molecular weight buffer components in the mobile phase, as described below.

1.1.3. GCF in protein / peptide analysis

To generate continuous linear pH gradients Anderson’s group has introduced a new technique called GCF where the mobile phases with multiple low molecular weights
buffer components mix externally to introduce the pH gradient at the column inlet [32, 33]. A HPLC gradient system is used to generate linear pH gradients by progressively increasing the percentage of elution buffer to application buffer which is imposed onto a weak anion-exchange column. GCF technique has extended operational capabilities over conventional CF due to its versatility in generating linear pH gradients of differing sloped and different buffer concentrations [34, 35].

The slope of the external pH gradient is not easy to control in the conventional techniques using a step change. However in GCF the slopes can be easily controlled and manipulated by adjusting the ratios of elution buffer to application buffer introduced on the ion-exchange column by the gradient program. Its ability to control slopes and to produce linear pH gradients with common buffer components achieves optimized protein separation (Figure 1.1).
Figure 1.1. Set up of Gradient anion chromatofocusing system.
GCF generally employs a weak anion-exchange column (positively charged stationary phase) equilibrated with a basic application buffer to achieve a negative net charge on the injected sample proteins. These anionic species are retained on the columns which are then eluted by a decreasing pH linear gradient. Elution of the proteins occurs theoretically when the pH of the mobile phase is equal to their pI values although experimentally there are deviations from the pI. GCF ion-exchange experiments resolved the proteins by giving sharper peaks, which means there is a higher sensitivity and more proteins that can be separated. Studies also showed that there is a dramatic increase in separation as the buffer concentration is increased. Anderson’s group has recently proved that GCF can be interfaced with mass spectrometer in the separation and detection proteins yielding molecular masses information [36].

1.1.4. RP chromatography in protein / peptide analysis

RP chromatography has applications in both analytical as well as the preparative field. Molecules with some hydrophobicity like proteins, peptides, and nucleic acids can be separated with good resolution. The central mechanism involves in the RP is mainly based upon the hydrophobic binding interaction between the solute molecule in the mobile phase and the immobilized hydrophobic ligand (stationary phase). The initial solute binding conditions used in RP chromatography are primarily aqueous which indicates a high degree of organized water structure surrounding both the solute as well as stationary ligand. Protein structure is sensitive to environment. Extreme pH levels and higher organic concentrations can lead to the denaturation of the protein and may cause irreversible binding to the column [6, 37].
Critical parameters that make up the RP column are the chemical composition of the base matrix, particle size of the packing material, type of the stationary phase ligand, and column length. The base matrix commercially available for the reverse phase media is generally composed of silica or synthetic organic polymer such as polystyrene. Silica support matrix is very popular for reverse phase packing materials. The main drawback is that it is soluble at pH above 7.5, whereas polymeric bases have a greater operational pH range (pH 2-13). However it is not possible to determine exactly which ligand is ideal for a solute without preliminary investigation. A rule of thumb is: the more hydrophobic the molecule to be purified, the less hydrophobic the ligand needs to be used and vice versa [38, 39].

Particle size varies from 1.5 to 10 microns and desired flow rates usually depend upon the internal diameter of the column and the particle size used. The range of flow-rates for different column internal diameters is given in Table 1.1 [40, 41]. C4 columns are the most utilized stationary phase for the RP separation of proteins and C4-C18 phases are used for peptides, depending on peptide’s hydrophobicity [37].

Biomolecules usually adsorb to the surface of a RP matrix under aqueous conditions, they desorb from the RP stationary phase within a narrow window of organic modifier concentration. The use of gradient in organic modifier is required to affect the elution of proteins. Separation takes place because of the different adsorption affinities of the protein for the RP ligands thus eluting at different organic modifier concentrations. As in IEC column length is not critical in the separation of biological macromolecule being more effected by organic mobile phase.
Oleg V. Krokhin research group recently published a paper on peptide separation based on linear – solvent – strength theory (LSS), which reported data regarding the elution organic modifier strength vs. slope of the gradient based for a multitude peptides. It was found that peptidic compounds elute from alkyl-bonded media in a very narrow range of acetonitrile concentrations. The retention of the large molecules is very sensitive to small changes in mobile phase composition at the critical organic modifier strength and hence large molecules desorb in narrow organic modifier range. Thus gradient elution is required in the RP chromatography involving macromolecules [42].
Table 1.1. Flow rates used for different HPLC column diameters

<table>
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<tr>
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<th>Column i.d. (mm)</th>
<th>Detection Enhanced</th>
<th>Flow Rates</th>
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<td>0.5 -2 mL/min</td>
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<tr>
<td>Narrow bore</td>
<td>2.1 mm</td>
<td>5</td>
<td>200 – 4 µL/min</td>
</tr>
<tr>
<td>Micro bore</td>
<td>0.8 – 1.0 mm</td>
<td>20 – 25</td>
<td>25 – 60 µL/min</td>
</tr>
<tr>
<td>Capillary</td>
<td>100 – 500 µm</td>
<td>80 – 2000</td>
<td>1 – 15 µL/min</td>
</tr>
<tr>
<td>Nano</td>
<td>&lt; 100 µm</td>
<td>2000 – 10,000</td>
<td>&lt; 1 µL/min</td>
</tr>
</tbody>
</table>
When the HPLC system is connected to a UV detector, solvents must be UV transparent. Acetonitrile (ACN), methanol (MeOH) are commonly used organic modifiers in RP separation. MeOH is economical, widely available and provides good separation capability of proteins, peptides. Because of their complexity, the separation of biological macromolecules from its complex mixtures is an important and critical step in the protein study [42]. Gallardo et al. characterized a protein mixture of beta-globulins in hemoglobin using a tryptic-digested samples. Samples were separated on C4, RP-HPLC column then further ionized and analyzed using ESI-MS setup [43].

An example of typical RP separation of proteins or peptides biological sample showing in complete resolution is from a research group from University of California reporting a UV elution profile of a polypeptide mixture from spinach on a C18 RP column. Proteins were separated using 5 – 100% linear gradient with a 60% formic acid in water application mobile phase and 2-propanol elution mobile phase with the results shown in Figure 1.2. Each peak consists of more than one peptide. Peak “PS2-F”, and peak “C” reported as a group of three peptides in each. Peak D2 (33.9min) 280 nm which had another peptide at the tail (PS2-1, 37.1min) [44].
Figure 1.2. A C18 RP-HPLC separation using formic acid and 2-propanol gradients
A single HPLC technique such as IEC, RP, CF or GCF is not sufficient in separating protein / peptides. A complimentary technique was needed in terms of characterizing proteins / peptides in mixtures. A combination of complimentary techniques is needed for separation of complex mixtures of proteins and peptides. Anderson’s group has developed a GCF technique that has unique capabilities. In this current work, we compare GCF with RP performance in separating proteins.

1.2. Experimental

1.2.1. Materials

1.2.1.1. Proteins

α-chymotrypsin (catalog no. L7651), insulin (catalog no. I5500), β–lactoglobulin A (catalog no. L7880), β–lactoglobulin B (catalog no. L8005), myoglobin (catalog no. M1882), cytochrom C (catalog no. C2506), lysozyme (catalog no. L7651), trypsin inhibitor (catalog no. T9003), conalbumin (catalog no. C-0755), albumin from rat serum (catalog no. A6272) were purchased from Sigma-Aldrich (St.Louis, MO, USA). Protein G (catalog no. Z02007), proteinase K (catalog no. Z02003), streptavidin (catalog no. Z02043) were purchased from Genscript (Piscataway, NJ).

1.2.1.2. Buffer components / chemicals / other procedures

Ammonium bicarbonate (catalog # 09830), acetic acid (catalog # 695092), lactic acid solution (catalog # 252476), 4-methylpyridine (catalog # 239615), pyridine (catalog # 270970), 4-chlorophenyl acetic acid (catalog # 139262) were purchased from Sigma-
Aldrich (St.Louis, MO, USA). Bis-tris methane (catalog # BR 301-100) from Fisher (Fair Lawn, NJ, USA). HPLC grade methanol and acetonitrile were from Fisher Scientific (Fair Lawn, NJ, USA). ACS grade ammonium acetate was from Sigma Aldrich (St. Louis, MO, 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 0.45µ cellulose ester membrane filters from Millipore (Billerica, MA, USA).

1.2.1.3. Instrumentation

Beckman Coulter SYSTEM GOLD 126 pumps with SYSTEM GOLD 168 Detector interfaced with SYSTEM GOLD 508 auto sampler (Brea, CA, USA). All the data collected and analyzed using 32 Karat Gold software (version 8). Agilent 1100 series HPLC binary mode-capillary pumps (Santa Clara, CA, USA) interfaced with a Brukers HCT 3000 Mass Spectrometer equipped with electro spray ionization (Brea, CA, USA). Shimadzu 10 AT-VP capillary HPLC pump (Columbia, MD, USA). All the data collected and analyzed using Bruker Daltonics esquire 5.1 SR 1 software and ChemStation. The pH measurements of the buffer components throughout the analysis were measured using Denver Instrument, Model 250 pH meter (Bohemia, NY, USA). A HPLC column (100 x 4.6 mm) packed in our facility using bulk anion-exchange material (Protein-Pak DEAE 8HR, 8µm diameter, 1000 Å pore diameter, DEAE functionalized polymethacrylate) was from Waters (Milford, MA, USA). C4, Vydac, 250mm × 0.3 mm, 5 µ, 300Å (Columbia, MD, USA). C4 and PEI HPLC columns were purchased from The Nest Group, 100 x 2.1 mm, 5 µ, 300Å (Southborough, MA, USA).
1.2.2. RP experimental studies

1.2.2.1. MS infusion studies

Infusion experiments were conducted on each standard protein (10 µM) using a Bruker esquire HCT-MS. Each protein standards was prepared in 20% ACN, 50% ACN, 20% MeOH and 50% MeOH with 0.1% formic acid in each to identify the optimum conditions for better MS signal. These experiments determined optimized MS parameters and MS detectability of the individual proteins. These experiments determined optimized MS parameters and MS detectability of the individual proteins. From these experiments an electrospray (ESI)-MS conditions were optimized for all the proteins, including a scan range of 800 – 2400 m/z, entrance potential (30 V), collision exit potential (60 V), ion spray voltage (4500 V), and 5 L/min nitrogen was used as nebulizer gas (300°C). The maximum accumulation time was 20 ms and the infusion rate was 20 µL/min to perform extracted ion analysis.

1.2.2.2. Vydac-RP column experiment

The mixture of eleven proteins was subjected to RP-HPLC separation using a C4 Vydac capillary column using 2 – 90% MeOH linear gradient. Each protein was injected at 100 µM concentration in mixture. Protein peak widths w0.5, resolutions were calculated using extracted ion current. All the chromatographic conditions used for this experiment can be seen below.

**Chromatographic conditions**

**HPLC system:** Agilent 1100, binary mode-capillary system.
**Column details:** C4, Vydac, 250 mm × 0.3 mm, 5 µm, 300 Å.

**Mobile phase:** 2% methanol, 0.1% formic acid in water for the application mobile phase and 90% methanol, 0.1% formic acid in water for the elution mobile phase.

**Flow rate:** 7 µL / min

**Time program:** 0.1 – 90% linear gradient in 35 minutes, held at 90% for 5 minutes, 90%-0.1% in 2 minutes. Due to the gradient delay time, each run was monitored until 65 minutes.

**Injection volumes:** 10 µL

### 1.2.2.3. Nest Group-RP experiment

The nine protein standards were characterized using the new The Nest Group RP-HPLC C4 columns. Each protein was prepared at 100 µM concentration in the mixture. All the chromatographic conditions used for this experiment can be seen below.

**Chromatographic conditions**

**HPLC system:** Beckman Coulter SYSTEM GOLD HPLC with 126 pumps with SYSTEM GOLD 168 PDA Detector.

**Column details:** C4, 100mm × 2.1 mm, 5 µ, 300Å.

**Mobile phase:** 0.1% TFA in DIW as application mobile phase and 0.1% TFA in 100% ACN as elution mobile phase.

**Flow rate:** 0.3 mL/min.

**Time program:** 0% of elution mobile phase for first 5 minutes, 0-100% elution mobile phase in 44 minutes, 100% elution mobile phase for 10 minutes and 100 – 0% elution
mobile phase in 6 minutes. Total run time was 65 minutes. Each run was equilibrated for 60 minutes prior to the protein injection.

**Injection volume:** 10 µL

**Wave length:** 280 nm, 254 nm.

### 1.2.3. GCF experimental studies

The selected nine proteins were prepared in 100 µM each in DIW and injected on to a weak anion-exchange, PEI HPLC column. The proteins were eluted by a pH gradient in the mobile phase going from pH 6.5 – 3.5. Proteins separated on PEI column using an optimized linear pH gradient. Hence, this section is subdivided into two deferent sections, optimization of pH gradient and protein characterization using optimized pH gradient 1.2.3.1, 1.2.3.2 respectively.

#### 1.2.3.1. Optimization of pH gradient

In order to enhance the resolution and peak half widths of the proteins on GCF, an optimized linear pH gradient was developed. Producing a smooth linear pH gradient in GCF is challenging. Our group has been successful in producing a smooth linear pH gradient with a combination of different organic and inorganic low molecular weight volatile buffers during late 1990s.

The pH gradient generated using the previously used buffer components (application buffer: 5 mM ammonium bicarbonate, 5mM pyridine; elution buffer: 25 mM acetic acid, 25 mM lactic acid) in the preliminary results did not produce a smooth linear gradient. In this order a concept called “bridging” has been introduced. The idea behind
bridging is nothing but an addition of one of the lower pKa buffer components of application buffer to the elution buffer and addition of one the highest pKa buffer components to the application buffer which is discussed more in detailed in coming paragraphs.

Various buffer components having pKa values in regular intervals (spacing) have been used to generate an even buffer capacity throughout the chromatographic run. As a first attempt, an application buffer with 5mM ammonium bicarbonate pKa = 6.37, 9.2, 10.3, 10 mM pyridine pKa = 5.19 along with 15mM of acetic acid and elution buffer with 25 mM acetic acid pKa = 4.76, 25 mM lactic acid pKa = 3.81 along with 5 mM of pyridine in it. Based on the experimental results the minimum concentration of 25 mM of elution buffer components was mandatory to elute all the proteins. Lower concentrations of application, elution buffers resulted either no retention or irreversible retentions.

After enormous number of attempts, our group has identified a set of volatile buffers which can produce a super smoother pH gradient within the pH range of 6.5 - 3.8. The application buffer was made up of 10 mM Bis-Tris methane (6.5), 10 mM 3-Methyl pyridine (5.68) with 5mM of acetic acid in it as a bridging agent and the elution buffer was with 10 mM Acetic acid (4.76), 10 mM Lactic acid (3.81) along with 5mM of 3-methyl pyridine as a bridging agent.
1.2.3.2. Protein characterization using optimized pH gradient

The optimized pH gradient has been successfully applied to analyze a nine-protein mixture (chosen by their pI values to fit into the mobile phase buffers pH range). All the chromatographic conditions used for this study can be seen below.

