Capillary HPLC Separation of Selected Neuropeptides

by

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Neuropeptides play a pivotal role in brain and peripheral nervous system function. As high performance liquid chromatography (HPLC) becomes the central tool in the separation and characterization of peptide and protein samples, its selectivity optimization has attracted increasing attention. This research program aims to develop useful, quantitative analysis methods for neuropeptides and their hydrolysis fragments by capillary HPLC. Related peptide pairs are successfully separated, such as leu-enkephalin and [Des-Tyr1] leu-enkephalin, dynorphin A and dynorphin B, galanin and its fragment Gal1-16. The hydrolysis of leu-enkephalin to [Des-Tyr1] leu-enkephalin by organotypic hippocampal slice cultures (OHSCs) can be monitored by the same HPLC system. The separation of seven hippocampal neuropeptides with similar hydrophobicity, Bj-PRO-5a, [Des-Tyr1] leu-enkephalin, leu-enkephalin, pentagastrin, Antho-RW-amide I, dynorphin A 1-6 and angiotensin II, is accomplished by thermally tuned tandem capillary columns (T^3C). The chromatographic selectivity is continuously, systematically and significantly optimized by individual adjustment of each column's temperature. The T^3C concept is applied for the first time with capillary columns, which is an important step towards optimization of selectivity for separations of small samples by liquid chromatography.

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PREFACE

First of all, I would like to thank Dr. Weber for his very helpful guidance and generous support. His passion about chemistry and dedication to scientific research has been a tremendous inspiration for me as well as many others. He is always willing to provide valuable advices for science as well as life. I feel blessed to have him as my advisor and mentor.

I also want to thank the past and present members of Weber group. Xiaomi Xu and Manyan Wang introduced me to the bioanalytical chemistry world. I learned a lot of things from them. Yansheng Liu and Jing zhang taught me the basic experiment steps when I just joined the peptide project. Their patience helped me get used to the new research quickly.

For the rest of the group members, Dujuan, Juanfang, Amy, Si, OU, Hong, Yanhong, Ling, Yifat and Amir, I feel proud to work with them. On the way to challenge the unknowns, I work in a team but not by myself.

Finally, I want to thank cordially my parents for their persistent care and encouragement, no matter where I am, how I am and what I am. I have been missing them so much!

1.0 INTRODUCTION

Neuropeptides play pivotal roles in brain and peripheral nervous system functions[1-17]. The sphere of influence of peptides is mainly determined by corresponding neuropeptidases, which inactivate, and sometimes activate, peptides by hydrolysis of amide bonds[18-21]. My work focuses on neuroprotective and anti-inflammatory species such as dynorphins, enkephalins and galanin. Dynorphins are opioid peptides.[22] Enkephalins are involved in the inflammatory response regulation.[22,23] Galanin (Gal, a 29-amino acid peptide for rat) is implicated in a number of physiological studies including central cardiovascular regulation, epilepsy treatment and feeding[8,24]. Galanin is also known to have neuroprotective properties under stroke-like conditions[25-28]. It can be enzymatically hydrolyzed to fragments[29,30] such as Gal 1-14, 1-16, 1-19, 17-30, 2-11, 26-30, 1-29, etc., some of which prove to be physiologically active[31-33].

This project involves quantitatively measuring the enzymatic activities of ectopeptidases in the hydrolysis of neuropeptides. The amount of peptides and their fragments are determined in the extracellular fluid of hippocampal tissue cultures.[34,35] My immediate goal is to develop a useful, quantitative analysis method for neuropeptides and their fragments by capillary HPLC. The ultimate goal is integrating our results to find out the peptides' degradation rate in key regions of the hippocampus, and to determine if this rate changes under oxygen/glucose deprivation.

Over the past 10 years, HPLC have gained rapid advances and became the central tool in the characterization of peptides and proteins[36]. Its most significant feature is providing excellent resolution[37]. Reversed-phase liquid chromatography (RPLC) and ion-exchange chromatography (IEC) are the most commonly used HPLC modes for peptide separation. These complementary separation techniques offer synergistic capabilities and can be used together to improve separation efficiency.

For the samples that have limited availability, packed capillary columns are preferred. Capillary HPLC has the inherent capability to concentrate very dilute aqueous sample solutions due to its pre-focusing effect. Samples can be loaded from a very large volume of a predominantly aqueous solution and then be eluted as highly concentrated bands by a higher percentage organic solvent. Capillary HPLC columns with inner diameters (ID) between 0.1 and 0.5 mm are routinely used in laboratories and their construction is easy and inexpensive[38]. Moreover, capillary column is especially suitable for high temperature and temperature programming chromatography due to its low heat capacity resulting from very small column ID. Little radial temperature gradient along with very small radial retention and viscosity gradients eliminate the cause of band broadening and splitting[39]. Capillary columns are extremely suitable for trace analysis of expensive biomolecules (neuropeptides in our case) under high temperature.

In the research below, neuropeptide couples with similar structures such as leu-enkephalin and [Des-Tyr1] leu-enkephalin, dynorphin A (DynA) and dynorphin B (DynB), Gal and its fragment Gal1-16 are successfully separated on capillary columns with electrochemical detection. The hydrolysis of leu-enkephalin to [Des-Tyr1] leu-enkephalin by OHSC is also monitored by capillary HPLC system. Then the separation seven hippocampal neuropeptides with similar hydrophobicity, Bj-PRO-5a (pEKWAP), [Des-Tyr1] leu-enkephalin (GGFL), leu-

enkephalin (YGGFL), pentagastrin (βAWMDF), Antho-RW-amide I (pESLRW), dynorphin A 1-6 (YGGFLR) and angiotensin II (DRVYIHPF), are accomplished by thermally tuned tandem capillary columns. "Thermally tuned tandem column (T^3C)" is a novel approach to optimizing chromatographic selectivity by continuous adjustment of the stationary phase[40]. The basic idea is combining two different stationary phases serially and then the analytes retention is tuned by individual temperature programming for each column. Tandem techniques such as LC-LC, LC-MS/MS and GC-MS manifest great advantages and have been widely used for separation and identification. Invented in the year 2000, the potential application of thermally tuned tandem column is being gradually recognized. In this work, T^3C concept is applied for the first time on capillary column, which we believe is an important step towards delicate temperature manipulation in LC. There is very few published work for this. More detailed illustration will be included in the background section.

2.0 BACKGROUND

2.1 PEPTIDE RETENTION MECHANISM

Understanding the peptide retention mechanism is important in conducting peptide separation. RPLC and IEC are premier separation techniques for peptides and proteins. RPLC depends on hydrophobic binding between the solutes and column surface, while IEC relies on electronic interaction[41]. Basic residues (arginine, histidine and lysine) are positively charged under normal chromatographic conditions for biomolecules (pH $\leq$ 3) and acidic residues (aspartic acid and glutamic acid) are deprotonated when the mobile phase pH is higher than their pK_a values.

On RPLC, partition is the primary retention mechanism that involves all the amino acid (aa) residues for small peptide molecules (up to 20 residues). While for polypeptides and proteins, their retention follows an adsorption/desorption model as described in reference[42]. Only a part of the molecule is adsorbed on the stationary phase and retention is determined to be a function of the number (Z) of solvent molecules required to desorb the solute[43]. Thus for polypeptide/protein separation, a narrow window of organic modifier concentration is observed[42], within which desorption takes place.

Two major factors contribute to good selectivity of RPLC. One is the high precision of the B% (the concentration of organic modifier) required for desorption. The other is the strong

hydrophobicity dependence of the solute-column binding affinity. Good separations are often achieved by gradient RPLC[44,45]. However, the critical concentration may change with temperature, pH and matrix composition. The initial steps are sometimes long term hit-or-miss trials which are unpreferable especially for limited sample amount. Also, the reproducibility of gradient elution tends to be instrument dependent.

Since early 1980s, a number of efforts have been made to the prediction of RPLC peptide retention time on the basis of amino acid sequence and side chain hydrophobicity[46-49]. These studies have gained partial success for peptides containing up to 20 residues. However tertiary structure and conformational factors (especially for those large polypeptide molecules) confine the solute-column interaction to only a portion of the molecule and cause discrepancies in the prediction of most models. In the year of 2004, a series of sequence-specific retention calculator (SSRCalc) algorithms came up on the basis of off-line HPLC-MALDI MS data of thousands of peptides. SSRCalcs take ion-pairing reagent, pI, nearest-neighbor effects, helical structure and stationary phase pore size into consideration[50-54]. They provide sound models for peptide retention prediction and become well accepted and widely applied in both proteomics studies and classical HPLC fields[53].