Chromatographic conditions

**HPLC system:** Beckman Coulter SYSTEM GOLD 126 pumps with SYSTEM GOLD 168 Detector.

**Column details:** PEI, 100mm × 4.6mm, 5 µ, 300Å.

**Mobile phase:** Application buffer (pH: 6.5): 10 mM Bis-Tris methane (6.5), 10 mM 3-Methyl pyridine (5.68) with 5 mM of acetic acid in it as a bridging agent; Elution buffer (pH: 3.8): 10 mM Acetic acid (4.76), 10 mM Lactic acid (3.81) along with 5 mM of 3-methyl pyridine as a bridging agent.

**Flow rate:** 1 mL/min

**Time program:** 0 - 26.64% of elution buffer in first 12 minutes, 26.64 - 41.64% in 10 minutes, 41.64 – 57.74% in 5 minutes, 57.74 - 100% in 19.03 minutes, 100 - 0% in 5 minutes. Total run time was 65 minutes. Column equilibrated for 60 minutes prior to each protein injection.
1.3. Results and Discussion

1.3.1. RP experiments

1.3.1.1. Infusion studies

Infusion experiments were conducted on each standard proteins (10 µM) using a Bruker esquire HCT ESI-MS using 20% ACN, 50% ACN, 20% MeOH and 50% MeOH with 0.1% formic acid in each infusion sample. Eleven protein standards were selected based upon their molecular weight range of 5500 Da – 32,000 Da and iso-electric point range of 9.2 – 2.6. One of the eleven proteins was at a higher pI and above the GCF buffer pH range (Lysozyme: 11.0) to observe the nature of elution outside the pH range.

An example spectrum of the “Cytochrome C” in 20% methanol with 0.1% formic acid is in the Figure 1.3 below. The deconvoluted spectrum gave a molecular weight as 12,229.51 Da. that is close its theoretical mass.
Figure 1.3. An infusion mass spectrum of 100 µM cytochrome C with 0.1% formic acid; m/z values: 644.62 (19+), 680.33 (18+), 720.33 (17+), 765.29 (16+), 816.25 (15+), 874.49 (14+), 941.64 (13+), 1020.05 (12+).
1.3.1.2. Vydac-RP experiments

The mixture of eleven proteins were subjected to RP-HPLC separation using a C4 Vydac capillary column using 2 – 90% MeOH linear gradient with the chromatogram given in Figure 1.4. Results from extracted ion chromatograms of each protein from the protein mixture were done to determine retention times, as given in Figure 1.4 and peak widths $w_{0.5}$, and separation factors ($\alpha$), and resolution (R) for adjacent peaks, given in Table 1.2.
Figure 1.4. Separation of protein mixture using a Vydac C4 RP-HPLC column.

Table 1.2. Figure-of-merit for eleven protein mixture on Vydac-RP column

<table>
<thead>
<tr>
<th>Protein</th>
<th>Separation factor ($k_2/k_1$)</th>
<th>Peak width $w_{0.5}$ (min)</th>
<th>Resolution $\alpha$</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-chymotrypsin A</td>
<td>1.3</td>
<td>1.3</td>
<td>-</td>
</tr>
<tr>
<td>Insulin</td>
<td>1.126</td>
<td>1.3</td>
<td>1.227</td>
</tr>
<tr>
<td>β-Lactoglobulin A</td>
<td>1.15</td>
<td>1.1</td>
<td>1.782</td>
</tr>
<tr>
<td>β-Lactoglobulin B</td>
<td>1.239</td>
<td>1.0</td>
<td>3.75</td>
</tr>
<tr>
<td>Myoglobin</td>
<td>1.055</td>
<td>5.8</td>
<td>0.329</td>
</tr>
<tr>
<td>Cytochrome C</td>
<td>1.022</td>
<td>7.1</td>
<td>0.073</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>1.111</td>
<td>7.0</td>
<td>0.342</td>
</tr>
<tr>
<td>Proteinase K</td>
<td>1.087</td>
<td>1.1</td>
<td>0.524</td>
</tr>
<tr>
<td>Trypsin Inhibitor</td>
<td>1.035</td>
<td>3.7</td>
<td>0.349</td>
</tr>
<tr>
<td>Protein G</td>
<td>1.017</td>
<td>5.0</td>
<td>0.108</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td>1.085</td>
<td>3.300</td>
<td>0.857</td>
</tr>
<tr>
<td><strong>Median</strong></td>
<td>1.071</td>
<td>1.700</td>
<td>0.346</td>
</tr>
</tbody>
</table>
1.3.1.3. Nest Group-RP experiments

The results obtained by Vydac-RP gave some valuable information regarding the protein separation order and peak widths $w_{0.5}$, resolutions of the proteins. However, in order to obtain more accurate values of peak widths, resolutions of proteins in a RP column, the same protein standards were run using a different manufacturer, The Nest Group. Each protein was prepared at 100 $\mu$M concentration in the mixture. All the chromatographic conditions used for this experiment can be seen experimental section. The chromatogram of nine-protein mixture and an overlapped ACN gradient can be seen in Figure 1.5. Peak widths, resolutions and separation factors of the nine protein mixture using on a Nest Group-RP can be seen in Table 1.3.
Figure 1.5. Mixture of nine protein standards using The Nest Group C4 HPLC column overlapped with ACN gradient.; 1. streptavidin, 2. insulin, 3. protein G, 4. rat albumin, 5. conalbumin, 6. trypsin inhibitor, 7. myoglobin, 8. β-lactoglobulin B, 9. β-lactoglobulin A.
Table 1.3 Figure-of-merit for nine protein mixture on Nest Group-RP column

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Separation factor ($k_2/k_1$)</th>
<th>Peak width $w_{0.5}$ (min)</th>
<th>Resolution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptavidin</td>
<td>NA</td>
<td>0.877</td>
<td>NA</td>
</tr>
<tr>
<td>Insulin</td>
<td>1.192</td>
<td>0.16</td>
<td>12.1</td>
</tr>
<tr>
<td>Protein G</td>
<td>1.024</td>
<td>0.17</td>
<td>2.3</td>
</tr>
<tr>
<td>Rat Albumin</td>
<td>1.018</td>
<td>0.54</td>
<td>3.5</td>
</tr>
<tr>
<td>Conalbumin</td>
<td>1.066</td>
<td>0.68</td>
<td>0.6</td>
</tr>
<tr>
<td>Trypsin Inhibitor</td>
<td>1.037</td>
<td>3.2</td>
<td>0.4</td>
</tr>
<tr>
<td>Myoglobin</td>
<td>1.010</td>
<td>0.3</td>
<td>0.02</td>
</tr>
<tr>
<td>β-Lactoglobulin-A</td>
<td>1.019</td>
<td>0.3</td>
<td>1.3</td>
</tr>
<tr>
<td>β-Lactoglobulin-B</td>
<td>1.007</td>
<td>2.28</td>
<td>0.08</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td><strong>1.046</strong></td>
<td><strong>0.950</strong></td>
<td><strong>2.500</strong></td>
</tr>
<tr>
<td><strong>Median</strong></td>
<td><strong>1.021</strong></td>
<td><strong>0.540</strong></td>
<td><strong>0.900</strong></td>
</tr>
</tbody>
</table>
1.3.1.4. Comparison study of Vydac and Nest Group RP columns

The results obtained from both Vydac and Nest Group columns were fundamentally close to each other. The elution pattern is almost close. However, some of the proteins have different elution order. Whereas, peak widths, resolutions of the proteins gave some different values. After a careful examination of the results, Nest Group-RP has shown superiority in the protein resolution as well as in peak widths compared for the most of the proteins compared to Vydac-RP column. Them median, average peak width, resolution values can be seen in Table 1.4.
Table 1.4 Comparison study of Vydac-RP versus Nest Group-RP

<table>
<thead>
<tr>
<th></th>
<th>Peak width $w_{0.5}$ (min)</th>
<th>Resolution (R)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vydac-RP</td>
<td>Nest Group-RP</td>
</tr>
<tr>
<td>Average</td>
<td>1.81</td>
<td>0.54</td>
</tr>
<tr>
<td>Median</td>
<td>1.20</td>
<td>0.95</td>
</tr>
</tbody>
</table>
1.3.2. GCF experiments

1.3.2.1. Optimizing pH gradient for GCF experiments

In order to enhance the resolution and peak half widths of the proteins on GCF, an optimized linear pH gradient was developed. The pH gradient generated using the previously used buffer components (application buffer: 5 mM ammonium bicarbonate, 5mM pyridine; elution buffer: 25 mM acetic acid, 25 mM lactic acid) in the preliminary results did not produce a smooth linear gradient as shown in the Figure 1.6.

In this order a concept called “bridging” has been introduced. The idea behind bridging is nothing but an addition of one of the lower pKa buffer components of application buffer to the elution buffer and addition of one the highest pKa buffer components to the application buffer which is discussed more in detailed in coming paragraphs.

Various buffer components having pKa values in regular intervals (spacing) have been used to generate an even buffer capacity throughout the chromatographic run. As a first attempt, an application buffer with 5mM ammonium bicarbonate pKa = 6.37, 9.2, 10.3, 10 mM pyridine pKa = 5.19 along with 15mM of acetic acid and elution buffer with 25 mM acetic acid pKa = 4.76, 25 mM lactic acid pKa = 3.81 along with 5 mM of pyridine in it. Based on the experimental results the minimum concentration of 25 mM of elution buffer components was mandatory to elute all the proteins. Lower concentrations of application, elution buffers resulted either no retention or irreversible retentions.
Figure 1.6 pH gradient used in DEAE-GCF experiment. Application buffer with (pH: 9.2) 5 mM ammonium bicarbonate, 5mM pyridine and an elution buffer with (pH: 2.6) 25 mM acetic acid, 25 mM lactic acid.

Time program used: 0-38% of elution buffer in first 4 minutes, 38%-41% by 14 minutes, 41%-51% in 18 minutes, 51%-61% in 28 minutes, 61%-95% in 42 minutes, 95% - 100% in 50 minutes and 100% of elution buffer until 60 minutes.
After enormous number of attempts, our group has identified a set of volatile buffers that can produce a super smoother pH gradient within the pH range of 6.5 - 3.8. The application buffer was made up of 10 mM Bis-Tris methane (6.5), 10 mM 3-Methyl pyridine (5.68) with 5mM of acetic acid in it as a bridging agent and the elution buffer was with 10 mM Acetic acid (4.76), 10 mM Lactic acid (3.81) along with 5mM of 3-methyl pyridine as a bridging agent (Figure 1.7).

Different time programs were tried to get a good linear pH gradient, initial pH gradient plots were not satisfactory. Few pH drops and rises have been noticed in the gradients. The reason for these drops might be due to lack of sufficient buffering ability of the buffer components in those particular pH regions of the gradients. To maintain even buffer capacity throughout the pH gradient, bridging was introduced between application and elution buffers (as described earlier in sections), which spans and provides excellent buffering capacity over the drops in the buffer ranges.
Figure 1.7. A linear pH gradient used in optimized GCF experiment; Application buffer (pH: 6.5): 10 mM Bis-Tris methane (6.5), 10 mM 3-Methyl pyridine (5.68) with 5 mM of acetic acid in it as a bridging agent; Elution buffer (pH: 3.8): 10 mM Acetic acid (4.76), 10 mM Lactic acid (3.81) along with 5 mM of 3-methyl pyridine as a bridging agent.

Time program used: 0 - 26.64% of elution buffer in first 12 minutes, 26.64 - 41.64% in 10 minutes, 41.64 – 57.74% in 5 minutes, 57.74 - 100% in 19.03 minutes, 100 - 0% in 5 minutes. Total run time was 65 minutes.
1.3.2.2. Nine-protein mixture analysis using optimized pH gradient

The optimized pH gradient has been successfully applied to analyze nine-protein mixture. All protein standards were chosen depending upon their pI values (pI values fit into the pH range of mobile phase buffers). The chromatogram of nine protein mixture with overlapped pH gradient can be seen in Figure 1.8. The confirmation of the protein elution at their respective pI values along with the band-width ($w_{0.5}$) of the protein in pH units can be seen in Table 1.5.
Figure 1.8 Mixture of nine protein standards using The Nest Group PEI- HPLC column overlapped with optimized linear pH gradient.

### Table 1.5 Comparison of protein elution pH with its pI values

<table>
<thead>
<tr>
<th>Proteins listed in elution order</th>
<th>Literature pI</th>
<th>Elution pH</th>
<th>Peak width, $W_{0.5}$ pH units</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myoglobin</td>
<td>7.16</td>
<td>7.05</td>
<td>NA</td>
</tr>
<tr>
<td>Streptavidin</td>
<td>6.53</td>
<td>6.98</td>
<td>0.16</td>
</tr>
<tr>
<td>Conalbumin</td>
<td>5.9</td>
<td>5.91</td>
<td>0.33</td>
</tr>
<tr>
<td>Insulin</td>
<td>5.3</td>
<td>5.54</td>
<td>0.3</td>
</tr>
<tr>
<td>Rat Albumin</td>
<td>5.7</td>
<td>5.12</td>
<td>0.23</td>
</tr>
<tr>
<td>Beat Lactoglobulin A</td>
<td>5.34</td>
<td>4.58</td>
<td>0.09</td>
</tr>
<tr>
<td>Beta Lactoglobulin B</td>
<td>5.1</td>
<td>4.53</td>
<td>0.05</td>
</tr>
<tr>
<td>Protein G</td>
<td>4.19</td>
<td>4.48</td>
<td>0.02</td>
</tr>
<tr>
<td>Trypsin Inhibitor</td>
<td>5.0</td>
<td>4.45</td>
<td>0.02</td>
</tr>
</tbody>
</table>
1.3.3. Comparison studies of RP versus GCF columns

PEI-GCF (linear pH gradient on anion exchange column) was compared with Nest Group-RP (linear gradient in ACN) employing the same gradient slope for each (44 min gradient). Peak width $w_{0.5}$, resolutions of proteins can be seen in Table 1.6, Table 1.7 respectively. By summarizing the results, GCF gave superior resolution, more than double the resolution of RP, primarily due to the greater separation of the peaks from one another. All RP protein peaks were eluted in a narrower region of the gradient compared to GCF, in which the proteins peaks were distributed along the entire length of using a nine-protein mixture. Regarding peak widths, some had narrower widths with RP, others with GCF. The median peak width for both techniques was similar. The average and median resolution of GCF is much greater that RP, which shows the separation of the GCF peaks, is good when compared with RP experimentation.
### Table 1.6 Comparison of protein peak widths ($w_{0.5}$) RP with GCF

<table>
<thead>
<tr>
<th>Proteins</th>
<th>RP peak width ($w_{0.5}$), min</th>
<th>GCF peak width ($w_{0.5}$), min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptavidin</td>
<td>0.877</td>
<td>0.43</td>
</tr>
<tr>
<td>Insulin</td>
<td>0.16</td>
<td>0.39</td>
</tr>
<tr>
<td>Protein G</td>
<td>0.17</td>
<td>0.37</td>
</tr>
<tr>
<td>Rat Albumin</td>
<td>0.54</td>
<td>0.79</td>
</tr>
<tr>
<td>Conalbumin</td>
<td>0.68</td>
<td>1.25</td>
</tr>
<tr>
<td>Trypsin Inhibitor</td>
<td>3.2</td>
<td>0.37</td>
</tr>
<tr>
<td>Myoglobin</td>
<td>0.3</td>
<td>0.28</td>
</tr>
<tr>
<td>β-lactoglobulin-A</td>
<td>0.3</td>
<td>0.54</td>
</tr>
<tr>
<td>β-lactoglobulin-B</td>
<td>2.28</td>
<td>0.65</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td><strong>0.95</strong></td>
<td><strong>0.56</strong></td>
</tr>
<tr>
<td><strong>Median</strong></td>
<td><strong>0.54</strong></td>
<td><strong>0.43</strong></td>
</tr>
</tbody>
</table>
Table 1.7 Comparison of protein resolutions of RP with GCF

<table>
<thead>
<tr>
<th>Protein peak elution order numbers</th>
<th>RP resolution of adjacent peaks</th>
<th>GCF resolution of adjacent peaks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 &amp; 2</td>
<td>12.1</td>
<td>24.9</td>
</tr>
<tr>
<td>2 &amp; 3</td>
<td>2.3</td>
<td>2.3</td>
</tr>
<tr>
<td>3 &amp; 4</td>
<td>3.5</td>
<td>11.4</td>
</tr>
<tr>
<td>4 &amp; 5</td>
<td>0.6</td>
<td>3.1</td>
</tr>
<tr>
<td>5 &amp; 6</td>
<td>0.4</td>
<td>1.4</td>
</tr>
<tr>
<td>6 &amp; 7</td>
<td>0.02</td>
<td>1.8</td>
</tr>
<tr>
<td>7 &amp; 8</td>
<td>1.3</td>
<td>2.6</td>
</tr>
<tr>
<td>8 &amp; 9</td>
<td>0.08</td>
<td>1.1</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td><strong>2.5</strong></td>
<td><strong>6.5</strong></td>
</tr>
<tr>
<td><strong>Median</strong></td>
<td><strong>0.9</strong></td>
<td><strong>2.3</strong></td>
</tr>
</tbody>
</table>
1.4. Conclusion

The purpose of this specific aim is to compare and evaluate the performance of GCF technique with RP-HPLC technique. In order to evaluate the superiority of one of these techniques, GCF (linear pH gradient on anion exchange column) was compared with reversed-phase (linear gradient in ACN) employing the same gradient slope for each (44 min gradient). After conducting a series of studies using different HPLC columns from different manufacturers, GCF gave superior resolution, more than double the resolution of RP, primarily due to the greater separation of the peaks from one another. All RP protein peaks were eluted in a narrower region of the gradient compared to GCF, in which the proteins peaks were distributed along the entire length of the gradient. Regarding peak widths, some had narrower widths with RP, others with GCF. The median peak width for both techniques was similar. Overall, GCF has shown some potential advantages when compared with RP technique.
1.5. References


2.1. Introduction

2.1.1 Current characterization protocols

The development of column technology for HPLC has focused on the generation of advanced chromatographic efficiencies. Characterization of a new HPLC column is an important prior to its use so that any degradation of column performance can be assessed throughout the column’s use by repeating the characterization study. It is also important to predict chromatographic conditions for a given group of analytes. Characterization protocols of reversed-phase columns are mature compare to that for ion-exchange column characterization protocol is well established following the Tanaka protocol [1-5]. In this protocol the following are characterized for a reversed-phase column: surface area and coverage of the stationary phase, number of theoretical plates, hydrophobicity, shape
selectivity, hydrogen-bonding capacity, ion-exchange capacity and acidic ion-exchange capacity. In addition aromatic selectivity can be assessed [6].

Characterization of ion-exchange is less developed. Determination of number of ion-exchange sites on an ion exchange column can be quantified by elemental or chemical determination [7-10], frontal analysis employing acid/base solutions [11-13] and sodium nitrate [12] and frontal analysis measuring the time of the transient pH effect [14, 15]. Frontal analysis has also been done employing protein solutions, however results are reported as binding capacity for proteins on the column rather than the number of ion-exchange sites [16-19].

There is no standard analyte used in the determination of column efficiency (number of plates or plate height) in ion-exchange HPLC as is in the Tanaka protocol for characterizing the efficiency of reversed-phase columns (using n-pentylbenzene) [20]. Usually investigators determine the column efficiency from injection of compounds for which the ion-exchange HPLC being developed. Studies have been published, however, on the influence of retention on plate height [21] and the determination of plate height in the gradient chromatography of proteins [22] in ion-exchange chromatography. In practice, manufacturers usually confirm the quality of the packing of the column by the injection of non-ionic compounds onto the ion-exchange column, noting peak width, which is assessing only the quality of the packing of the column.
2.1.2 Ion-exchange column characterization needs

What is lacking in ion-exchange characterization is simple test involving injection of a standard ionic analyte that can give a measure of both the column’s efficiency and its ion-exchange capacity. The chromatographic figures-of-merit of retention time for the standard ionic compound will give a measure of the binding site capacity, while the peak width will give a measure of its efficiency. Values for retention time and peak width of an ionic compound standard at the onset of using a new column will serve as indicators of optimal performance to measure against as the column is used. In the present work we document the use of a NaNO$_3$ standard for this purpose.

Another parameter in ion-exchange columns that has been under-addressed is quantitative assessment of binding strengths of ions for the ion-exchange sites. Selectivity coefficients quantify the relative binding of competing ions to the ion-exchange ligand site according to the ion-exchange reaction given in equation 1:

\[
\text{L}^+ - \text{Cl}^- + \text{Al}^- \rightarrow \text{L}^+ - \text{Al}^- + \text{Cl}^- \quad \text{Eq. 1}
\]

where L$^+$ is the ion-exchange site ligand, Cl$^-$ is the counter ion, Al$^-$ is the analyte ion and K is the selectivity coefficient for the displacement reaction of Cl$^-$ by Al$^-$ binding to L$^+$. [23]

Work in determining selectivity coefficients of ions binding to ion-exchangers, however, has been relegated to a very limited number of studies done in the past. In these studies, equilibrium analysis is done in batch experiments in which the ion-exchange resin, with a counter ion, are equilibrated in solutions of one or more ions of known
concentration, with the final concentration of ions in the solution, after equilibrium is reached, being determined by titration. From this data selectivity coefficient are determined \([23-25]\). Other studies determine distribution coefficients \([26, 27]\) for ions on ion exchangers which is different than selectivity coefficient, being a ratio of one ion in the resin to that in the solution. In these studies distribution coefficients were determined by weighing out resins and doing batch equilibrium studies determining cations in the solution and the resin. Two other published studies do not directly determine selectivity coefficients but estimate them by employing more complex formulas \([28, 29]\).

Therefore, for the most part, scientists utilize a ranking of order given in textbooks, ranking displacement strengths of the ions in ion-exchange chromatography that may or may not be applicable to a particular ion-exchange packing material. The ranking of anions according to displacement strength in anion-exchange chromatography is:

\[
\text{Citrate}^{2-} > \text{SO}_4^{2-} > \text{oxalate}^{2-} > \Gamma > \text{NO}_3^- > \text{CrO}_4^{2-} > \text{Br}^- > \text{SCN}^- > \text{Cl}^- > \text{formate}^- > \text{acetate}^- > \text{OH}^- > \text{F}^- 
\]

This ranking lacks quantitative information of the relative binding strength of the ions with the different ion-exchange ligands. What is missing in this ranking order is comparing ions in the ranking, i.e. a quantitative measure of how much the binding strength differs for the different ions. Thus one can only design chromatographic experiments qualitatively, not quantitatively. A theoretical model is presented below for measuring strengths of interactions of competing ions for ion-exchange sites, as well as determining number of ion-exchange sites through frontal analysis experiments.
2.1.3 General aspects of frontal analysis experiments

In the present work we are proposing a new way to use frontal analysis experiments to determine number of binding sites and the selectivity coefficient in the ion-exchange binding of ions, deriving a mathematical model from the equilibrium reaction given by Equation 1. In the frontal analysis experiments done in the present work a mobile phase consisting of a given concentration $\text{AI}^-$ and $\text{Cl}^-$ is pumped through the column. The column prior to this is equilibrated with $\text{Cl}^-$. The $\text{Cl}^-$ concentration is the same concentration in all the experiments, including equilibration of the column. $\text{AI}^-$ competes with $\text{Cl}^-$ for $\text{L}^+$ site. As the $\text{AI}^-/\text{Cl}^-$ mobile phase continues to pump through the anion-exchange column, $\text{AI}^-$ is removed from the mobile phase displacing $\text{Cl}^-$ from $\text{L}^+$ (the extent determined by the concentrations of $\text{Cl}^-$ and $\text{AI}^-$ and the value of K). Eventually when all the $\text{L}^+$ sites which are to bind $\text{AI}^-$ do so, then $\text{AI}^-$ will “breakthrough” and the amount $\text{AI}^-$ bound is directly determined from the volume of the particular $\text{AI}^-$ concentration mobile phase that passed through the column.

Given in Figure 2.1 is the breakthrough curve that is noted at the detector, with the detector monitoring the concentration of $\text{AI}^-$. As shown in Figure 2.1 the breakthrough time (converted to volume by multiplying by the flow rate) is determined as the midpoint of the rise in the breakthrough curve. Also shown in this figure is the correction for a step change in the gradient to reach the detector from the pumps without the column in place, which must be subtracted from breakthrough curve volume determined with the column in place. This is a system volume of tubing prior to the column that must be subtracted. In addition, the void volume of the column must also be subtracted to from the breakthrough volume.
Figure 2.1. Model chromatograms of breakthrough curves overlapped with and without column in place
A series of frontal analysis experiments are done varying concentration $\text{Al}^-$ in mobile phase (all at the same $\text{Cl}^-$ concentration) and determining the amount $\text{Al}^-$ bound. The additional inclusion of $\text{Cl}^-$ in the mobile phase, a novel approach done in this work that has not been done in previous frontal analysis studies, allows for the determination of not only the number of ion-exchange ligand sites in the packed column but also determines the value for the selectivity coefficient $K$ for the ion-exchange reaction, according to the equations derived in section 2.1.4 below.

### 2.1.4 Frontal analysis model for the quantitative determination of number of ion-exchange sites and their binding strength of interaction with ions

The selectivity coefficient $K$ for the ion-exchange binding reaction given in equation 1 can be written as given in equation 2:

$$K = \frac{\{L^+ - \text{Al}^-\} \cdot [\text{Cl}^-]}{\{L^+ - \text{Cl}^-\} \cdot [\text{Al}^-]}$$  \hspace{1cm} \text{Eq. 2}

where $\{L^+ - \text{Al}^-\}$ is the surface concentration of ion-exchange ligand with bound analytical ion (µmole/m²), $\{L^+ - \text{Cl}^-\}$ is the surface concentration of ion-exchange ligand with bound counter ion (µmole/m²), $[\text{Cl}^-]$ mobile phase concentration of the counter ion (mM), $[\text{Al}^-]$ mobile phase concentration of the analyte ion (mM), and $K$ is the selectivity coefficient for the ion-exchange reaction. Note the units can be any prefix of the molar terms, provided that the solution concentration of ions are the same units and the surface concentration units are the same units.
The total amount of ion-exchange ligand sites on the column is given by Equation 3:

\[ n_{L_{\text{total}}} = n_{L_{\text{AI}}} + n_{L_{\text{CI}}} \quad \text{Eq. 3} \]

where \( n_{L_{\text{total}}} \) is the total amount of ion-exchange ligand sites in the column (µmoles), \( n_{L_{\text{AI}}} \) is the total amount of ion-exchange ligand sites with bound AI\(^+\) (L\(^+\)-AI\(^-\) in µmoles), and \( n_{L_{\text{CI}}} \) is the total amount of ion-exchange ligand sites with bound CI\(^-\) (L\(^+\)-CI\(^-\) in µmoles).

Multiplying each of the surface area terms by the total surface area of the packing material in the column (m\(^2\)), Equation 2 can be rewritten for total amount of each of the bound ligand types in the column, and by incorporating Equation 3, the following Equation 4 can be written:

\[ K = \frac{n_{L_{\text{AI}}}[\text{CI}^-]}{(n_{L_{\text{total}}}) - n_{L_{\text{AI}}})[\text{AI}^+] } \quad \text{Eq. 4} \]

Equation 4 is rearranged to yield Equation 5:

\[ \frac{[\text{CI}^-]}{((n_{L_{\text{total}}})(K))} \times (1/[\text{AI}^+]) + 1/ n_{L_{\text{total}}} = 1/ n_{L_{\text{AI}}} \quad \text{Eq. 5} \]

Equation 5 is in the form \( mx + b = y \) (since [CI\(^-\)] is held constant throughout the experiments), which means a linear regression analysis of plot of 1/ \( n_{L_{\text{AI}}} \) (y) versus 1/[AI\(^+\]) (x) from data obtained from the breakthrough experiments, yields an intercept giving the reciprocal value of the amount of ion-exchange ligand sites on the column.
[nL_{\text{total}}] \text{ and the slope yields the reciprocal value for the selectivity coefficient } K, \text{ after plugging in values for } [\text{Cl}^-]\text{ and } nL_{\text{total}}, \text{ which are other factors in the slope term.}

2.2. Experimental

2.2.1. Instrumentation

A polyethylenimine (PEI) WAX HPLC column (2.1mm x 50mm 5µ, 1000Å) from Poly LC (Columbia, MD, USA) was used for the chromatographic analysis. A Beckman Coulter System Gold HPLC instrument (Brea, CA, USA) interfaced with a Beckman Coulter PDA detector. All the samples were injected using System Gold 508 Auto sampler. 32 Karat Gold Software (version 8.0) used to collect and analyze the data.

2.2.2. Materials

A.C.S. grade sodium nitrate, toluene, and HPLC grade methanol were purchased from Fisher Scientific (Fair Lawn, NJ, USA). HPLC grade ammonium formate was purchased from Sigma Aldrich (St. Louis, MO, USA). HPLC grade water was generated using Barnstead Nano system with a Nanpure Diamond Pack Organic Free DI cartridge from Thermo Scientific (West Palm Beach, FL, USA). All the mobile phases were filtered through 0.45µ cellulose ester membrane filters from Millipore (Billerica, MA, USA).
2.2.3. Frontal analysis (breakthrough) studies

2.2.3.1. Frontal analysis studies on anion-exchange column

25 mM ammonium formate (pH: 6.56, no pH adjustment) was used as the equilibrating mobile phase and different concentrations of NaNO₃ (0.8, 4, 15, 40, 100 mM) in 25 mM ammonium formate was used as breakthrough mobile phase (no pH adjustment). The column was equilibrated using 25 mM ammonium formate for 80 minutes after each NaNO₃ breakthrough and then a step change to elution buffer in 0.1 minutes. The breakthrough mobile phase was pumped through column for 100 minutes and switched back to application mobile phase in 0.1 minutes to re-equilibrate the column using equilibrium mobile phase. The flow rate was 0.4 mL/min. The detector wavelength was set to 300 nm.