Both temperature and mobile phase have an effect on RPLC selectivity although sometimes these effects are minimal[55-57]. Software enables automated selectivity optimization based on B% and temperature. On the basis of equation (2) and (3), computer optimization requires only two exploratory experiments, such as Drylab[58]. In equation (2), studies showed that S tends to increase with the increase of *k'*, which indicates the small influence of mobile phase on tuning band spacing[59,60]. The effect of temperature on selectivity demonstrates similar pattern: $\Delta H°$ correlates with the solute retention so that temperature alone has small selectivity-modifying

capability at constant mobile phase composition. But for some ionic solutes or molecules retained by mix-mode mechanism, the effect may be much larger[61].

2.2 HIGH TEMPERATURE SEPARATION

In liquid chromatography, temperature is an essential variable that can affect a series of physical parameters such as solubility, viscosity, diffusivity and vapor pressure which may in turn change retention, selectivity, column efficiency, solute structure, column back pressure and stationary phase properties. That is to say, almost all the critical parameters involved in LC are more or less influenced by temperature[39,62]. More than forty years ago, researchers started to realize the importance of temperature in separation speed and sample throughput[63-66]. A 15-20 fold decrease in analysis time was achieved simply by increasing temperature from 25℃ to 200℃[67]. Viscosity and diffusivity can be changed by temperature to enhance linear velocity thus shorten analysis time. For the HPLC separation of peptides or proteins, elevated temperature has been first advocated with the aim of accelerating analysis and improving column efficiency[67]. However the combination of low pH (< 2) mobile phase with high temperature can lead to a very short life for commonly used alkyl-silica columns[68]. Until recently a so-called "sterically protected" RP material was developed and obtained well-spread application in high temperature peptide and protein separation[69].

The temperature dependence of retention is accurately described by a van't Hoff type equation:

$$\ln k' = \frac{\Delta H°(T)}{RT} + \frac{\Delta S°(T)}{R} + \ln\beta \qquad (1)$$

where k' is the retention factor at a specific condition, $\Delta H°$ and $\Delta S°$ are respectively the standard enthalpy and entropy change for solute transfer, T is the absolute temperature, β is the phase ratio of the column (the volume of stationary phase divided by the volume of mobile phase) and R is the universal gas constant. $\Delta H°$ and $\Delta S°$ can be assumed to be independent of temperature within a narrow temperature range as is usually the case for neutral compounds, so that the van't Hoff equation can be written as:

$$\ln k' = C - \frac{\Delta H°}{R}\frac{1}{T} \qquad (2)$$

which demonstrates the linear correlation between solute retention and temperature. Here C, $\Delta H°$ and R remain constant for a given solute.

The adsorption/desorption model explains high sensitivity of peptide retention to B%, which makes isocratic elution difficult because each solute requires a specific eluent composition for elution. Gradient elution is preferred for RPLC peptide separation especially for polypeptide samples. It has been shown that a change in gradient steepness can be quite effective in changing peak spacing and resolution[70-74]. One of the most useful, however approximate, equations that describe the relationship between retention and eluent composition is given by equation 3:

$$\log k' = \log k'_w - S\phi \qquad (3)$$

where ϕ is the volume fraction of organic modifier in the eluent and k'_w is the extrapolated value of k' when pure water is used as the mobile phase. S is a constant for a given solute.

Then we differentiate Eq. (1) with respect to T and Eq. (3) with respect to ϕ , the ratio of changes required in temperature and in B% to obtain the same effect on retention factor is shown as follows:

$$\frac{dT}{d\phi} = -\frac{SRT^2}{\Delta H°} \qquad (4)$$

Eq. 4 is only valid at a fixed mobile phase composition and temperature where parameters $\Delta H°, \Delta S°, k'_w$ and S are independent of T and ϕ. Research shows that for small analytes such as alkylbenzenes, an approximately 1% increase in ACN concentration is equivalent to an approximately 5℃ increase in temperature. And for large molecules like lysozyme, the temperature increase to attain a same effect as 1% ACN increment varies from 6 to 16℃[75]. Overall, adjustment of temperature and B% tends to impact in a very similar manner to solute retention. Apparently, varying temperature is a more convenient way especially for capillary columns, compared to gradient elution with possible issues such as preparing different mobile phases, non-negligible dwelling time, uneven mixing and pressure fluctuation.

With the trend of miniaturization, narrow-bore (i.d. ≤ 2 mm) and capillary columns are implicated in more and more separation works. Temperature programming becomes a good choice in method development for capillary HPLC simply because of the negligible radial temperature gradient and excellent heat transfer with small column ID[76-79]. Actually, stable solvent gradients are difficult to obtain for miniaturized columns and the dwell volume (The total volume inside the components located between the gradient mixing point and the column inlet) is quite big when columns are small. Under these situations, temperature programming could be more useful than gradient elution.

2.3 THERMALLY TUNED TANDEM COLUMN CONCEPT

The primary goal of any chromatographic method development is to find the condition that provides an acceptable separation. The separation of peaks *i* and *j* is described in terms of

resolution (R_s) which is usually derived to the following format for method-development purpose,

$$R_s = \frac{\sqrt{N}}{4} \times \frac{k'_j}{k'_j+1} \times \frac{\alpha-1}{\alpha} \qquad (5)$$

This equation stresses the dependence of R_s on the number of theoretical plates (N), the retention factor of the late eluted analyte (k'_j), and the selectivity factor ($\alpha = \frac{k'_j}{k'_i}$). Resolution directly reflects how well the species have been separated; it not only relates to the separation of band centers but also takes peak widths into account. The parameters α, *k'* and N are treated as independent variables. Their influence on the resolution is demonstrated in the graph below according to reference[80].

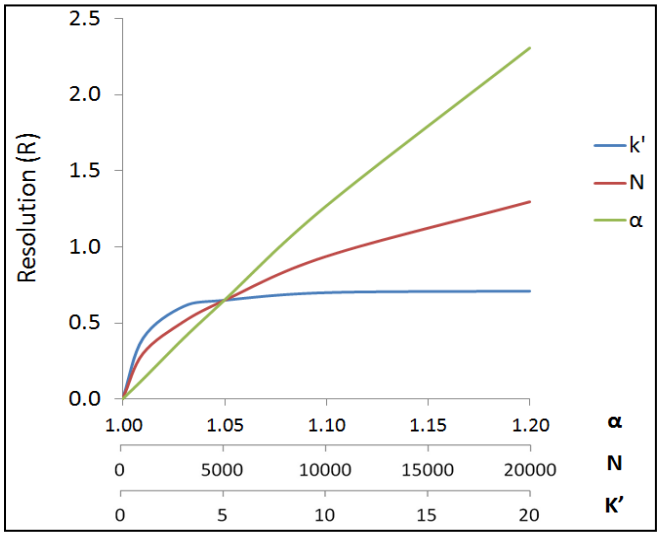


Figure 1. *Plot of resolution vs. selectivity factor, plate number and retention factor based on Eq. (5).*

In Fig. 1, it can be concluded that for low retained solutes (*k'*<5), increasing retention factor has the most evident contribution to resolution, however at the point of *k'*=5 further increase will only lead to very slight changes in R_s. Plate number has the intermediate effect. From the Eq. (5) we know that in order to double the resolution, there must be a four-fold increase in plate number based on the square root relationship. Apparently, selectivity factor is

most powerful in enhancing R_s as shown above, a small increase of α from 1.05 to 1.10 increase R_s by a factor of 2 and there is almost no change to the slope. Therefore, if the sample has a reasonable number of species (<10~15) with considerable retention (t_r is significant larger than t_0), altering selectivity would be the most efficient way to achieve better separation in LC.

Among all the chromatographic variables, such as the type of stationary phase, mobile phase, temperature and pH, mobile phase type/composition and stationary phase type were found to have greatest impact on selectivity[81]. For peptide separation, the interaction between the amino acid residues and the mobile phase is complicated and thus the choice of solvent is limited. Only three are commonly used: acetonitrile, isopropanol and ethanol. Acetonitrile is the most universally utilized organic modifier in RPLC analysis of peptides and proteins, while the use of isopropanol is restricted by its relatively high viscosity and ethanol is often used for process scale purifications. In addition, trial-and-error efforts to find the critical B% are quite time-consuming and strongly experience dependent. Nevertheless, as varieties of novel stationary phases have been synthesized (e.g. coated-polymer phase, polymeric coated silica, carbon based particles and etc.), studies showed that largest changes in selectivity were brought about by varying the stationary phase type[40].