2.2.3.2. Correction of breakthrough volume

The breakthrough volume must be corrected for the system volume from the mixing chamber of the gradient pump to the column by doing breakthrough experiment described in section 2.2.3.1 without a column in place, connecting the auto sampler exit tubing directly to the detector. This breakthrough volume is subtracted from the breakthrough volume form the column frontal analysis experiment. Each concentration of NaNO₃ was run in these correction studies matching with column studies.

In addition, column void volume was determined by injecting 20 µL of 20 µM toluene in methanol. Methanol was used as a mobile phase at a flow rate 0.4 mL/min. This void volume was also subtracted from the breakthrough volume (corrected for
system volume) giving the true volume of NaNO₃ mobile phase that passes through to the column up to the breakthrough point. From the volume and concentration of NaNO₃ one can calculate the nmoles of NO₃⁻ that are retained on the ion-exchange ligand sites.

2.2.4. Injection of toluene and NaNO₃ to assess column quality

2.2.4.1. Toluene

The manufacturer’s column test was performed at the onset of the use of the new column and after extended use that led to column degradation. The manufacturer’s protocol was injection of 10 µL of 20 mM toluene sample in methanol into a 100% methanol mobile phase at a flow rate of 0.2 mL/min. Detector wavelength was 254 nm.

2.2.4.2. NaNO₃

A proposed ionic compound, NaNO₃ was used to test column quality. Triplicate 20 µL volume of different concentrations of NaNO₃ in mobile phase were injected into 25 mM ammonium formate set at 0.4 mL/min. The detector wavelength was set to 300 nm. Retention times and peak-width-at-half-height of NaNO₃ at all the concentrations were calculated. The NaNO₃ concentration chosen for assessment of column performance was that which demonstrated it was within the linear adsorption isotherm, at which peak parameters are unaffected by a change in concentration of the analyte in the injected sample.
2.3 Results and Discussion

2.3.1 Quantitative determination of number of ion-exchange ligand sites [$n_{L\text{-}}\text{NO}_3$] and selectivity coefficient (K) of nitrate and formate for these sites

The result of the frontal analysis experiments of pumping various concentrations of NaNO$_3$ in 25 mM ammonium formate is tabulated in Table 2.1 and plotted in Figure 2.2. Normally the amount of ion-exchange ligand sites in the column is given by the plateau level of the plot. As can be seen in the Figure 2.1, the plateau region of the plot is still increasing slightly at the highest concentration studied. In fact it is difficult to know what is the true asymptote level of the plot. Determining at which concentration saturation is reached is problematic in the frontal analysis determination of the amount of ion-exchange sites. The proposed model addresses this estimation issue.

Applying the model derived in section 2.1.4, the data can be fit to Equation 5 plotting $1/n_{L\text{-NO}_3}$ versus $1/[\text{NO}_3^-]$. The value for $n_{L\text{(total)}}$ is obtained from the intercept and K for NO$_3^-$ displacing formate from the PEI anion-exchange can be calculated from the slope. This is plotted in Figure 2.3, yielding the regression line ($y = 1.0046x + 0.114$) with $R^2$ of 1. From this plot values of 8.77 µmol for the total number of ligand sites on the column and K (selectivity coefficient for NO$_3^-$ displacing formate on PEI ligand site) of 2.73. Using the column dimensions and a density value for packed column of 0.4 g/mL, the ion-exchange ligand coverage on the PEI-WAX PolyLC column is 127 µmol/g. This is within the manufactures stated range of 80 – 125 µmol/g for this packing material as determined by a picric acid assay [9].
Table 2.1. Number of moles at different concentrations of NaNO₃

<table>
<thead>
<tr>
<th>Conc. NaNO₃ (mM)</th>
<th>Calculated number of moles</th>
<th>Num. of µmoles (Mean) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Trial 1</td>
<td>Trial 2</td>
</tr>
<tr>
<td>0.8</td>
<td>730</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>2921</td>
<td>2913</td>
</tr>
<tr>
<td>15</td>
<td>5682</td>
<td>5604</td>
</tr>
<tr>
<td>40</td>
<td>7328</td>
<td>7392</td>
</tr>
<tr>
<td>100</td>
<td>7860</td>
<td>7360</td>
</tr>
</tbody>
</table>
Figure 2.2. Frontal analysis plot of amount of ion-exchange sites on the column versus NaNO₃ concentration in mobile phase.
Figure 2.3. Plot of reciprocal μmoles of ion-exchange sites on column versus concentration of NaNO₃ in mobile phase.

\[
\frac{1}{n(L_{\text{total}})} \mu \text{mol}^{-1}
\]

\[
y = 1.0046x + 0.114 \\
R^2 = 1
\]
This work has broad applicability for characterizing ion-exchange packing materials. The frontal analysis approach described in the present work determines both the amount of ion-exchange sites on the column and the strength of interaction of ions for the ion-exchange sites as a selectivity coefficient. The advantage of the model is that it uses multiple data points to determine each of these values. In determining $n_{L, (total)}$ one is not estimating the asymptote level but using linear regression parameters.

In addition, this frontal analysis is vastly superior to other procedures in determining selectivity coefficients for ions. The frontal analysis approach used in the present work determines the selectivity coefficient values by pumping solutions of the ions through the column, far easier than batch experiments, and much more practical for characterizing HPLC packing materials. Studies determining selectivity coefficients of ions for different ion-exchange columns have been sparsely done in the past and have not been done for years. In particular, there has only been one study done estimating selectivity coefficient (K) for an ion-exchange HPLC columns to date, but this study only determined a pseudo K which they termed a global selectivity coefficient [28]. This work opens up the opportunity to conduct these determinations on any ion-exchange column without disturbing the contents of the column. The other significant advantage of the frontal analysis approach in determining selectivity coefficients is that multiple data points are used to more accurately determine its value, whereas the batch experiment typically uses only one concentration of the ions in solution equilibrating with the packing material.

In conclusion, the present work opens up the opportunity to readily do accurate characterization studies on a variety of ion exchange columns.
2.3.2 Injection of NaNO$_3$ as a standard to assess anion-exchange column performance

2.3.2.1 Determination of concentration threshold of injected NaNO$_3$ to be within the linear adsorption isotherm

A series of varying concentrations of NaNO$_3$ was injected (20 µL) onto the PEI anion-exchange column to determine the upper threshold of the concentration range of NaNO$_3$ in which the chromatography occurs within the linear adsorption isotherm. In the linear adsorption isotherm a change in concentration of the NaNO$_3$ does not affect figures-of-merit of the peak. Retention time and peak-widths are compared for the different concentrations of NaNO$_3$ injected in Table 2.2 and plotted in Figure 2.4. The upper threshold NaNO$_3$ concentration for the linear adsorption isotherm is injection of 20 µL of 200 µM for the concentrations studied, as it is noted that peak retention time and peak width remains relatively constant at concentrations less than or equal to this. From this study it was determined that injection of 20 µL of 100 µM NaNO$_3$ was well within the linear adsorption isotherm region and was thus used to document column performance.
Table 2.2 Retention times (RT) and peak-widths (PW) of NaNO₃ peaks (n = 3)

<table>
<thead>
<tr>
<th>Conc. of NaNO₃ (µM) (20 µL injected)</th>
<th>Mean RT ± SD (min)</th>
<th>Mean PW ± SD (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>3.461 ± 0.002</td>
<td>0.15 ± 0.09</td>
</tr>
<tr>
<td>40</td>
<td>3.464 ± 0.003</td>
<td>0.16 ± 0.04</td>
</tr>
<tr>
<td>80</td>
<td>3.471 ± 0.011</td>
<td>0.16 ± 0.00</td>
</tr>
<tr>
<td>200</td>
<td>3.467 ± 0.007</td>
<td>0.16 ± 0.06</td>
</tr>
<tr>
<td>400</td>
<td>3.455 ± 0.010</td>
<td>0.17 ± 0.00</td>
</tr>
<tr>
<td>800</td>
<td>3.427 ± 0.003</td>
<td>0.18 ± 0.03</td>
</tr>
<tr>
<td>1000</td>
<td>3.425 ± 0.017</td>
<td>0.19 ± 0.08</td>
</tr>
<tr>
<td>2000</td>
<td>3.385 ± 0.009</td>
<td>0.23 ± 0.00</td>
</tr>
<tr>
<td>4000</td>
<td>3.332 ± 0.012</td>
<td>0.30 ± 0.06</td>
</tr>
</tbody>
</table>
Figure 2.4. Plots of retention time (RT) versus log of NaNO₃ concentration (A), peak-widths, \( w_{0.5} \) (PW) versus log of NaNO₃ concentration (B).
2.3.2.2 Use of NaNO₃ standard to document change in PEI anion-exchange column performance compared to toluene standard

Peak figures-of-merit results from injection of the 100 µM NaNO₃ standard were compared to that of injections toluene recommended by the manufacturer at the beginning of column use and after extensive use. Results are given in Table 2.3, with the chromatograms for the NaNO₃ given in Figure 2.5. It can be seen that the NaNO₃ was a more sensitive indicator of the decrease in column efficiency, as the peak width increases by 50% for the NaNO₃ while the increase in toluene peak width is 30%. More importantly, the NaNO₃ indicates a loss of functional binding sites on the ion-exchanger by the decrease in the retention time of the NaNO₃. Toluene does not probe the ion-exchange sites and thus does not detect a loss of the column’s binding capacity. Subtracting the void volume time (0.30 min) from the NaNO₃ retention times at the beginning of the column lifetime and at its present condition one can determine the extent of binding sites lost on the column. The retention time with void time subtract at the beginning of column loss was 3.17 min and after extensive use was 1.52 min. There was thus a 52% loss of function ion-exchange sites.
Table 2.3. Peak figures-of-merit results from injection of the 100 µM NaNO₃ standard compared with injections of 20 mM toluene recommended by the column manufacturer at the beginning of column use and after extensive use.

<table>
<thead>
<tr>
<th></th>
<th>R.T. ± SD, mins</th>
<th>Peak width (w₀.₅) ± SD, mins</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>20 mM Toluene</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PEI column as in new condition</td>
<td>0.305 ± 0.005</td>
<td>0.08 ± 0.00</td>
</tr>
<tr>
<td>PEI column after extensive usage</td>
<td>0.297 ± 0.004</td>
<td>0.10 ± 0.00</td>
</tr>
<tr>
<td><strong>100 µM NaNO₃</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PEI column as in new condition</td>
<td>3.467 ± 0.004</td>
<td>0.16 ± 0.00</td>
</tr>
<tr>
<td>PEI column after extensive usage</td>
<td>1.821 ± 0.022</td>
<td>0.24 ± 0.02</td>
</tr>
</tbody>
</table>
Figure 2.5. Chromatogram of 20 µL of 100 µM NaNO₃ injected onto PEI anion-exchange column when new (A) and used (B).
2.4. Conclusion

Characterization of ion-exchange is less developed. Conventional characterization methods were done by elemental or chemical determination. And these are either time consuming or involvement of complex mathematical equations. Current work demonstrates a novel procedure to accurately characterize a polyethyleneimine (PEI) WAX HPLC column (2.1 x 50 mm 5 µ, 1000 Å, Poly LC) with simple, in-expensive buffers (ammonium formate, sodium nitrate (NaNO₃)).

The experimentation involved two main steps, frontal analysis and injections of NaNO₃ to assess column performance. Frontal analysis studies were done to determine the number of ion-exchange binding sites in the HPLC column. 25 mM ammonium formate (pH: 6.56, no pH adjustment) was used as equilibrating mobile phase and different concentrations of NaNO₃ (0.8, 4, 15, 40, 100 mM) in 25 mM ammonium formate as breakthrough mobile phase (no pH adjustment). The obtained results from the frontal analysis were fit into a simple mathematical model of ion-exchange reaction to identify the selectivity coefficient of the NaNO₃ which helps in quantifying relative binding of competing ions to the ion-exchange ligand site. NaNO₃ injections were done using different concentrations of NaNO₃ to determine the upper threshold of linear adsorption isotherm range of NaNO₃.

The results of the frontal analysis derived from the mathematical model gave a regression line \(y = 1.0046x + 0.114\) with \(R^2\) of 1. From this plot values the total number of ligand sites are calculated as 8.77 µmol. The column selectivity coefficient is 2.73. Using the column dimensions and a density value for packed column of 0.4 g/mL, the
ion-exchange ligand coverage on the PEI-WAX PolyLC column is 127 µmol/g which is within manufacturer state range (manufacturer stated range: 80 – 125 µmol/g).

On the other hand, different concentrations of NaNO3 injections have shown that after certain concentration of nitrate the analyte losing its linear isotherm state where the retention time started shifting and broadening of peak width occurred. By assessing this threshold range of linear isotherm, one can smartly manage their sample loading to achieve better chromatographic conditions. Peak figures-of-merit results from injection of the 100 µM NaNO3 standard were compared to that of injections toluene recommended by the manufacturer (20 mM toluene) has reveled that the peak width increases by 50% for the NaNO3 while the increase in toluene peak width is 30%. And, the shift in the retention time of 100 µM NaNO3 from 3.17 mins to 1.52 mins beginning and after extensive use respectively has shown that 52% of function ion-exchange sites has been lost after an extensive usage.
2.5. References


3.1. Introduction to neuroprotective agents

Neuroprotection involves prevention of neuronal death by inhibiting one or more of the pathophysiological steps in the processes that follow brain injury or ischemia due to occlusion of a cerebral artery or neurodegenerative processes [1]. The concept of neuroprotection is now important in many diseases that were once only treated symptomatically and in which a disease-modifying approach is desirable. Mostly these agents play a keen role in the management of neurodegenerative disorders as well as cerebral ischemia, and hypoxia/ischemia during surgical procedures [2]. These protective strategies are also required to protect the brain against toxic effects of chemicals and drugs. There are numerous neuroprotective agents from several pharmacological as well as nonpharmaceutical categories [3].

 Approximately more than eighty categories are popular under neuroprotection research area. Some of those are listed below. Translation of neuroprotective benefits from the laboratory bench to the emergency room has not been successful.
Table 3.1. A list of neuroprotective agents under clinical trails

<table>
<thead>
<tr>
<th>Category</th>
<th>Agents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenosine reuptake blockers</td>
<td>Dipyridamole, Propentofylline</td>
</tr>
<tr>
<td>Antioxidants or free radical scavengers</td>
<td>Tocopherol (vitamin E)</td>
</tr>
<tr>
<td></td>
<td>Ascorbic acid (vitamin C)</td>
</tr>
<tr>
<td></td>
<td>Beta carotene (precursor of vitamin A)</td>
</tr>
<tr>
<td></td>
<td>Cannabidiol (a cannabis compound)</td>
</tr>
<tr>
<td></td>
<td>Cerovive</td>
</tr>
<tr>
<td></td>
<td>Dexanabinol</td>
</tr>
<tr>
<td>MAO-A&amp;B inhibitors</td>
<td>Lazabemide</td>
</tr>
<tr>
<td></td>
<td>Selegiline</td>
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<tr>
<td>Neuropeptides</td>
<td>Thyrotropin-releasing hormone</td>
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<td></td>
<td>Vasoactive intestinal peptide</td>
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<tr>
<td>Osmotic diuretics</td>
<td>Frusemide</td>
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<td></td>
<td>Mannitol</td>
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<tr>
<td>Proteins and peptides</td>
<td>Fused protein transduction domain of the HIV Tat protein with FNK</td>
</tr>
<tr>
<td></td>
<td>Glatiramer acetate</td>
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<tr>
<td></td>
<td>Osteopontin</td>
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</table>
A recent review stated that there are more than 100 out of 178 controlled clinical trials that were published in the neuroprotection related literature and a few of them approved and successfully applied as neuroprotectives in clinical practice [4]. The principles and methods of drug discovery for the neuroprotective therapeutics are applicable to most of the neurological disorders including Parkinson’s disease [5], Alzheimer disease [6], Stroke and other neuro related issues [7]. The modern drug-discovery programs have become advantageous because of the involvement of combinatorial chemistry and high-throughput screening applications. Different pathways will be targeted for different diseases, for example: Rho kinases (ROCKs) are serine/threonine kinases, crucial in fundamental processes of cell proliferation, migration, and survival. Mueller et all reported that hyper induction of the ROCK pathways has been observed in various disorders of the CNS injury to the adult vertebrate brain and spinal cord activates ROKCs, thereby inhibiting neurite growth and sprouting [8].

The capacity to generate neurons, from human embryonic stem cell (hESC) lines, offers great potential for developing cell-based therapies, and a potential opportunity for neuroscientists interested in mechanisms of neuroprotection and neurodegeration. Potentially unlimited generation of well-defined functional neurons from hESC and patient–specific induced pluripotent cells (iPSC) offers new systems to study disease mechanisms, receptors pharmacology, and potential targets for neuroprotection within a human cellular environment [9].

In this order, high-throughput drug screening system can deliver drug molecules against all known drug targets in the brain slices, rather than against just the few targets that have previously been implicated in the brain cell protection. Since there is no pre-
selection bias, one is able to evaluate the widest possible range of drug targets. With the use of genomic and proteomic technologies, several drug targets have now become available. Based upon the complexity of the brain structure and other known and unknown factors the evaluation criteria to identify a neuroprotective agent became challenging. Most of the times the summarized characteristics below are keen in a neuroprotective drug development.

By summarizing all the clinical failures of the neuroprotective agents, penetration of the drug molecule through the BBB and the complexity of the brain structure are the potential reasons most of the times a special strategy needed to address neurodegenerative issues. Especially, large molecules are challenging to pass through the BBB due to their hydrophilic nature [10-13]. Due to these above reasons, a careful monitoring of the preclinical data is essential. Any inconsistencies in preclinical studies may cause inconvenience in further studies. In some cases mechanism of action remains unknown through out the basic studies, which might lead to some potential drawbacks in clinical studies. Selecting the route of administration and optimal dosage is important. Especially dosage should be safe and tolerable to humans. Efficacy studies are important in the early stages using proper animal models.

3.2. Bioanalytical methods

3.2.1. Introduction

Bioanalytical methods come under category of pre-clinical trails in the drug discovery process, used to quantify drug candidates and their metabolic products in biological matrices [14, 15]. Preclinical development represents a critical stage in the progression
from discovery to marketed pharmaceutical drug candidates that have passed initial
discovery screening and are identified to posses some drug-like properties. Extensive
resources and huge financial commitments are being made to vigorously test the drug
candidates before they enter clinical trials [16-18]. The guidelines are very clear from the
regulatory point of view in terms of ensuring the welfare of the volunteers and patients
participating in the clinical trials by vigorous testing and safety using appropriate animal
models [18, 19]. A careful examination of all above guidelines it is clear that there are
series of questions concerned about the toxicity, PK parameters, safety assessment,
formulation optimization, and so on need to be answered.

As advancement after the animal study, sometimes the same methods could be
applied to humans [20, 21]. Bioanalytical method employed for the quantitative
determination of drugs and their metabolites in biological fluids, plays a significant role
in the evaluation and interpretation of pharmacokinetic (PK), and toxicology studies [22].
which further helps evaluating the safety, efficacy and optimal dosing of the drug
candidate.

Bioanalytical method validation in the pharmaceutical industry is influenced by
regulations from the US Food and Drug Administration (FDA), the UK Medicines
Control Agency (MCA) and similar bodies from Canada, Japan and other countries. A
consensus on the requirements for analytical method validation was reached by a panel of
experts at the Washington conference on Analytical Methods Validation: Bioavailability,
Bioequivalence and Pharmacokinetic Studies in 1990 and reported by Shah and
colleagues, 1992 [23]. This is a report with the guidelines for analytical method
developments, validation and application to drug analysis in biological matrices.
According to the guidelines, the choice of sampling media and sample preparation steps is usually determined by the nature of the drug candidate. For example, drug levels in a clinical pharmacokinetic study demand the use of blood, urine, and possibly saliva. A bioavailability study may require drug level data in blood or urine. Steps involved in the estimation of drugs in biological fluid are collection of the sample, sample treatment and separation of the compound of interest from the matrix and analysis.

Most of the times the above drug estimations from various matrices are part of the drug development and are dependent upon the accurate quantification of drugs and endogenous components in biological samples. Unlike its disciplines of analytical chemistry such as drug substance and drug product analysis, one very unique feature of contemporary bioanalysis is that its measurement target is always at very low concentration levels, typically at low ng/mL concentration range and even at pg/mL for highly potent medicines. Sometimes, compounded by coexisting endogenous or exogenous compounds with similar chemical structures to the target analytes at a much higher concentration (typically at µg/mL to mg/mL range), that challenges bioanalytical scientists to accurately and definitively measure the analytes of interest.

### 3.2.2. LC-MS (MS/MS, MRM) application in bioanalysis

Numerous analytical techniques as well as their suitable combinations have been developed to perform bioanalysis, which include immunoassays [24, 25], gas chromatography-flame ionization detection (GC-FID)[25], gas chromatography-mass spectrometry (GC-MS), high performance liquid chromatography-ultraviolet (HPLC-UV), high performance liquid chromatography-mass spectrometry (HPLC-MS or LC-
MS), and high performance liquid chromatography- tandem mass spectrometry (HPLC-MS/MS or LC-MS/MS), Figure 3.1. Among all above analytical techniques advancement in drug development process LC-MS has become very popular after 1980s and has rapidly become standard instrumentation in any well-equipped bioanalytical laboratory due to it’s high accuracy, sensitivity [26].

In brief, LC-MS is a combination of the physicochemical separation capabilities of liquid chromatography (LC) and the mass (MS or MS/MS) separation/detection capabilities of mass spectrometry. In LC-MS bioanalysis, assay selectivity can be readily achieved by different stages of separation of the analyte(s) of interest from unwanted components in the biological matrix: sample extraction (protein precipitation, liquid–liquid extraction, solid-phase extraction, etc.), HPLC chromatographic separation techniques (RP-HPLC, IX-HPLC), and tandem mass spectrometric detection in selected reaction monitoring (SRM) or multiple reaction monitoring (MRM) mode [24-26].

MRM is a mass spectrometry technique based on selection of a fragment ion and one or more characteristic fragment ions at the same time. The MRM mode is a proven as an outstanding technique providing high specificity, accurate, and reproducible between laboratories and analysts [27, 28]. Through the use of retention time windows, MRM-based quantitation can also be multiplexed to analyze and quantitate hundreds of analytes per run, thus increasing the throughput of this type of assay and making it rapid enough for drug discovery as well as clinical applications where proteins and peptides studied [29-32]. Although MRM-based quantitation is typically used for the metabolic studies in critical biological matrices of new drug discover applications, researchers take advantage of MRM in most of the analytical method developments and validations by monitoring
multiple fragments of analytes in order to get more accurate results while avoiding matrix interferences [33].

Typically, MRM mode involves a series of single reactions (precursor / fragment ion transitions where the collision energy is tuned to optimize the intensity of the fragment ions of interest) are measured sequentially, and the cycle is looped throughout the entire time of the HPLC separation. MRM transitions are determined from the MS/MS spectra of the existing analyte. Typically, doubly charged precursors (or triply charged in some instances) are selected. Two transitions per analyte, corresponding to high intensity fragment ions, are then selected and the collision energy optimized to maximize signal strength of MRM transitions by using automation software. By using scheduled MRM (available in latest mass spectrometer softwares), where MRM transitions are based on retention time, we can effectively monitor several hundreds of analytes in a single run [34].

However, factors, including matrix effect, ion suppression, and insource breakdown of labile metabolites, can compromise the reliability of a LC-MS bioanalytical assay [35-38]. These factors should be carefully evaluated during method development. The focus of LC-MS bioanalysis in the pharmaceutical industry is to provide a quantitative measurement of the active drug and/or its metabolite(s) for the accurate assessment of pharmacokinetics, toxicokinetics, bioequivalence, and dose–response (pharmacokinetics/pharmacodynamics) relationships. All the factors mentioned above come across at different stages of drug discovery process (Figure 3.2).
Figure 3.1. Block diagram of LC-MS or MS/MS
Figure 3.2. Flow diagram of steps in drug discovery and development
The quality of these studies, which are often used to support regulatory filings and other evaluations, is directly related to the conduct of the underlying bioanalysis. Therefore, the application of best practices in bioanalytical method development, validation, and associated sample analysis is key to an effective discovery and development of a drug product.

Natural products were more traditional way of source of active compounds before the technique called combinational chemistry. In traditional ways once the active ingredient identified it used to be isolated and its chemical structure characterization done using NMR, MS, IR, and derivatization or selective degradation chemistry [39]. Screening entailed an assessment of bioactivity and physicochemical data compared to known databases.

High-resolution mass spectrometry played a critical role allowing molecular formula searches from accurate mass data. Similarly, spectral databases allowed positive confirmation or class assessments. This process helped to ensure that novel compounds were selected. Since the introduction of combinatorial chemistry 20 years ago, the analyst’s role in early drug discovery has shifted to the development of highly efficient LC-MS analytical methods to support quantitative analysis. The drug discovery process begins with compound library development and ends with the selection of preclinical drug candidates for preclinical safety assessment. LC-MS bioanalysis plays an important role throughout this process.
3.3. References


4.1. Introduction

There is current interest in thiazolidinedione (TZD) derivatives or glitazones, which are a class of compounds with demonstrated pharmacological activity for a variety of conditions. TZD compounds have shown promise as antidiabetic, antibiotic, antifungal and neuroprotective agents [1-4].

The first generation TZD, troglitazone, an antidiabetic, showed a rare but severe hepatotoxicity side effect [5]. This led to the development of the TZD derivative pioglitazone, which is a popular drug of choice used in the treatment of diabetes. Pioglitazone has also been shown to have a neuroprotective effect in the MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) rodent model of Parkinson's disease, demonstrating the following neurologic protection effects: protection against dopaminergic cell loss with preservation of striatal dopamine concentrations, improved cognitive performance and preservation of motor behavior [4, 6,7,8].
Mitochondrial processes have been implicated in neurodegenerative diseases. For example, abnormal mitochondrial activity in neuronal cells leading to a susceptibility to changes in intracellular energy has been reported in Parkinson’s and Alzheimer’s diseases [9-11]. Therapeutic agents targeting the mitochondria as neuroprotective agents are thus an active area of drug discovery research for neurodegenerative diseases.

MitoNEET, a recently discovered mitochondrial protein, has been identified as a target of TZD containing compounds [12]. The structure of mitoNEET has been elucidated, with a redox-active iron-sulfur cluster being identified [13-16]. MitoNEET is important in maintaining the maximal oxidative capacity of the mitochondria [17]. Recently, Geldenhuys et al. used computational docking and binding assay studies to design and then synthesize a novel TZD derivative NL-1 \{5-[(3,5-di-tert-butyl-4-hydroxyphenyl)methyl]-1,3-thiazolidine-2,4-dione\}, which binds to a specific site on mitoNEET [18]. In this study, NL-1 was shown to decrease mitochondrial respiration, with evidence of reduced generation of reactive oxidative species. This could explain the neuroprotective effects noted in mitochondrial toxin experiments.

The mitochondrial effect of NL-1 and other TZD compounds interacting with mitoNEET is a newly proposed mechanism for the neuroprotective activity. Previous studies involving pioglitazone have implicated PPAR-γ (peroxisome proliferator activated receptor-gamma) activation or inhibition of monoamine oxidase B mechanisms to explain these neuroprotective effects [8]. The interaction of NL-1 with its presumed target mitoNEET, results in a neuroprotective effect, as well as other potential effects. Further study of NL-1 is warranted, with the potential for discovering new therapeutic pathways in drug development.
Previous LC-MS/MS methods have been developed determining the TZD derivatives pioglitazone [19], rosiglitazone [20] and troglitazone [21]. In order to study the pharmacokinetics and other clinical trial aspects of NL-1, a sensitive quantitative method is needed to evaluate this compound in vivo. The present work is the first report of a validated LC-MS/MS technique for the determination of NL-1 in mouse serum. This method was successfully applied in a preliminary pharmacokinetic (PK) study of the parent compound NL-1 in mouse serum.

4.2. Experimental

4.2.1. Materials

The analyte NL-1 (Figure 4.1A) and the internal standard (IS) NL-2 \{5-[(4-hydroxy-3,5-dimethyl-phenyl)methyl]thiazolidine-2,4-dione\} (Figure 4.1B) were synthesized and purified according to published procedures [22,23]. HPLC grade methanol and acetonitrile were from Fisher Scientific (Fair Lawn, NJ, USA). ACS grade ammonium acetate was from Sigma Aldrich (St. Louis, MO, 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 0.45µ cellulose ester membrane filters from Millipore (Billerica, MA, USA). Six different lots of Non Swiss Albino Mouse serum were from Innovative Research (Novi, MI, USA). Dosed mouse serum samples were obtained at the indicated time points. All animal procedures were approved by the Institute Animal Care and Use Committees (IACUC) at the Northeast Ohio Medical University facility.
4.3.1. Preparation of stock and working solutions

A set of NL-1 working solutions were prepared by a serial dilution with acetonitrile of a 0.5 mg/mL stock solution in acetonitrile. The IS working solution at 100 ng/mL was obtained similarly from a NL-2 stock solution at 0.5 mg/mL in acetonitrile. All the solutions were stored at -20°C.