Significant elution order changes are often observed with changing the column type. However, discontinuousness is the major practical issue that makes varying stationary phase the last choice for many chromatographers in method development. Changing stationary phase type means manually replacing columns, rebuilding all the separation conditions and slow equilibrium. Thus a strategy by which selectivity can be tuned continuously, systematically and significantly is strongly desired.

Thermally tuned tandem column (T^3C) is a novel technique introduced by Mao Yun from Dr. Peter Carr's group. This method realized the continuous adjustment of selectivity based on stationary phase type. The principle is schematically shown in Fig. 2. Two columns packed with radically different stationary phases are serially coupled and held in two independent heating zones. In this way the temperature of each column can be individually controlled in order to modify its contribution to the total retention.

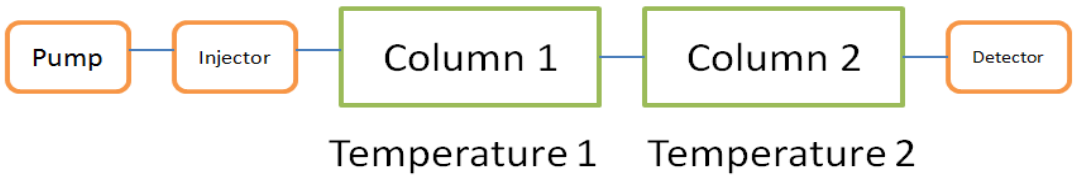


Figure 2. *Block diagram of the thermally tuned tandem column system.*

As mentioned in 2.2, the main effect of raising temperature is accelerating the separation. Enhancing the temperature of a certain column is quite analogues to shortening it, which in turn emphasize the role of another column in the system. The observed selectivity is somewhere between the selectivities of each individual column in series. According to Mao's work, temperature can change the overall selectivity of T^3C dramatically, continuously and conveniently when two radically different phases are coupled. T^3C is considered as an optimal way to optimize selectivity based on both stationary phase type and temperature.

The effect of temperature on selectivity for a single column was discussed in section 2.2. Temperature variation alone is not an effective way to optimize selectivity because its influence on α is much smaller than that on retention time. While for T^3C system, the selectivity change depends on the selectivity difference between the two columns. As for in GC, the retention time for the two columns are additive[40], the selectivity factor of T^3C (α_n) can be written as:

$$\alpha_n = \frac{\theta_1 k'_{1,i}}{k'_{n,i}}\alpha_1 + \frac{\theta_2 k'_{2,i}}{k'_{n,i}}\alpha_2 \qquad (6)$$

where the first subscript refers to the column 1, 2 or the net T^3C system and the second subscript denotes the solute *i* or *j*, $\alpha_n = \frac{k'_{n,j}}{k'_{n,i}}$, $t_{n,0} = t_{1,0} + t_{2,0}$, and θ_1 and θ_2 are the dead time fractions of the 1st column ($\frac{t_{1,0}}{t_{n,0}}$) and 2nd column ($\frac{t_{2,0}}{t_{n,0}}$), respectively. It can be further simplified to

$$\alpha_n = f_{1,i}\alpha_1 + f_{2,i}\alpha_2 \qquad (7)$$

if we define $f_{1,i} \equiv \frac{\theta_1 k'_{1,i}}{k'_{n,i}}$ and $f_{2,i} \equiv \frac{\theta_2 k'_{2,i}}{k'_{n,i}}$ then $f_{1,i} + f_{2,i} = 1$ and Eq. (7) can be rewritten as follows:

$$\alpha_n = f_{1,i}(\alpha_1 - \alpha_2) + \alpha_2 \qquad (8)$$

Selectivity factor on single column (α_1 and α_2) can be assumed to be independent of temperature. The enthalpy changes ($\Delta H°$) for closely retained solutes have very similar values due to similar retention mechanisms, so that same trend of their *k*' vs. termperature results in little selectivity change with temperature. Thereby the change of overall selectivity $\Delta\alpha_n$ under two different temperatures is:

$$\Delta\alpha_n = \alpha_n(T_2) - \alpha_n(T_1) = \left(f_{1,i}(T_2) - f_{1,i}(T_1)\right) * (\alpha_1 - \alpha_2) = \Delta f_{1,i}\Delta\alpha \qquad (9)$$

Eq. (9) clearly shows that in T^3C the selectivity change by varying the system temperature depends on the range of $f_{1,i}$ (the fraction of t_r of compound *i* on column 1) and the selectivity difference between the two tandem columns. Actually, the direct correlation between the total selectivity change $\Delta\alpha_n$ and the stationary phase difference $\Delta\alpha$ indicates the similarity of this method to traditional replacing columns of different packing phases. Moreover, the ability of tuning α in a continuous manner tends to be the major advantage and the most significant practical meaning of T^3C technique. Also Eq. (9) suggests that maximally different selectivities of the two columns are required in order to achieve significant $\Delta\alpha_n$ by varying temperature.

People use the so called 'κ-κ plot' to quantitatively compare the selectivity between phases[82,83]. 'κ-κ plot' is defined as a plot of logarithmic retention factors of different solutes measured on two columns in the same eluent, in which a good linear relationship means same or similar retention mechanism while a poor correlation implies different selectivities. Further explanation and the κ-κ plots obtained is included in section 4.2.2.

The advantage of T^3C system is easy to be summarized: It provides great ability to tune the system selectivity based on different retention mechanisms of the two columns; temperature control is convenient and fast, especially when dealing with capillary column series; compared to gradient elution, temperature control gives better reproducibility and is suitable for routine analysis of similar samples; the selectivity optimization is continuous; only a few exploratory experiments are needed then operation condition can be rapidly located by computer program, this will be further illustrated later. As long as the selected packing materials and the sample to be analyzed are thermally stable under the operating temperatures, T^3C is a good choice for selectivity optimization of samples that are difficult to resolve in single phase systems.

With all the advantages above, T^3C system has shown great potential in the separation of structure related analytes. The separation of ten trazine herbicides and twelve urea and carbamate pesticides were accomplished using bonded and carbon-type phases in the year 2000[84]. The separation of nine antihistamines was achieved based on a bonded silica and a polybutadiene-coated zirconia phase[85]. And in the year 2001, ten barbiturates and a group of phenylthiohydantoin-amino acides have been successfully separated on the combination of ODS and C-ZrO_2 phases[86]. Moreover, optimization of temperature and mobile phase on single column was compared with the T^3C approach[40]; the result showed superior tuning capacity of T^3C system that single column optimization can never compete. In this work, seven hippocampal

neuropeptides are resolved on the combination of a ODS and a polymeric-coated silica phase. This is the first time that T^3C concept is applied to capillary column series. A heater that is specific for capillary tandem columns is designed.

3.0 EXPERIMENTAL SECTION

3.1 INSTRUMENTS

All chromatographic experiments were conducted with a Waters 600 E Multisolvent Delivery System (Waters Corporation, MA) equipped with a quaternary pump and a helium degasser. The mobile phase was delivered to a simple tee to split the flow. After splitting, the mobile phase flowed through a VICI injector (Valco Instruments Co., Houston, TX) and then the capillary column at 0.5 μL/min. The capillary columns were packed using upward slurry method as described in reference[87,88] with a final pressure of ~7000psi. The columns were varied in dimensions according to experiment requirements.

Both UV and electrochemical detectors were utilized to monitor the HPLC chromatogram. The UV absorbance detector is a Waters ACQUITY UPLC Tunable UV Detector with a 10nL cell (Waters Corporation, MA). Measurements were made at a wavelength of 214 nm. The signal was collected and converted by a PeakSimple Chromatography Data System (SRI Model 202, SRI Instruments, Inc., Las Vegas, NV). The principle of the electrochemical detection is a rapid complexation reaction between biuret reagent (0.24 M carbonate buffer, 12.0mM disodium tartrate and 2.0 mM copper sulfate, pH 9.76) and peptide backbone. The product copper-peptide complexes are electroactive[89-92] which can be oxidized in a BAS cross-flow cell (assembled with a self-made glassy carbon working electrode block) at an applied

potential of +0.55 V vs. Ag/AgCl. Potential was controlled by a BAS Epsilon potentiostat (W. Lafayette, IN). Biuret reagent was delivered by a Picoplus syringe pump (Harvard Apparatus, Holliston, MA) at 0.3 μL/min. A homemade Y-shape post-column reactor[93,94] was installed after the column with an inlet ID = 50 μm and outlet ID = 75 μm. Both detection methods can provide adequate sensitivity for the sample mixture. Although electrochemical cell is more sensitive[89], the post-column reactor brings about excess ex-column volume and band broadening. Peak height and peak area (PeakSimple version 3.93-32bit, SRI Instruments, Inc.) are proportional to concentration.