4.3.2. Preparation of calibration and quality control (QC) standards

Working calibration serum solutions were prepared by spiking 10 µL of corresponding NL-1 working solutions in 200 µL of mouse serum (mixture of 6 lots) to give concentrations of 1, 2, 5, 10, 20, 50, 100 ng of NL-1 per mL. Working QC serum solutions were prepared in a similar manner at the concentrations of 2.5, 15 and 80 ng/mL, which served as low, mid and high QC working serum solutions respectively. The working calibration and QC solutions, as well as the undiluted dosed serum samples, were stored at -20°C until sample preparation and LC-MS/MS analysis, as described below.
Figure 4.1. The chemical structures of NL-1 (A) and NL-2 (B) with fragmentation pattern
4.3.3. Serum sample preparation

The following protein precipitation procedure was employed in the sample preparation of the calibration standards, QC standards, blanks and the dosed serum samples. The working calibration and working QC serum solutions (section 4.3.2), as well as the dosed serum samples were thawed to room temperature and taken through the sample preparation steps described below. Single (used for diluting dosed serum samples) and double (used in the selectivity studies) blanks were prepared by adding 10 µL of acetonitrile into 200 µL commercial mouse serum. Then 10 µL of IS working solution was spiked into each of the 200 µL serum standards/samples/blanks, except for the double blank, in which 10 µL of acetonitrile was added. Following a 20 seconds vortex, 800 µL of acetonitrile was added into each of the above prepared standards/samples/blanks, then sonicated for 10 minutes at room temperature (Figure 4.2). After sonication, all of the samples were centrifuged at 13,000 g for 20 minutes. The supernatants were transferred into HPLC auto sampler vials for LC-MS/MS analysis.

4.3.4 Dosed serum samples

A series of dilutions were done (10-fold or 100-fold) with single blanks (prepared from 6 mixed commercial lots) in order to adjust the concentration of NL-1 in the mouse dosed serum samples to be within the validated linear range of the calibration standards. QC standards (2.5, 15 and 80 ng/mL) were run with the dosed serum samples to confirm acceptable performance of the method.
Figure 4.2. Flow diagram of serum sample preparation
4.3.5. LC-MS/MS analysis

A Shimadzu UPLC system (Columbia, MD, USA) consisted of a Prominence DGU – 20A3R inline degasser, two LC-30AD pumps, a SIL-30AC auto sampler, and a CBM-20A controller. The UPLC system was interfaced to an AB Sciex QTrap 5500 mass spectrometer (Figure 4.3) equipped with an electrospray ionization source (Framingham, MA, USA). Following the sample preparation procedure described above, 10 µL of each supernatant was injected onto a Columbus C-18 HPLC column (2 x 50 mm, 5 µm) with a C-18 guard cartridge from Phenomenex (Torrance, CA, USA). An optimized gradient of mobile phase A: 15 µM ammonium acetate in 2% methanol and mobile phase B: 100% methanol at 0.2 mL/min was developed (see Table 1). The run time for each injection was 10 minutes, with a pre-equilibrium time of 11 minutes.

Negative ionization was selected for MS detection. A multiple reaction monitoring (MRM) function was employed for quantification, with the transitions set at m/z 334 → 263 for NL-1 and 250 → 179 for IS. The data was acquired and processed using Analyst software version 1.6.1 (AB SCIEX). The mass spectrometric conditions were optimized for both NL-1 and NL-2 (IS), including declustering potential (-75 V), entrance potential (-10 V), collision exit potential (-75 V), collision energy (-30 V), ion spray voltage (-4500 V), and nebulizer temperature (450°C). Nitrogen was used as the nebulizing gas.

4.3.6. Analytical method validation

A full method validation was performed in mouse serum according to FDA BioAnalytical Method Guidelines [24] and other references [25,26]. The entire assay was
validated for linearity, precision, accuracy, absolute extraction recovery, selectivity, lower limit of quantification (LLOQ), matrix effect and stability.

4.3.6.1. Calibration

The calibration curves were established by plotting the peak area ratios of NL-1 to IS (y) versus the spiked NL-1 concentrations (x) of the calibration standards (n=2 for each of the seven calibrators, with the average for each calibrator plotted). The slope and correlation coefficient of the calibration curve were calculated using weighed (1/x) linear least squares regression. Two sets of calibrators (one at the beginning of the run and the other at the end) were run each time an experimental run was done (24 to 35 hours duration) for all the validation studies and analysis of the dosed serum samples.

4.3.6.2. Selectivity, matrix effect and LLOQ

Six double blanks matched to six LLOQ serum standards at 1 ng/mL were prepared from six different individual lots of mouse serum to evaluate the matrix interference and LLOQ.

The relative matrix effect [25] was assessed by comparing the peak area ratio of double blank serum, to which NL-1/IS is added post-preparation for three NL-1 concentrations (2.5, 15, 80 ng/mL), with the peak area ratio determined for the same concentration standard solutions (80% ACN) spiked with NL-1 and IS.

In addition, a multiple MRM transition study was done to confirm that there was not a significant matrix effect present in the NL-1 MRM analysis transition (m/z 334→263). Prepared dosed serum samples (14.7 and 16.9 ng/mL) from two different mice (n=2 for
each mouse serum sample) and freshly prepared calibrators [n=2 for each concentration (1, 2, 5, 10, 20, 50, 100 ng/mL)] were measured at two different NL-1 MRM transitions [MRM1: m/z 334 → 263 (the analysis transition) and MRM2: m/z 334 → 231], as determined from the product ion spectra experiment (Figure 2A). The average ratio of MRM1/MRM2 peak areas for NL-1 for each dosed mouse serum sample was compared with the overall average of the MRM1/MRM2 peak area ratios for NL-1 of the calibrators (average of all seven calibrators).
Figure 4.3. Block diagram of QTRAP 5500 mass spectrometer
4.3.6.3. Precision, accuracy and extraction recovery

Intra-assay (within a day) and inter-assay (5 days) precision and accuracy studies were conducted using the three QC standards (n=5). Accuracy was determined as follows: QC standards of 2.5, 15, and 80 ng/mL were prepared, the NL-1 concentrations experimentally determined and these results compared to the theoretical spiked values.

The absolute extraction recovery was assessed by comparing the experimentally determined NL-1 peak areas of QC standards (2.5, 15, 80 ng/mL), prepared and analyzed normally (NL-1 added prior to sample preparation), with the NL-1 peak areas of double blank prepared solutions to which NL-1(2.5, 15, 80 ng/mL) was added after sample preparation (post-preparation sample).

4.3.6.4. Stability studies

Stability studies (n=3) were done using two different QC standards (2.5 and 80 ng/mL), which were kept at or exposed to the following storage regimens: 6 hours at room temperature, 2 months at -20 °C, and three freeze-thaw cycles over a three day period. The stability results of these NL-1 QC standards were compared with theoretical values.

Stock solution stabilities for NL-1 analyte and IS were also assessed. Stock solutions of NL-1 were stored for 8 months at -20 °C. QC standards at two different concentrations (2.5 and 80 ng/mL) were then prepared from the stored and freshly prepared stock solutions, as described previously, and experimentally determined concentrations of NL-1 compared (n=3 for each sample).
4.4. Results and discussion

4.4.1. Optimization of mass spectrometric parameters

Both positive and negative ionization modes were evaluated for the detection of NL-1 and NL-2 IS signals. Negative ionization showed a much higher intensity and was thus chosen. The negative product ion spectra in Figure 4.4 A and 4.4 B for NL-1 and NL-2 IS respectively. The MRM transitions of m/z 334 → 263 for NL-1 and 250 → 179 for the NL-2 IS were selected for quantification. The presumed fragmentation generating the daughter ions of the MRM transitions are shown in Figure 1 for NL-1 and NL-2 IS.

4.4.2. Optimization of HPLC conditions

Isocratic mobile phases with different concentrations of methanol or acetonitrile were tested but found to be unsatisfactory with respect to peak broadening and exhibiting a substantial carry-over effect. Various linear gradients of water (A) and methanol (B) or water (A) and acetonitrile (B) provided sharper peaks with higher intensities, but the carry-over issue remained. A steep linear gradient (Table 4.1) from 26.5% to 95.1% methanol (taking into account that mobile phase A is 2% methanol), with addition of ammonium acetate to mobile phase A, was found to give optimal performance, with no carry over.
Figure 4.4. Product ion spectra of NL-1 (A) and NL-2 (B)
Table 4.1. HPLC gradient program

Mobile phase-A was 15 µM in 2% methanol and mobile phase-B was 100 % methanol

<table>
<thead>
<tr>
<th>Minutes</th>
<th>B%</th>
</tr>
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<tbody>
<tr>
<td>0-3</td>
<td>25 (isocratic)</td>
</tr>
<tr>
<td>3-3.5</td>
<td>25-95 (linear)</td>
</tr>
<tr>
<td>3.5-10</td>
<td>95 (isocratic)</td>
</tr>
</tbody>
</table>
4.4.3. Linearity, selectivity and LLOQ

The calibration plot was confirmed to be linear at least in the range of 1-100 ng/mL, with a correlation coefficient of $r = 0.9996$. The least squares fit equation (1/x weighting) was $y = 0.923x - 0.0767$, where $y$ is the peak area ratio NL-1 to IS and $x$ is the NL-1 concentration in serum (Figure 4.5). The selectivity and LLOQ of the method were evaluated using the double blank and LLOQ samples (1 ng/mL). As shown in the Figure 4.6, NL-1 and IS retention times were 5.76 and 5.24 min, respectively. No interferences appeared in these regions for the blanks, noting that the scale for LLOQ signals (Figure 4.6) is two orders of magnitude greater than the blank response scale (Figure 4.6). The coefficient of variation (%CV) and accuracy for the LLOQ were 8% and 99%, respectively, meeting FDA guidelines. A representative dosed serum sample of 13 ng/mL NL-1 (concentration after dilution) is also shown in Figure 4.6.
Figure 4.5. Calibration curve of NL-1 between 1 -100 ng/mL
Figure 4.6. Representative MRM chromatograms of NL-1 (A1, B1, C1) and NL-2 (A2, B2, C2) for double blank (A1 and A2), LLOQ standard (B1 and B2), and a dosed sample at 13 ng/mL mouse serum (C1 and C2).
4.4.4. Precision and accuracy

The intra-assay and inter-assay precision and accuracy were assessed using high, medium and low QC standards As can be seen in Table 4.2, the %CVs ≤ 3.5% and the relative errors (%RE) were in a range of -2.7 to 2.0%, indicating that the precision and accuracy of this assay fulfill the criteria of the FDA guidelines.

4.4.5. Absolute extraction recovery studies

The absolute extraction recoveries were determined for QC standards (2.5, 15.0, 80.0 ng/mL). As indicated in Table 4.3, excellent recoveries were obtained for all three concentrations using the protein precipitation procedure. The absolute extraction recoveries of NL-1 from mouse serum matrix using the given sample preparation procedure were 96 – 115%, with a %CV ≤ 10%. This compares favorably to previous LC-MS/MS determinations of other TZD compounds, which gave recoveries of 88 - 95% [19], 80% [20], and 63 – 81% [21].
Table 4.2. Precision and accuracy of intra-assay and inter-assay for the quantification of NL-1 in mouse serum (n=5)

<table>
<thead>
<tr>
<th></th>
<th>Intra-Assay</th>
<th></th>
<th></th>
<th>Inter-Assay</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Spiked NL-1 conc. (ng/mL)</td>
<td>Measured NL-1 conc. (ng/mL)</td>
<td>Precision %C V</td>
<td>Accuracy %RE</td>
<td>Measured NL-1 conc. (ng/mL)</td>
</tr>
<tr>
<td>Low QC</td>
<td>2.5</td>
<td>2.54±0.09</td>
<td>3.54</td>
<td>1.60</td>
<td>2.55±0.07</td>
</tr>
<tr>
<td>Mid QC</td>
<td>15</td>
<td>14.8±0.43</td>
<td>2.90</td>
<td>-1.33</td>
<td>14.6±0.42</td>
</tr>
<tr>
<td>High QC</td>
<td>80</td>
<td>78.5±1.63</td>
<td>2.07</td>
<td>-1.87</td>
<td>78.1±1.45</td>
</tr>
</tbody>
</table>

%CV = (Standard Deviation/Mean) x 100%;
%RE = [(Measured – Spiked)/Spiked] x 100%
Table 4.3. Absolute extraction recovery of NL-1 in mouse serum (n=3)

<table>
<thead>
<tr>
<th>NL-1 conc. (ng/mL)</th>
<th>Mean recovery ± SD</th>
<th>%CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>115±6.0</td>
<td>5.2</td>
</tr>
<tr>
<td>15</td>
<td>96±9.6</td>
<td>9.9</td>
</tr>
<tr>
<td>80</td>
<td>107±4.1</td>
<td>3.9</td>
</tr>
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</table>

%CV = (Standard Deviation/Mean) x 100%
4.4.6. Matrix effect studies

The relative matrix effect (RME) was assessed by comparing the NL-1/IS peak area ratio for each of three double blank samples to which NL/IS has been added post-preparation (NL1 at 2.5, 15, 80 ng/mL) with the NL-1/IS peak areas ratio for each of the same concentration standard solution (80% acetonitrile), multiplied by 100. Acceptable values ranging from 85 to 95% were found (Table 4.4), indicating minimal matrix effect. The absence of a significant matrix effect was further confirmed by a multiple MRM experiment, in which the results of ratio of peak areas for two NL-1 MRM transitions (MRM1: m/z 334 → 263 and MRM2: m/z 334 → 231) for the dosed serum samples from two different mice were compared with the average peak area ratio for the seven calibrators. A matrix effect is identified in a particular dosed serum sample if the MRM ratio (Equation 1) of the dosed serum sample is significantly different (> 20%) than the MRM ratio of the calibrators.

\[
\frac{\text{Peak area MRM1}}{\text{Peak area MRM2}} = \text{MRM Ratio} \quad \text{Equation 1}
\]

where MRM1 is m/z 334 → 263 and MRM2 is m/z 334 → 231 for NL-1.
Table 4.4. Relative matrix effect of NL-1 in mouse serum (n=3)

<table>
<thead>
<tr>
<th>NL-1 conc. (ng/mL)</th>
<th>Relative Matrix Effect (RME) ± SD (%)</th>
<th>%CVb</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>85.3±2.2</td>
<td>2.6</td>
</tr>
<tr>
<td>15</td>
<td>89.8±0.8</td>
<td>0.9</td>
</tr>
<tr>
<td>80</td>
<td>95.3±4.7</td>
<td>4.9</td>
</tr>
</tbody>
</table>

RME = [(post-preparation NL-1/IS peak area ratio)/(NL-1/IS peak area ratio in 80% ACN) x 100%

%CV = (Standard Deviation/Mean) x 100%
Results from this experiment found the MRM ratio was $17.7 \pm 2.6$ and $17.6 \pm 0.2$ (\pm range) for the two dosed serum samples from different mice, while the average MRM ratio of the seven calibrators was $15.3 \pm 1.9$ (\pm SD). This is within the acceptability criterion, thus confirming the absence of significant matrix interference in the two mouse serum samples.