Two heaters were installed for the tandem column system. One was a foil-like Kapton heating unit (Minco Products, Inc., Minneapolis, MN) to heat the injection valve as described in reference[87].The other was a sandwich-shape column heater designed specifically for capillary T^3C experiments. The design is quite different from the heating apparatus for commercial size columns. Capillary columns are length adaptable, freestanding and fragile. The column itself can be bended. The heater must be able to hold column couples of diverse dimensions together and independently adjust the temperature for each individual column.

Figure 3 is the block diagram of the instrumental set-up where the structure of the heater is specified. The heater consists of two trapezoidal aluminum blocks with heating pads attached at the bottom. Two complementary trapezoids are capable of heating column couples of different relative lengths, and columns can be bended when necessary. Temperatures are individually controlled by two temperature controllers (Dwyer Instruments, Inc., IN) according to the feedback from the sensors inside. The aluminum heaters and the columns coupled by a connector (Upchurch Scientific Inc., WA) are held inside the blue insulation frame to prevent thermal convection. Glassy cotton was placed between the Al blocks to prevent mutual temperature

interference. Then a cap made of same insulation material covers and fastens all the components together. These heating apparatus can heat the columns up to 100 ℃.

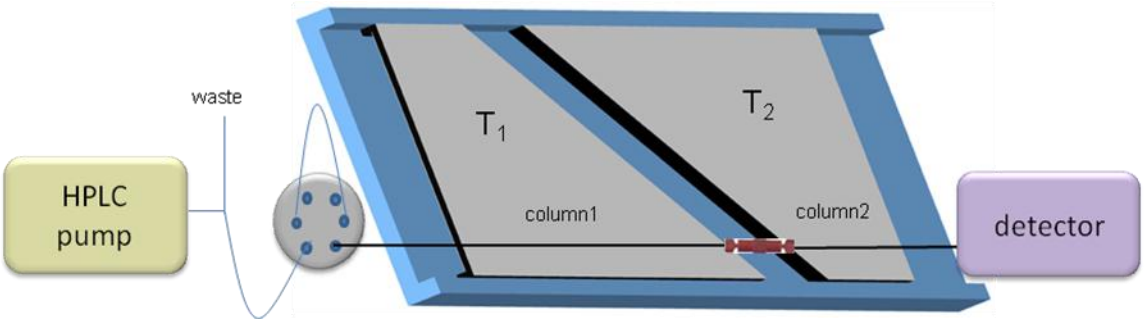


Figure 3. *Diagram of instrumental set-up for capillary T^3C system.*

3.2 CHEMICALS AND MATERIALS

All chemicals used in the experiment were HPLC grade or higher. Source of chemicals are as follows: acetonitrile (ACN), trifluoroacetic acid (TFA), ammonium acetate, dynorphin A (porcine), dynorphin B (porcine), galanin fragment 1-16 (porcine, rat), Bj-PRO-5a (pEKWAP), pentagastrin (βAWMDF), Antho-RW-amide I (pESLRW), and angiotensin II (DRVYIHPF) were from Sigma (St. Louis, MO); Na_2CO_3, $NaHCO_3$ (EM Science, Gibbstown,NJ); copper sulfate pentahydrate, acetic acid (HOAc), 1-propanol (J.T. Baker, Phillipsburg, NJ); leu-enkephalin (YGGFL), [Des-Tyr1] leu-enkephalin (GGFL), dynorphin A 1-6 (YGGFLR), (American Peptide Company Inc., CA); galanin was purchased from Abbiotec, LLC (San Diego, CA). Copper sulfate pentahydrate was recrystallized once from water and disodium tartrate dehydrate (Baker) was recrystallized from diluted NaOH, all the other chemicals were used as received. All aqueous solutions were prepared with purified water from a Millipore Synthesis

A10 system (Millipore Corporation, Billerica, MA). Fused silica capillaries were purchased from Polymicro Technologies, LLC (Phoenix, AZ).

3.3 ANALYTICAL COLUMNS

In the T^3C experiment, a 20 cm × 148 μm i.d. capillary column packed with 2.6 μm XTerra C18 MS particle (Waters, Milford MA) was connected with a 10 cm × 148 μm i.d. capillary column packed with 5 μm HC-COOH particle donated from Dr. Peter Carr's group (University of Minnesota). XTerra C18 MS is a kind of hybrid particle with 1/3 silanols replaced by methyl groups, which could stand high temperature (>80 ℃), large pH range (pH1~12) and provide superior peak shapes. The HC-COOH is a carboxylate functionalized hyper-crosslinked (HC) phase based on silica which is further introduced in 4.2 section.

For the separation of Gal and Gal1-16, DynA and DynB, Leu-enkephalin and [Des-Tyr1] Leu-Enkephalin from OHSCs, columns were packed with 2.6 μm XTerra C18 MS particle using 100 μm i.d. capillary with column lengths in the range of 7~12 cm. Particular lengths are provided in figure legends.

3.4 CHROMATOGRAPHIC CONDITIONS

The injection volume is 0.5-μL with analyte concentrations of 1~25 μM dissolved in ultrapure water from Cayman Chemical Co. (Ann Arbor, MI). The experiments were operated at pressure up to ~4500psi. Mobile phases were varied for different analytes and stationary phases.

The dead time was determined by negative solvent peak for each chromatogram. Retention factors were calculated based on the dead times measured at corresponding temperatures and the values at other temperatures were interpolated from the equation established by known data points. The extra column time (t_{ex}) was determined to be 0.239min by injecting uracil (25mg/L) without column under chromatographic conditions with otherwise were the same. Except gradient elution experiments, the mobile phases were premixed before use. All solutions were passed through Nylon filters (Fisher Scientific, Pittsburgh, PA) with 0.20 μm pores to remove impurity.

4.0 RESULTS AND DISCUSSIONS

4.1 SEPARATIONS OF GAL/ GAL 1-16, DYNA/ DYNB AND YGGFL/ GGFL SAMPLED FROM OHSCS

For polypeptide like Gal which shows obvious adsorption/desorption retention mechanism, its critical mobile phase concentration is difficult to find. Studies show that under gradient elution of commercial RP columns, the retention time for Gal is more than 30 mins.[95,96] Similar t_r is observed for the initial attempts using standard galanin on a 9-cm capillary column (Fig. 4).

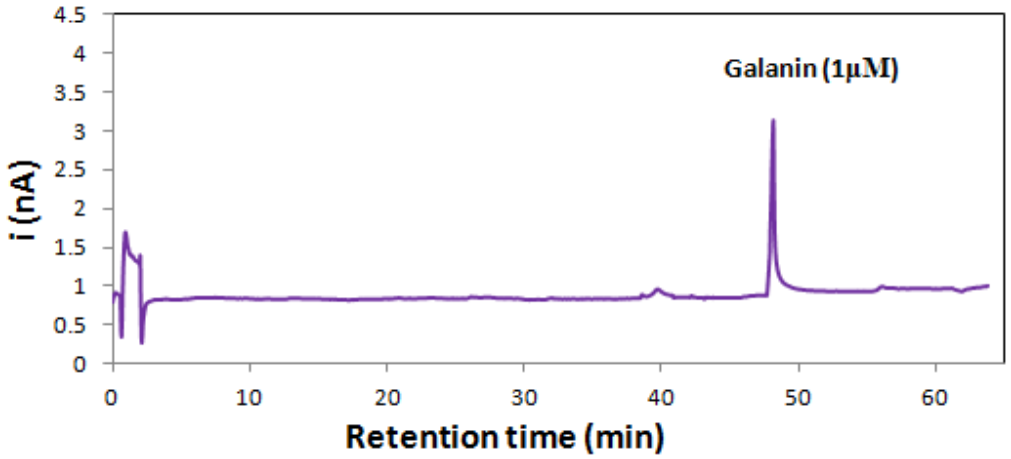


Figure 4. *Gradient elution of Gal (A: 0.1%TFA in H_2O; B: 0.1%TFA in 80%ACN; gradient: 20%B~100%B in 60 mins).*

After trial-and-error experiments, elution time for Gal was shortened within 10 mins by simultaneously reducing the column length and increasing the starting B%. The separation of Gal

and Gal 1-16 was achieved on a 7-cm capillary RP column (Fig. 5). Significant tailing is observed from isocratic elution (C) because of very strong interaction between Gal molecule and column surface, which is always the case for isocratic chromatogram of big peptides and proteins under ambient temperature. Mobile phase gradients definitely benefit the peak shapes. However under gradient elution, the peak spacing is decreased (A and B in Fig.5), which implies reduced peak capacity compared to isocratic condition. It is interesting to note that Gal 1-16 was eluted later than Gal although it has 13 amino acid residues less than Gal. The prediction of the relative hydrophobicity based on SSRCalc gives the same trend (36.89 for Gal1-16, 32.32 for Gal). This indicates that the overall hydrophobicity of a solute molecule can be cancelled out by internal hydrophilic groups and the steric/conformational effects on retention become more evident for bigger molecules.