4.4.7. Stability

The stability of NL-1 in serum samples was determined for various storage conditions by measuring the concentrations of NL-1 in QC standards after storage and comparing the results with the theoretical values. As seen in the Table 4.5, there was no degradation or loss of NL-1 in serum after storing at room temperature for 6 hours, at -20 °C for 2 months, and after 3 freeze-thaw cycles (101 – 110% recovery for all tested storage conditions). Also, no changes in NL-1 concentration were found for serum samples taken through the preparation steps and stored for 24 hours at room temperature. The stock solution stability was determined to be 89%, with a %CV of 9%, for storage for 8 months at -20°C, indicating good stability.
Table 4.5

Stability studies of NL-1 in mouse serum (n=3)

<table>
<thead>
<tr>
<th>Condition</th>
<th>Spiked NL-1 conc. (ng/mL)</th>
<th>Measured NL-1 conc. (ng/mL) Mean ± SD</th>
<th>Stability (% Recovery)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 Freeze thaw cycles</td>
<td>Low 2.5</td>
<td>2.75±0.1</td>
<td>110</td>
</tr>
<tr>
<td></td>
<td>High 80</td>
<td>84.5±0.1</td>
<td></td>
</tr>
<tr>
<td>6hr at RT</td>
<td>Low 2.5</td>
<td>2.64±0.1</td>
<td>106</td>
</tr>
<tr>
<td></td>
<td>High 80</td>
<td>82.7±0.3</td>
<td>103</td>
</tr>
<tr>
<td>2 months at -20°C</td>
<td>Low 2.5</td>
<td>2.73±0.1</td>
<td>109</td>
</tr>
<tr>
<td></td>
<td>High 80</td>
<td>80.7±3.5</td>
<td>101</td>
</tr>
</tbody>
</table>
4.4.8. Carry-over

A carry-over problem was encountered in the development of this LC-MS/MS method, as was noted in a previous LC-MS/MS method for TZD compounds [21]. Various strategies were attempted to address this issue including using C8 and phenyl HPLC columns, as well as utilizing various gradient and isocratic programs employing mobile phases consisting of various concentrations of methanol or acetonitrile. Different auto sampler solutions with various proportions of methanol and acetonitrile with HPLC grade water to wash the syringe also failed to solve the problem. Finally, addition of ammonium acetate to achieve a final concentration of 15 µM in mobile phase A addressed this carry-over problem. {Mullangi, 2012 #24620}

4.4.8. Application to parent drug NL-1 pharmacokinetic studies

The validated LC-MS/MS method was applied to the measurement of NL-1 in serum samples from mice before or after dosing at seven different time points including 0, 1/2, 1, 2, 4, 6, and 24 h. A single-dose of 10 mg/kg of NL-1 was administered to seven different mice through an intraperitoneal route. The 10 mg/kg dosing was established by previous studies in a MPTP parkinsonian mouse model, in which a neuroprotective effect for NL-1 was documented to occur at this dosage [27].

The serum concentration-time profile of NL-1 is plotted in Fig. 4. The preliminary pharmacokinetic curve shows NL-1 reaching its maximum level within 30 minutes [5850 ng/mL (17.5 µM) at 30 min], decreasing to 650 ng/mL (1.9 µM) at 2 hr and 15 ng/mL (0.04 µM) at 6 hr. The NL-1 concentration measured in mouse serum in the present work
is within the activity concentration range for NL-1 binding to its presumed mitoNEET target [28]. In this previously published study, an IC50 concentration for NL-1 of 7 µM was found to competitively displace [3H]-rosiglitazone from mitoNEET.

Although it is premature for testing NL-1 in humans, it is reasonable to postulate that the therapeutic concentration range of NL-1 in human serum will be similar to that found in the present study in mice. This hypothesis is based on the already established therapeutic concentration range in human serum for the anti-diabetic TZD drug pioglitazone, which has also been tested in humans for a neuroprotective action [29,30]. The administered pioglitazone dosage for the neurological trials was exactly the same as that administered in the treatment of diabetes (15 – 45 mg/ day). Thus the human serum concentrations of pioglitazone found for the diabetes clinical trials are the same as would be measured for the use of pioglitazone for neurological conditions.

Relating known pioglitazone concentrations in serum to what would be expected for NL-1 concentrations in human serum is possible from experiments measuring binding constants of the two compounds to mitoNEET. These experiments found that NL-1 and pioglitazone have similar binding constants for the target mitoNEET [28]. It thus follows that therapeutic serum concentrations of pioglitazone can approximate the therapeutic serum concentrations of NL-1 in the treatment of neurodegenerative disorders, although this assumes NL-1 and pioglitazone have similar distribution and metabolism kinetics in humans.

Published human clinical trials for pioglitazone, with the oral administration being 45 mg/day, yielded serum concentration maximums of 1500 ± 630 ng/mL (4.2 ± 1.8 µM)
This is approximately the same magnitude (a factor of 4 lower) as was found for serum NL-1 in the present mouse study, with a maximum concentration of 5850 ng/mL (17.5 µM). Thus the mouse NL-1 studies can be considered to approximate what will be found for humans. Therefore it is likely that the present technique will be applicable for the concentration range in human serum samples measured in future NL-1 clinical trials.
Figure 4.7. Serum dose response curve (concentration vs. time) of NL-1 after a single 10 mg NL-1/kg intraperitoneal administration to mice.
4.5. Conclusion

A new highly sensitive LC-MS/MS method has been developed and validated to quantify the thiazolidinedione novel ligand NL-1 in mouse serum. A carry-over issue was addressed by addition of ammonium acetate to the application mobile phase. The present method is accurate, precise and robust. A preliminary experiment has been done showing suitability of the technique to pharmacokinetic studies of the parent drug NL-1.
4.6. References


25. Liu, Y., Ma, B., Zhang, H. Ying, J. Li, Q. Xu, D. Wu, Y. Wang, Development and validation of a sensitive liquid chromatography/tandem mass spectrometry method for the determination of raddeanin A in rat plasma and its application to a


5.1. Introduction to pharmacokinetics

The term pharmacokinetics is defined as the study of the time course of drug absorption, distribution, metabolism, and excretion. Pharmacokinetic principles ensure the safety and efficacy of the therapeutic management of drugs. The ultimate goals of pharmacokinetics are enhancing efficacy and decreasing toxicity of a patient’s drug therapy. The development of strong correlations between drug concentrations and their pharmacologic responses has enabled clinicians to apply pharmacokinetic principles to actual patient situations and a drug’s effect on patient is often related to its concentration at the site of action, so it would be useful to monitor this concentration [1, 2]. Receptor sites of drugs are generally inaccessible to our observations or are widely distributed in the body, and therefore direct measurement of drug concentrations at these sites is not practical. For example, the receptor sites for digoxin are thought to be within the myocardium. Obviously we cannot directly sample drug concentration in this tissue. However, we can measure drug concentration in the blood or plasma, urine, saliva, and
other easily sampled fluids [3-5]. Pharmacokinetics provides a mathematical basis to assess the time course of drugs and their effects in the body. It enables the following processes to be quantified:

- Absorption
- Distribution
- Metabolism
- Excretion

This pharmacokinetic process often referred to as ADME, determine the drug concentration in the body when medicines are prescribed. A fundamental understanding of these parameters is required to design an appropriate drug regimen for a patient. The effectiveness of a dosage regimen is determined by the concentration of the drug in the body [6].

The drug concentration should be measured at the site of action of the drug that is, near the receptor. However, drug concentrations are normally measured in whole blood from which serum or plasma is generated due to inaccessibility of the site of action. Other body fluids such as saliva, urine and cerebrospinal fluid (CSF) are sometimes considered. It is assumed that drug concentrations in these fluids are in equilibrium with the drug concentration at the receptor [7, 8]. The measured drug concentrations in plasma, serum or other body fluids as mentioned above are often referred to as drug levels which gives an understanding of total drug concentration in the physiological system, i.e. a combination of bound and free drug that are in equilibrium with each other. In routine clinical practice, serum drug level monitoring and optimization of a dosage regimen require the application of clinical pharmacokinetics. A number of drugs show a narrow
therapeutic range and for these drugs therapeutic drug level monitoring is required. A
flow diagram of the physiological drug path in a physiological system can be seen in the
Figure 5.1.

A variety of techniques are available for representing the pharmacokinetics of a drug.
The most usual is to view the body as consisting of compartments between which drug
moves and from which elimination occurs. The transfer of drug between these
compartments is represented by rate constants, which are considered below.

5.1.1. Rates of reaction

To consider the processes of ADME the rates of these processes have to be
considered; they can be characterized by two basic underlying concepts. The rate of a
reaction or process is defined as the velocity at which it proceeds and can be described as
either zero-order or first-order [9].

5.1.1.1. Zero-order reaction

Consider the rate of elimination of drug A from the body. If the amount of the drug,
A, is decreasing at a constant rate, then the rate of elimination of A can be described as:

\[ \frac{dA}{dt} = -k^* \]

\( (k^* = \text{zero-order rate constant}) \)
Figure 5.1. Flow chart of the physiological drug path in the body
The reaction proceeds at a constant rate and is independent of the concentration of a present in the body. An example is the elimination of alcohol. Drugs that show this type of elimination will show accumulation of plasma levels of the drug and hence nonlinear pharmacokinetics.

5.1.1.2. First-order reaction

If the amount of drug A is decreasing at a rate that is proportional to A, the amount of drug A remaining in the body, then the rate of elimination of drug A can be described as:

\[
\frac{dA}{dt} = -kA
\]

(k = first-order rate constant)

The reaction proceeds at a rate that is dependent on the concentration of a present in the body. It is assumed that the processes of ADME follow first-order reactions and most drugs are eliminated in this manner.

Most drugs used in clinical practice at therapeutic dosages will show first-order rate processes; that is, the rate of elimination of most drugs will be first-order. However, there are notable exceptions, for example phenytoin and high-dose salicylates. In essence, for drugs that show a first-order elimination process one can show that, as the amount of drug administered increases, the body is able to eliminate the drug accordingly and accumulation will not occur. If you double the dose you will double the plasma concentration. However, if you continue to increase the amount of drug administered then all drugs will change from showing a first-order process to a zero-order process, for example in an overdose situation.
5.1.2. Pharmacokinetic models

Pharmacokinetic models are hypothetical structures that are used to describe the fate of a drug in a biological system following its administration [2, 10, 11].

5.1.2.1. One-compartment model

Following drug administration, the body is depicted as a kinetically homogeneous unit (Figure 5.2). This assumes that the drug achieves instantaneous distribution throughout the body and that the drug equilibrates instantaneously between tissues. Thus the drug concentration–time profile shows a monophasic response. It is important to note that this does not imply that the drug concentration in plasma (Cp) is equal to the drug concentration in the tissues. However, changes in the plasma concentration quantitatively reflect changes in the tissues.

5.1.2.2. Two-compartment model

The two-compartment model resolves the body into a central compartment and a peripheral compartment (Figure 5.3). Although these compartments have no physiological or anatomical meaning, it is assumed that the central compartment comprises tissues that are highly perfused such as heart, lungs, kidneys, liver and brain. The peripheral compartment comprises less well-perfused tissues such as muscle, fat and skin.
Figure 5.2. One-compartment PK model; $ka = \text{absorption rate constant (1/h)}$, $k = \text{elimination rate constant (1/h)}$

Figure 5.3. Two-compartment PK model; $k_{12}$, $k_{21}$ and $k$ are first-order rate constants: $k_{12} = \text{rate of transfer from central to peripheral compartment}; k_{21} = \text{rate of transfer from peripheral to central compartment}; k = \text{rate of elimination from central compartment.}$
A two-compartment model assumes that, following drug administration into the central compartment, the drug distributes between that compartment and the peripheral compartment. However, the drug does not achieve instantaneous distribution, i.e. equilibration, between the two compartments.

5.1.3. Pharmacokinetics of TZD derivatives

The term neuroprotection deals with the pathological and pathophysiological issues with ischemic brain. Abrupt deprivation of oxygen and glucose to neuronal tissues elicits a series of pathological cascades, leading to neuronal death [12, 13]. A recent review stated that 100 out of 178 controlled clinical trials reported for stroke-related issues were focused on neuroprotection [14]. Neuroprotective agents proposed with different mechanism including glutamate receptor antagonists NMDA antagonists, calcium channel blocker [15-18], sodium channel blockers [19], anti-inflammatory agents [20] and free radical scavengers [21] did not make it through the clinical trials due to varying issues. Researchers have different views for the possible mechanism of action of promising compounds as well as probable reasons for their failure of these neuroprotective agents[22-27].

In general, however, the failure of these neuroprotective agents in preclinical or clinical trials may be attributed to multifactorial effects of these agents and/or to the complexity and multifactorial nature of the neurodegerative disorders. Thus research continues towards finding new neuroprotective agents. In particular, novel agents towards new targets need to be identified that have greater potency which will result in better clinical outcomes.
In this order a new approach to explore in neuroprotective agents is the link between mitochondrial processes and neurodegenerative diseases came into light. For example, abnormal mitochondrial activity in neuronal cells leading to a susceptibility to changes in intracellular energy has been reported in Parkinson’s and Alzheimer’s diseases[28-30].

Recently a novel mitochondrial protein, MitoNEET, has been identified as a target of thiazolidinedione (TZD) containing compounds[31]. Since MitoNEET is important in maintaining the maximal oxidative capacity of the mitochondria [32] it is a potential target to affect neuroprotection [33]. Recently, Geldenhuys et al. used computational docking and binding assay studies to design and then synthesize a novel TZD derivative NL-1 \{5-[(3,5-di-tert-butyl-4-hydroxyphenyl)methyl]-1,3-thiazolidine-2,4-dione\}[34], which binds to a specific site on mitoNEET. In this study, NL-1 was shown to decrease mitochondrial respiration, with evidence of reduced generation of reactive oxidative species. In addition, NL-1 in the MPTP mouse model showed that NL-1 (10 mg/Kg) could preserve striatal dopamine levels with a single injection. Thus NL-1, studied in the present work, is a potential neuroprotective drug candidate.

The objective of the present study is to investigate the pharmacokinetics and bioavailability of the novel TZD ligand, NL-1 in dosed mice serum and brain, essential for determining optimal dosing. A validated LC-MS/MS technique for the determination of NL-1 in mouse was developed (as discussed in the earlier chapter) and successfully applied in the pharmacokinetic (PK) study in the mice serum and brain.
5.2. Experimental

5.2.1. Materials

The analyte NL-1 and the internal standard (IS) NL-2 were synthesized and purified according to published procedures [22, 23]. HPLC grade methanol and acetonitrile were from Fisher Scientific (Fair Lawn, NJ). ACS grade ammonium acetate was from Sigma Aldrich (St. Louis, MO). 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). All the solvents were filtered through 0.45µ cellulose ester membrane filters from Millipore (Billerica, MA). Six different lots of Non Swiss Albino Mouse serum were from Innovative Research (Novi, MI) were mixed and used for preparation of standards, calibrators, QC standards, and for diluting samples. Dosed mouse serum samples were obtained at the indicated time points.