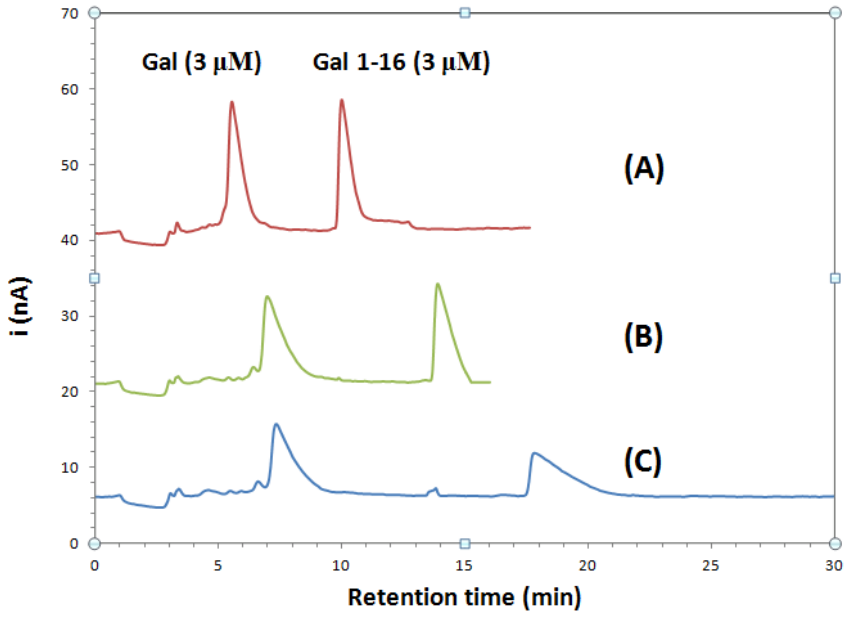


Figure 5. *Comparison of isocratic and gradient elution of Gal and Gal 1-16 fragment on single ODS column. (A): gradient 26%ACN~40%ACN in 20 mins; (B): gradient23%ACN~40%ACN in 20 mins; (C): isocratic 23%ACN. All mobile phases contain 0.1%TFA (v/v).*

DynA/ DynB and YGGFL/ GGFL were separated on the same column (2.6 μm Xterra particle packed, 11.2 cm L) under the same isocratic condition (23% ACN, 0.1% TFA, 3% 1-propanol). All peaks came out within 7 mins (Fig. 6). This is an excellent illustration of the adsorption/desorption model for polypeptides since radical difference in hydrophobicity between these two analyte couples did not necessarily lead to big difference in their retention. The R_s between the enkephalins (R_s=3.4) with only one aa difference is apparently larger than that between the dynorphins (R_s=1.5) with more than 10 aa difference. And the peak shape for enkephanlins are a lot better than dynorphin peaks. All the chromatographic features above suggest that partitioning is the major retention mechanism for small molecules like enhephalins. For polypeptides like dynorphins and galanins, although strongly retained under low B%, their elution is abruptly accelerated once the organic modifier reaches the critical concentration. The band spacing is small because these species interact slightly with the column surface once they were desorbed.

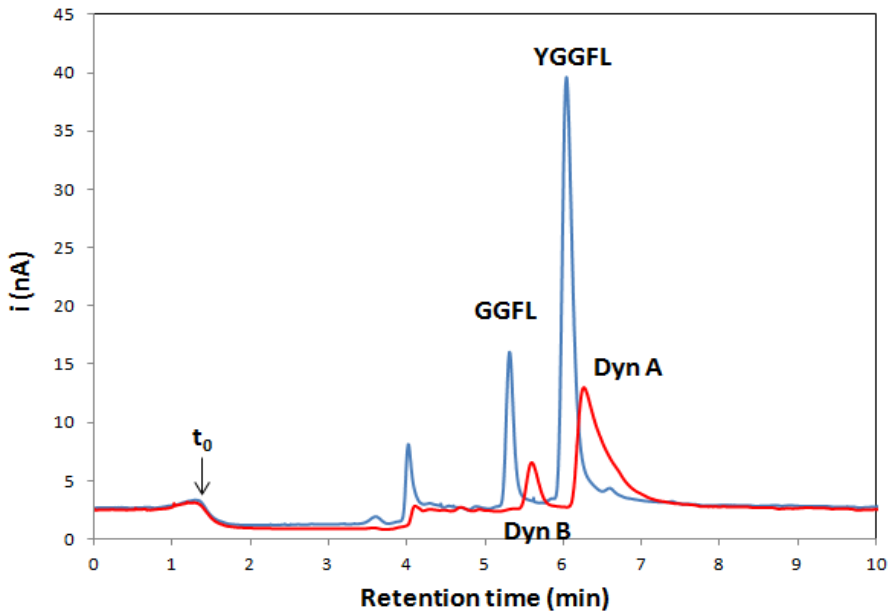


Figure 6. *Separation of DynA/DynB and YGGFL/GGFL under same isocratic condition.*

Samples obtained by electroosmotic sampling from OHSCs were analyzed by the system above with the method as illustrated in reference[34]. The hydrolysis of YGGFL to GGFL by peptidase in the tissue was monitored. The chromatograms of the standard solution and the extracellular fluid sampled are shown in Fig. 7. The sum of peak areas of GGFL and YGGFL is smaller than that of IS, indicating other decomposition processes may happen. The Michaelis-Menten constant (K_m) of the peptidase can be determined by adding different concentrations of YGGFL to OHSCs. The calibration curve of peak height is linear in the concentration range 0~35 μM of YGGFL with coefficient of correlation r^2=0.9989, validating the feasibility of our system for quantitation. YGGFL and $^{D}Y^{D}AG^{D}F^{D}L$ (IS) in standard solution (A in Fig. 7) were diluted to 30 μM before the HPLC analysis.

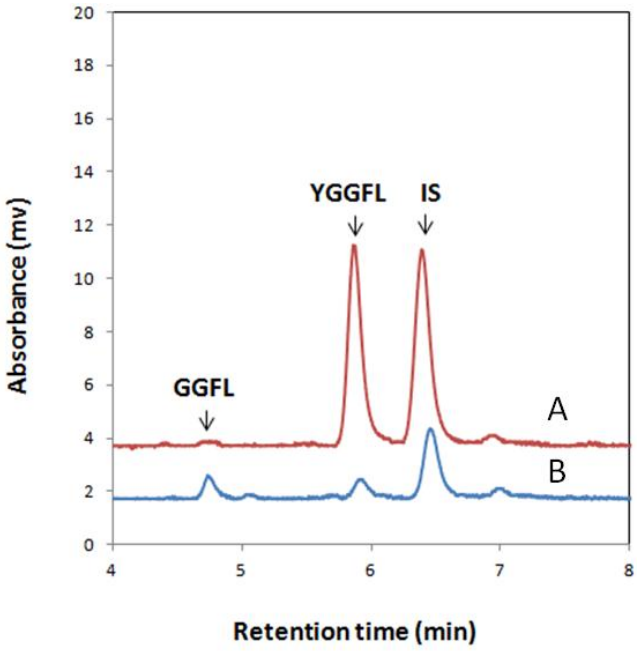


Figure 7. *Chromatograms of standard (A) and extracellular fluid sampled from hippocampus slice culture (B). Mobile phase, 23% ACN, 0.1% TFA, 3% 1-propanol; flow rate 1 μL/min; Column, 100 μm i.d. × 12 cm packed with 2.6 μm Xterra MS C18.*

The above results validate the feasibility of capillary HPLC in peptide separation and quantitation. It is not easy to find one system condition that universally suitable for a broad spectrum of peptides. Even for a simple peptide mixture, laborious trial-and-error can never be avoided. Small peptides favor isocratic elution under which large peptides show severe peak tailing and/or low peak capacity (Fig. 5 and Fig. 6), whereas large polypeptides perform better in gradient elution yet gradient dwell time (~15 min for my system) can be adverse to reproducibility as well as separation speed. A separation system that is able to robustly detect complex peptide samples needs to be discovered or proposed.

4.2 CAPILLARY T^3C SEPARATION OF HIPPOCAMPAL NEUROPEPTIDES

A group of selected neuropeptides (Table. 1): Bj-PRO-5a (pEKWAP), [Des-Tyr1] leu-enkephalin (GGFL), leu-enkephalin (YGGFL), pentagastrin (βAWMDF), Antho-RW-amide I (pESLRW), dynorphin A 1-6 (YGGFLR) and angiotensin II (DRVYIHPF) were proposed to be separated by capillary T^3 columns. These species are distributed in the hippocampus, some of which coexist and sometimes function together[97-99]. Composed of 4~8 amino acids, several of these peptides are similar in hydrophobicity (according to SSRCalc). T^3C technique can be useful because these peptides contain charged residues and terminal groups that further distinguish them from each other.

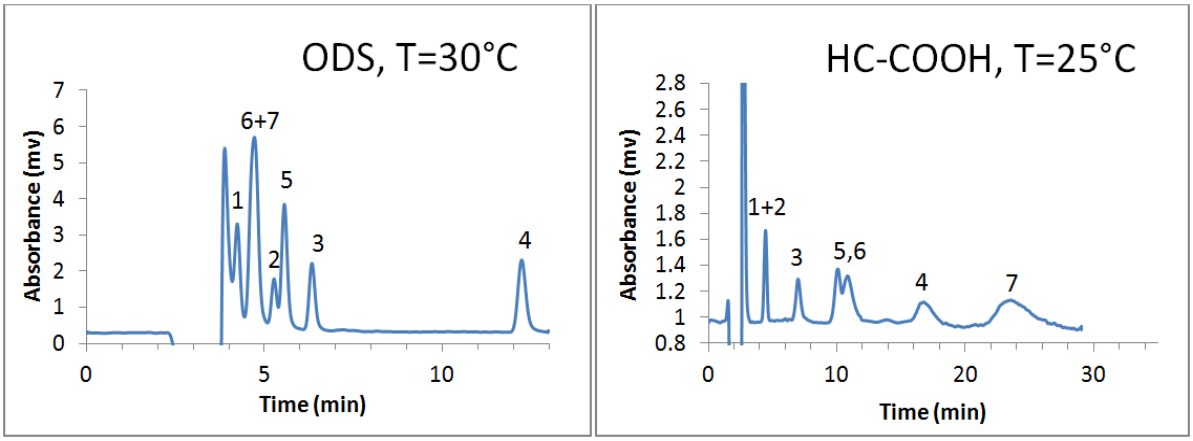
Table 1. Name, amino acid sequence, estimated charge and number denoting each peptide.

	Peptides	Sequences	Estimate charge at pH=3.80

1	Bj-PRO-5a	pE**K**WAP	0
2	[Des-Tyr1] Leu-enkephalin	GGFL	0
3	Leu-enkephalin	YGGFL	0
4	Pentagastrin	βAWM**D**F-NH_2	$+\frac{1}{2}$
5	Antho-RW-amide I	pESL**R**W-NH_2	+1
6	Dynorphin A 1-6	YGGFL**R**	+1
7	Angiotensin II	**DR**VYI**H**PF	$+1\frac{1}{2}$

4.2.1 Initial Separation of Seven Neuropeptides on ODS and HC-COOH Columns

Chromatograms of the sample mixture on an ODS (XTerra C18 MS) column at 30 ℃ and a HC-COOH column at 25 ℃ in 24/76 acetonitrile/15mM CH_3COONH_4 buffer with 0.05%TFA (v/v) (pH=3.80) are shown in Fig. 8. ODS phase is the most widely used aliphatic RPLC material for peptide separation.



A B

Figure 8. *Chromatograms of the seven selected neuropeptides on single ODS column (A) at 30 ℃ and HC-COOH column (B) at 25 ℃, respectively. Mobile phase, 24/76 acetonitrile/15mM CH3COONH4 buffer with 0.05%TFA (v/v), pH=3.80, flow rate 0.5 μL/min. Solutes: 1, Bj-PRO-5a (pEKWAP); 2, [Des-Tyr1] Leu-enkephalin (GGFL); 3, Leu-enkephalin (YGGFL); 4, Pentagastrin (βAWMDF); 5, Antho-RW-amide I (pESLRW); 6, Dynorphin A 1-6 (YGGFLR); 7, Angiotensin II (DRVYIHPF).*

HC-COOH is a mixed-mode RP/WCX (weak cation exchange) stationary phase[100]. It is a silica based particle with carboxylate functionalized polystyrene network coating. The extensive polymer network prevents the loss of bonded phase leading to superior thermal stability (>100 ℃) and some hydrophobicity. Its ion-exchange capability comes from the deprotonated carboxyl groups. Therefore the total retention factor for HC-COOH is written as follows:

$$k' = k'_{RP} + k'_{IEX} = k'_{RP} + \frac{B_{IEX}}{[C^+]_m} \qquad (10)$$

where k'_{RP} and k'_{IEX} are hydrophobic and ion-exchange contribution to retention respectively. $[C^+]_m$ is the concentration of ion displacer in mobile phase and B_{IEX} is a measure of the strength of ion-exchange interaction. The influence of ion displacer on peptide retention is shown in Fig. 9, where good linear correlations between k' and the reciprocal of NH_4^+ concentration are observed with positive intercepts. This validates the existence of both ion-exchange and hydrophobic mechanisms. Increase in $[NH_4^+]$ accelerates the elution of all six species to different extents (pEKWAP is not retained under this condition). The retention of angiotensin II is affected most by $[NH_4^+]$, followed by dynorphin A1-6 and pESLRW. These three were retained most on HC-COOH. Considering the charged residues (K, R and H) of these three peptides and the trends of their k' in Fig. 9, it is not difficult to conclude that cation-exchange was the primary mechanism operating on HC-COOH at pH=4.80. Acidic side chains (D) may weaken the net positive charge but had little effect on local charge density. The retentions of neutral ones are small and slightly changed. The $[NH_4^+]$ in mobile phase is fixed at 15 mM because this gives

good compromise between the *k'* for least retained solute (pEKWAP, *k'*=0.81 on T^3C) and the α for least resolved pair (α=1.18 between pESLRW and YGGFLR when $[NH_4^+]$=15mM as shown in Fig. 9).

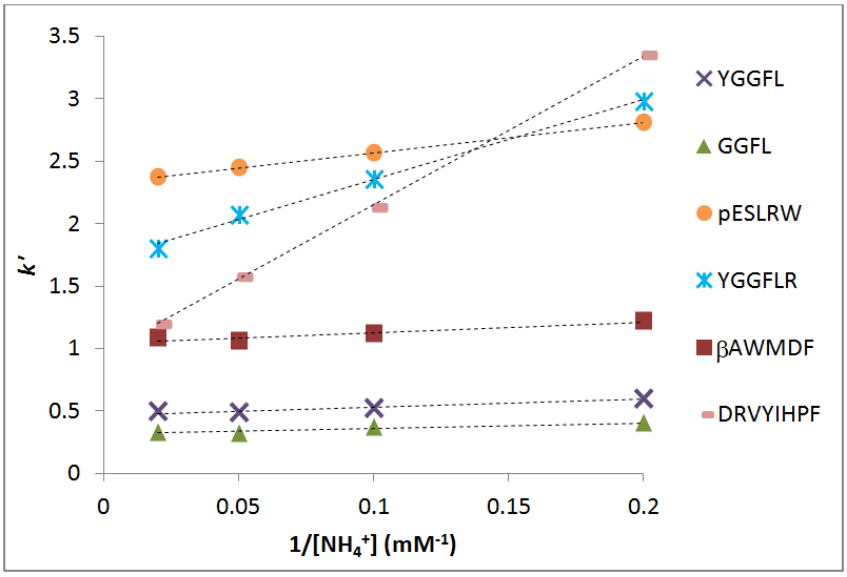


Figure 9. *Correlation between retention factors k' and reciprocal of concentration of NH_4^+ in mobile phase for the six peptides on HC-COOH column. Mobile phase, 40/60 acetonitrile/CH_3COONH_4 buffer with 0.05%TFA (v/v), pH~5.0, flow rate 1 µL/min.*