5.2.2. Animals and surgical procedures

Adult male C57BL/6 retired breeder mice (approximately six months old) were purchased from Harlan, Indianapolis. All the mice were housed individually had free access to food and water and were maintained under a 12 h light cycle with lights on 0600 hr. All treatments comply with the NIH guide for Care and Treatment of Laboratory Animals and were approved by the IACUC at Northeast Ohio Medical University. The mice serum was separated from the blood by collecting it into heparinized tubes, and centrifuged for 10 minutes at 1000x g and 4°C. The obtained serum was labeled accordingly and stored at -80°C until sample preparation.
5.2.3. LC-MS/MS analysis

A Shimadzu UPLC system (Columbia, MD) consisted of a Prominence DGU – 20A3R inline degasser, two LC-30AD pumps, a SIL-30AC auto sampler, and a CBM-20A controller was used. The UPLC system was interfaced to an AB Sciex QTrap 5500 mass spectrometer equipped with an electrospray ionization source (Framingham, MA). Following the sample preparation procedure described above, 10 µL of each supernatant was injected onto a Columbus C-18 HPLC column (2 x 50 mm, 5 µm) with a C-18 guard cartridge from Phenomenex (Torrance, CA). An optimized gradient of mobile phase A: 15 µM ammonium acetate in 2% methanol and mobile phase B: 100% methanol at 0.2 mL/min was developed. The run time for each injection was 10 minutes, with a pre-equilibrium time of 11 minutes.

Negative ionization was selected for MS detection. A MRM function was employed for quantification, with the transitions set at m/z 334 → 263 for NL-1 and 250 → 179 for IS. The data was acquired and processed using Analyst software version 1.6.1 (AB SCIEX).

5.2.4. Preparation of stock and working standard, calibration and QC standards

A set of NL-1 working solutions was prepared by a serial dilution with acetonitrile of a 0.5 mg/mL stock solution in acetonitrile. The IS working solution at 100 ng/mL was obtained similarly from a NL-2 stock solution at 0.5 mg/mL in acetonitrile. All the solutions were stored at -20°C. The calibration standards were prepared by spiking 10 µL of corresponding NL-1 working solutions in 200 µL of serum (mixture of 6 lots) to give concentrations 1, 2, 5, 10, 20, 50, 100 ng of NL-1 per mL serum. The QC standards were
prepared in a similar manner at the concentrations of 2.5, 15 and 80 ng/mL, which served as low, mid and high QC standards respectively. All the calibration standards, QC standards and undiluted dosed serum samples were stored at -20°C until sample preparation and LC-MS/MS analysis, as described below.

5.2.5. Homogenization of serum and brain samples

All the dosed serum and brain tissues were homogenized using commercial mouse serum and diluted phosphate buffer saline (PBS) respectively to a certain quantity in order to reduce the quantification errors due to the tiny sample sizes. Dosed serum tissue did not require any additional steps to homogenize and proceed to protein precipitation right after diluting with commercial serum, however the excessively hydrophobic brain tissues needed a pretreatment with diluted PBS as followed. One portion of mice brain was mixed with two portions of PBS and homogenized using a special homogenizing apparatus from KONTES GLASS CO. Each brain sample tissue was homogenized to a slurry consistency, takes two to three minutes of vigorous homogenization. Both serum and brain dosed samples were subjected to protein precipitation (can be seen in “sample preparation” section) along with other standards.

5.2.6. Sample preparation of calibration standards, QC standards, blanks, serum and brain tissues

A simple protein precipitation procedure was employed in the sample preparation of the calibration standards, QC standards, blanks and the homogenized dosed serum and brain samples. The calibration, QC and dosed samples were thawed to room temperature
and taken through the sample preparation steps described below. Single (used for diluting
dosed serum samples) and double (used in the specificity studies) blanks were prepared
by adding 10 µL of acetonitrile into 200 µL commercial mouse serum. Then 10 µL of IS
working solution was spiked into each of the 200 µL serum standards/blanks/samples,
except for the double blank, in which 10 µL of acetonitrile was added. Following a 20
seconds vortex, 800 µL of acetonitrile was added into each of the above prepared
standards/samples/blanks then sonicated for 10 minutes at room temperature. After
sonication, all of the samples were centrifuged at 13,000 g for 20 minutes. The
supernatants were transferred into HPLC auto sampler vials for LC-MS/MS analysis.

5.2.7. Dilution of dosed serum and brain samples

A series of dilutions were done (10-fold or 100-fold) using commercial serum in
order to ensure that the measured values were within the demonstrated linear range of the
calibration standards. QC standards (2.5, 15 and 80 ng/mL) were run with the diluted
dosed serum and brain samples to confirm acceptable performance of the method.

5.2.8. Pharmacokinetics

Both serum and brain concentrations were measured by plotted using Prism 5
(GraphPad). Area under serum or brain concentration-time curve (AUC) (from zero to
infinite) was calculated. The elimination half-life (t 1/2) was calculated by the equation:
t ½ = 0.693 / λ, where λ was estimated from the slope of the serum or brain concentration
versus time curve. The clearance (CL) was determined from Dose/AUC. Peak serum
concentration (Cmax) following after an intraperitonial administration. Analyses of both
serum and brain samples were performed at least three to seven mice and expressed as the mean of the measured concentrations of NL-1 at 0, 1/2, 1, 2, 4, 6, 24 h time points.

5.3. Results and discussion

5.3.1. Analytical part of NL-1

LC-MS/MS analysis was done using multiple reaction monitoring (MRM) of m/z 334 → 263 for NL-1 and 250 → 179 for IS was done MRM chromatograms of NL-1 & IS in blank, serum and brain matrix are shown separately in Figure 2. As determined in a previously (ref) the method had a linear range of at least 1-100 ng/mL in serum, the intra-assay and inter-assay precisions (%CV) were less than 4% and accuracy error ranged from -3% to 10%, and the absolute extraction recovery for NL-1 96 -115%.

5.3.2. Pharmacokinetics of NL-1 following an IP injection

The pharmacokinetic profile of NL-1 in its serum and brain concentration – time curves following a 10 mg/kg of NL-1 IP administration as shown in Figure 3. Analyses of both serum and brain samples were performed using at least three to seven lots of mice and expressed as mean of the measured concentrations of NL-1 at 0, 1/2, 1, 2, 4, 6, 24 h time points can be seen in Table 1. NL-1 has shown maximum serum as well as brain concentrations at 0.5 h and almost eliminated out of the system within 6 h with a T1/2 of 0.77 h. The resulted brain Tmax is 0.5 h, Cmax (µg/mL) is 3.15 ± 2.03 and AUC (µg/mL*h) is 0.240 as shown in Table 2.
Figure 5.4. Representative MRM chromatograms of NL-1 (A1, B1, C1) and NL-2 (A2, B2, C2) for blank mouse serum (A1 and A2), dosed mouse serum (B1 and B2), and a dosed mouse brain (C1 and C2).
Figure 5.5. Mouse serum, brain concentration – time curve of NL-1 following an intraperitoneal injection (10 mg/kg)
The objective of the current study is to identify the key PK parameters of NL-1 following administration to mice. The required mice serum and brain samples were obtained following an IP injection of NL-1 (10 mg/kg) and were analyzed by a newly developed and validated LC-MS/MS method in MRM mode along with QC standards. The mean plasma concentration versus time profile of NL-1 is depicted in Figure 5.5, and the mean concentrations of dosed serum, brain samples are summarized in Table 5.1.
Table 5.1. Average serum and brain concentrations of NL-1 (ng/mL) at seven different time points (n = 3-7).

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>NL-1 serum peak conc. (ng/mL) Mean ± SD</th>
<th>NL-1 brain peak conc. (ng/mL) Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Not measured</td>
<td>Not measured</td>
</tr>
<tr>
<td>1/2</td>
<td>4289.00±1942.93</td>
<td>3154.44±2029.48</td>
</tr>
<tr>
<td>1</td>
<td>3523.67±1325.22</td>
<td>2013.67±1044.70</td>
</tr>
<tr>
<td>4</td>
<td>1643.29±1064.44</td>
<td>695.00±266.33</td>
</tr>
<tr>
<td>6</td>
<td>223.13±129.86</td>
<td>386.67±486.64</td>
</tr>
<tr>
<td>12</td>
<td>131.87±209.73</td>
<td>82.15±118.61</td>
</tr>
<tr>
<td>24</td>
<td>1.49±1.62</td>
<td>1.34±2.84</td>
</tr>
</tbody>
</table>
NL-1 is rapidly absorbed after the injection and reached the peak serum concentration (Cmax) at approximately 3.15 ± 2.03 h, but eliminated relatively in a good time frame from the serum with a elimination half-time (T1/2) of 0.77 h, where Tmax is 0.5 h. The Cmax, T1/2 give a good understanding of the analyte physiological state in the rodent body. All the summarized results can be seen in Table 5.2.

According to the results one can predict the administration of analyte in large doses would not be potent or overdose since the analyte eliminates the body in a good time frame. And, since the T1/2 is relatively lower, one can administrate more number of doses as a daily intake dosage. Since the analyte is rapidly absorbed into the serum, the concentration of NL-1 was reaching to a significant level with a Cmax of 3.15 ± 2.03 ng/mL in first 30 mins and the corresponding mean area under curves (AUC) in serum was 0.240 ng h mL⁻¹.
Table 5.2. Key pharmacokinetic parameters obtained after 10mg/kg NL-1 intraperitoneal injection

<table>
<thead>
<tr>
<th>PK Parameter</th>
<th>Values (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$T_{\frac{1}{2}}$ (h)</td>
<td>0.77</td>
</tr>
<tr>
<td>$T_{\text{max}}$ (h)</td>
<td>0.5</td>
</tr>
<tr>
<td>$C_{\text{max}}$ (ng mL$^{-1}$)</td>
<td>3.15 ± 2.03</td>
</tr>
<tr>
<td>AUC (µg/mL*h)</td>
<td>0.240</td>
</tr>
</tbody>
</table>
The key pharmacokinetic parameters indicate a good serum exposure of NL-1. However, the same analyte has shown little lower exposure to brain tissue when compared with serum tissue using the same method. There might be a number of factors leading to the lower absorption into brain tissue. The complexity of the brain structure or the enzymes and hydrophobic neurochemicals in brain may have combined with the analyte and metabolite it into other components that may be readily absorbed and excreted from the brain cells. Accordingly, a more extensive metabolite studies should be performed as a part of future studies in order to investigate the absorption of analyte into the brain.

5.4. Conclusion

The objective of the study was to evaluate pharmacokinetics and bioavailability of a novel TZD ligand NL-1 in dosed mice serum and brain. A validated LC-MS/MS technique for the determination of NL-1 in mouse was developed and successfully applied in this pharmacokinetic (PK) study. The results shown that analyte has very good serum and brain bioavailability. At mean serum and brain concentrations at different time points have shown that the analyte reaches to target sites very quickly and offsets the physiological body in about 6 hours and eliminates out of the system within 24 hours. Extensive metabolic studies have to be performed in order to study the ADME pathways of the analyte.
5.5. References


CHAPTER VI

FUTURE DIRECTIONS

Gradient chromatofocusing (GCF) is a technique developed using linear pH gradients on weak-anion exchange HPLC columns. GCF technique greatly extends the capabilities of chromatofocusing by overcoming the shortcomings of the conventional chromatofocusing technique. The current work demonstrated the superiority of GCF technique over reversed-phase (RP) HPLC in protein resolution (R), peak width $w_{0.5}$, and separation factor ($\alpha$). All RP protein peaks were eluted in a narrower region of the gradient compared to GCF, in which the proteins peaks were distributed along the entire length of the gradient. Regarding peak widths, some had narrower widths with RP, others with GCF. Overall, GCF has shown some potential advantages when compared with RP technique.

As a future direction, the developed GCF technique will be applied to study serum and plasma tissue samples. The resolution of the live tissue proteins from the serum, plasma will help identifying proteins using GCF that are not possible to identify using RP chromatographic technique only.
Chapter 2 demonstrates a new procedure for characterizing weak anion-exchange (WAX) HPLC columns. Conventional characterization methods were done by elemental or chemical determination. And these are either time consuming or involvement of complex mathematical equations. This current work demonstrates a novel procedure to characterize using simple, in-expensive buffers.

The results of the frontal analysis and standard analyte injections gave ion-exchange ligand coverage on the PEI-WAX PolyLC column as 127 µmol/g that is within manufacturer state range (manufacturer stated range: 80 – 125 µmol/g). Peak figures-of-merit results of the 100 µM NaNO₃ standard injections were compared to that of injections of toluene recommended by the manufacturer (20 mM toluene) has revealed that the peak width increases by 50% for the NaNO₃ while the increase in toluene peak width is 30%. And, the shift in the retention time of 100 µM NaNO₃ from 3.17 mins to 1.52 mins beginning and after extensive use respectively has shown that 52% of function ion-exchange sites has been lost after an extensive usage.

All the experiments in this chapter were designed and performed using two different anions (formate and nitrate). In order to obtain more accurate results the same experimental conditions should be tested using more number of ions. Especially, the number of binding sites in column should not be affected while changing the anions including, citrate, sulfate, oxalate, acetate, chloride etc. And, more experiments should be performed to identify the selectivity coefficients between the ions.

Chapter 3 discusses about the neuroprotective agents and their bioanalytical applications. This chapter also discussed the clinical failures of the neuroprotective
agents due to their issues with penetration through blood-brain-barrier and the complexity of the brain structure. Further more, role of the LC-MS (MRM) in bioanalytical applications in help achieving high sensitivity and reproducibility in developing bioanalytical methods. Recent advances in MRM mode have made it possible to bring metabolomic profiling into quantitative metabolomics that permits precise measurements of comprehensive small-molecule pharmacokinetic (PK) and metabolic profiles.

Chapter 4 and 5 demonstrate developing a quantitative LC-MS/MS (MRM) method for determination of thiazolidinedione (TZD) mitoNEET ligand NL-1 in mouse serum and its pharmacokinetic application. This method has been applied to study key PK parameters of the parent analyte NL-1 in mouse serum. The results of PK studies of NL-1 in its serum and brain concentration – time have shown promise of NL-1 at seven different (0,1/2,1,2,4,6,24 h) time points. More extensive metabolite studies should be performed as a part of future studies in order to investigate the ADME fate of the analyte and further human application.
APPENDICES

Appendix 1

Sample sequencing order of NL-1 (including dosed mice serum and brain) while performing LC-MS/MS analysis for 24h, 6h, 4h samples in two different lots (lot 1 & 2).

CS = calibration standards, DB = double blank, QC = quality control, BR = brain tissue, SR = serum tissue, ND = no dilution, 10X = 10 times dilution of ND.

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Appendix 2

List of the protein and respective molecular weights considered for study

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