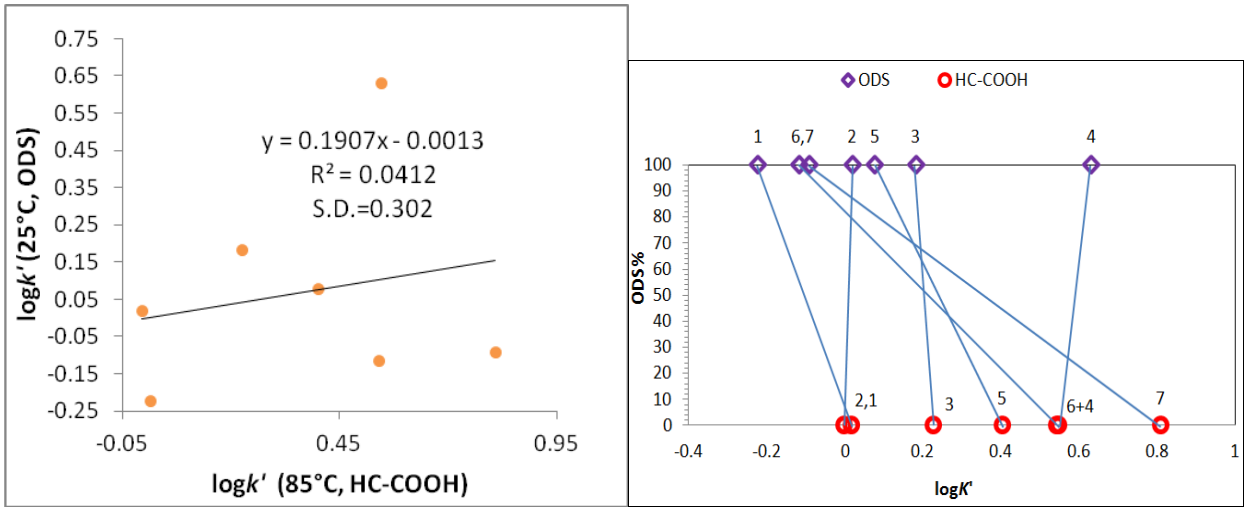
As seen in Fig. 8, neither phase could resolve the peptides under the indicated chromatographic conditions. The chromatogram of the ODS column (see Fig. 8A) shows that although comprised of different number of aa residues, the solute Dynorphin A 1-6 and Angiotensin II (6/7) are completely overlapped. Fairly symmetric and narrow peaks are observed on ODS chromatogram. And most species are much less retained on ODS phase as compared to the HC-COOH phase. Eluted at very early time, the first six peaks huddle together. Solute number 2 and 5 are not baseline resolved. The Bj-PRO-5a peak came out immediately after the injection step which makes quantitative analysis difficult. Furthermore, the big blank chromatogram between peak 3 and 4 is a waste of time and reagent. On HC-COOH column, the

solute pairs Bj-PRO-5a and [Des-Tyr1] Leu-Enkephalin (1/2) coeluted at 25 ℃, Antho-RW-amide I and Dynorphin A 1-6 (5/6) cannot be resolved. The elution order is dramatically different compared to that on ODS phase. The peak widths for late eluting species are more than 2 mins. Significant peak tailing is observed for peptide 7 (angiotenin II), the one that contains most aa residues and highest positive charge among the seven analytes. The wide and tailing peaks were caused by the particular strong electronic interactions between the charged basic groups of the analytes and the HC-COOH surface. Large HC-COOH particle diameter (5 μm) is also adverse to column efficiency resulting in wide peaks. Peak shapes can be significantly improved under elevated temperature on HC-COOH column. Overall, the solutes are more strongly retained on HC-COOH so that higher temperature of HC-COOH column is required in order to ensure *k*' values comparable to those on the ODS column.

4.2.2 Selectivity Comparison Between the ODS and HC-COOH Columns

To quantitatively compare the selectivity differences between ODS and HC-COOH column, a κ-κ plot is constructed based on the retention data in Fig. 11. The log *k*' values of the neuropeptides on ODS at 25 ℃ were plotted against the log *k*' on HC-COOH at 85 ℃ (Fig. 10A). Data from higher temperature on HC-COOH are used in order to ensure all the log *k*' values are comparable. As mentioned in section 2.3, the κ-κ plot compares the retention behaviors on each stationary phase. A good linear relationship means the same or similar retention mechanism operates on each column, while a poor correlation reflects a selectivity difference. Figure 10A clearly shows a minimal correlation of the retention on the two phases. The correlation coefficient is only 0.0412 and the average s.d. is calculated to be 0.302. This confirms our previous observation that these two stationary phases are quite different in terms of selectivity

towards our neuropeptide samples. Peptides were separated by hydrophobicity on ODS whereas largely by ionic binding on HC-COOH. We pick the solute pair dynorphin A 1-6 and leu-enkephalin as an example, dynorphin A1-6 was eluted faster than leu-enkephalin on the ODS column; however it has twice the retention of leu-enkephanlin on the HC-COOH column. Angiotensin II and pESLRW manifest similar retention manner versus neutral species due to cation-exchange interaction with HC-COOH.



A B

Figure 10. *Plot of logk' of the seven analytes on ODS column at 25℃ vs. logk' on HC-COOH column at 85 ℃ (A). Elution order comparison plot of the same series of peptides on the ODS phase and HC-COOH phase (B).*

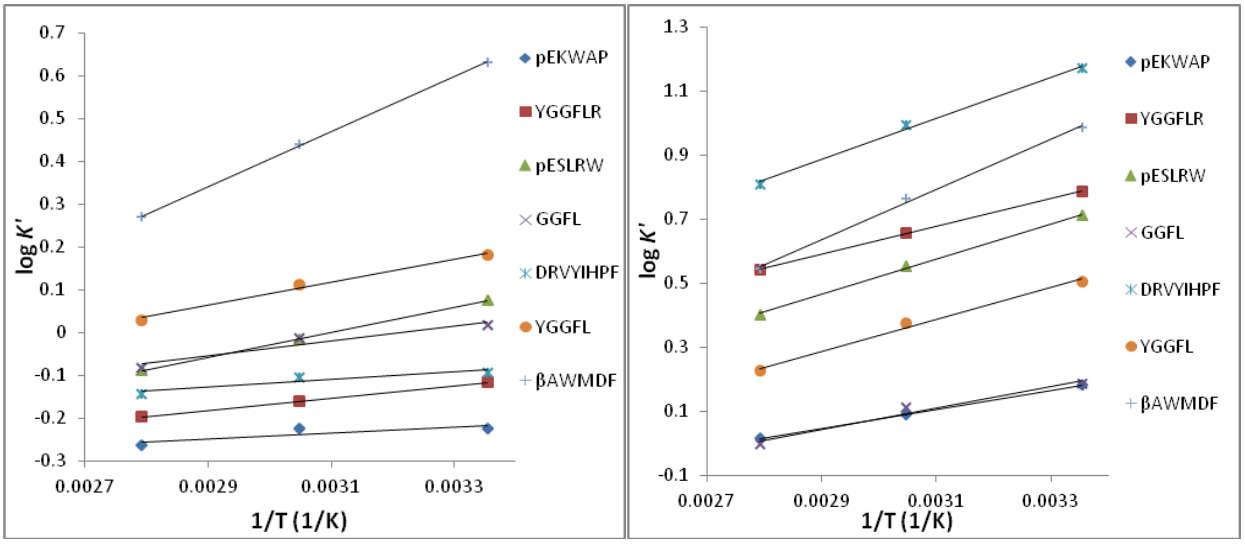
A detailed comparison of the retention order between the two columns is presented in Figure 10B, in which the log*k'* values in Fig. 10A are plotted on the same scale for each phase. The solid lines connect the same species and each crossover indicates a switching of elution order from one phase to another. The *y* axis represents percent retention contribution of ODS to the whole retention in terms of column length in T^3C system ($\text{ODS\%} = \frac{\text{Effective ODS column length}}{T^3\text{Column length}} \times 100\%$, where *$T^3$ column length* represents total retention on T^3C and *Effective ODS column length* refers to the actual extent to which solutes were

retained by ODS phase under corresponding temperature in T^3C.) As mentioned in Section 2.3, heating a column is analogous to shortening it. Single column separation can be treated as the extreme case of the dual column separation. For example, ODS%= 100% in a T^3C system means that the effect of HC-COOH is eliminated by temperature, thus the retention is exactly the same as that on single ODS column. In practical, the T^3C separation must be somewhere between the two extremes. Obviously all the solutes are more retained on HC-COOH. A total of 8 crossovers in Figure 10B indicate that coelution will happen under 8 conditions when selectivity is tuned from one phase to another.

In order to improve the overall separation, the critical pairs (least resolved pair of species) on the two phases must be sufficiently different (6/7 vs. 1/2 and 4/6) and every pair of analytes must be separated on at least one of the two columns. The significant selectivity variance from ODS to HC-COOH satisfies the above requirements, therefore this T^3C bears the potential to resolve every single component. However, the optimal separation condition is difficult to locate by trial-and-error because of the countless T℃ combinations across wide tunable ranges. Systematic optimization is necessary with computer assistance.

4.2.3 Effect of Temperature on *k'* and α for a Single Column

The selectivity of T^3C is optimized by simultaneously adjusting the temperature of each column. In order to predict the T^3C separation, the effect of temperature on solute retention was studied. Experiments were carried out under three temperatures (25 ℃, 55 ℃ and 85 ℃) for each peptide on each individual column. The correlation between peptide retention and temperature is clearly shown in Fig. 11, where the log*k*' of each analyte is plotted against the reciprocal of absolute temperature.



A B

Figure 11. *Effect of temperature on retention. Plot of logk' vs. 1/T on ODS column for the seven peptides (A) and that on HC-COOH column (B). Temperature values on each column refer to article.*

4.2.4 Selectivity Tuning in T^3C

As mentioned above, optimal separation condition for T^3C can be estimated by computer program instead of laborious hit-or-miss experiments. Based on the correlations in Fig. 11, retention time can be calculated at any other temperature. Then the net t_r on T^3C for each peptide at any specific temperature combination (T_1 and T_2) was calculated as

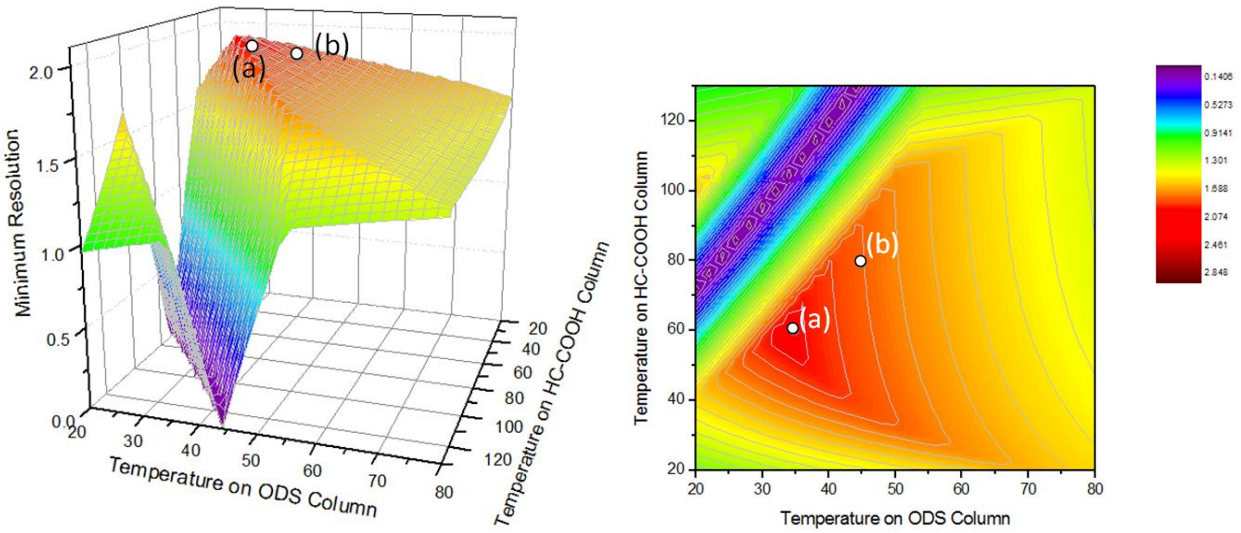
$$t_{n,i} = t_{1,i} + t_{2,i} - t_{ex} \qquad (11)$$

where $t_{n,i}$ is the net t_r on the T^3C column set, $t_{1,i}$ and $t_{2,i}$ are the retention times for solute *i* on the first and second column respectively, t_{ex} is the time that solute spends outside the column (extra column time). The correctness of this equation was validated in reference[40].

Finally the overall R_s of every solute pair on T^3C system was conveniently calculated by the following equation:

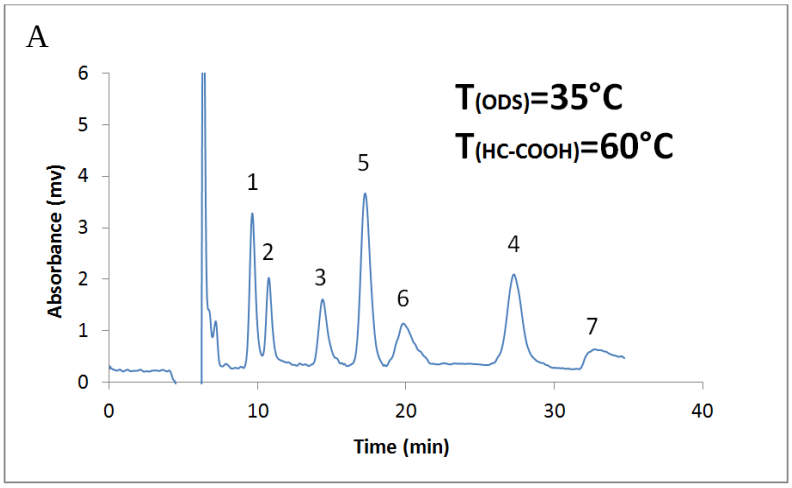
$$R_S = \frac{\sqrt{N}}{4}\frac{t_{n,j}-t_{n,i}}{t_{r,av}} \qquad (12)$$

in which R_s is resolution, $t_{r,av}$ is the simple average of the analyte retention times. N is the theoretical plate number which is assumed to be 3200 for each column and 6400 for the T^3C column set. We plot R_s for the critical pair against temperatures on ODS and HC-COOH columns and yield the two-dimensional window diagram shown in Fig. 12. In figure 12A the highest point corresponds to the best resolution that can be possibly achieved for the least resolved pair (peptide 5 and 6), where the temperature is 60 ℃ on HC-COOH and 32 ℃ on ODS with a predicted R_s of 2.02. Fig. 12B is the contour plot of the same set of data with R_s indicated by gradual color change. The big red and yellow regions (R_s>1.5) of both figures implies that satisfactory separation can be accomplished by many combinations of T_1 and T_2. Moreover, the relative flat roof in Fig. 12A indicates the robustness of the T^3C separation in this region. Therefore even if our estimation is not very accurate, a slight deviation of the operating temperature will not be destructive to the R_s. However when the HC-COOH temperature goes beyond 60 ℃ and the ODS temperature drops below 32 ℃, minimum R_s drops abruptly to the bottom of the "valley" in the graph. So in practice, 60 ℃ on HC-COOH and 35 ℃ on ODS was chosen (indicated by point (a) in Fig. 12) for the T^3C separation of sample mixture with the minimum R_s estimated to be 1.98. Then we obtained the chromatogram shown in Fig 13 (A).



A B

Figure 12. *Plot of resolution for critical pairs vs. temperature of ODS and HC-COOH columns: (A) three-dimensional plot; (B) resolution contour plot. The T^3C experiment was carried out at temperatures indicated by point (a) (b).*



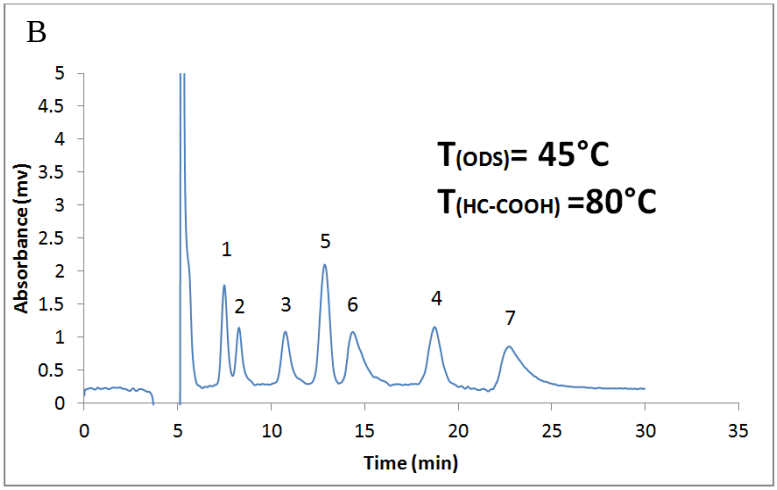
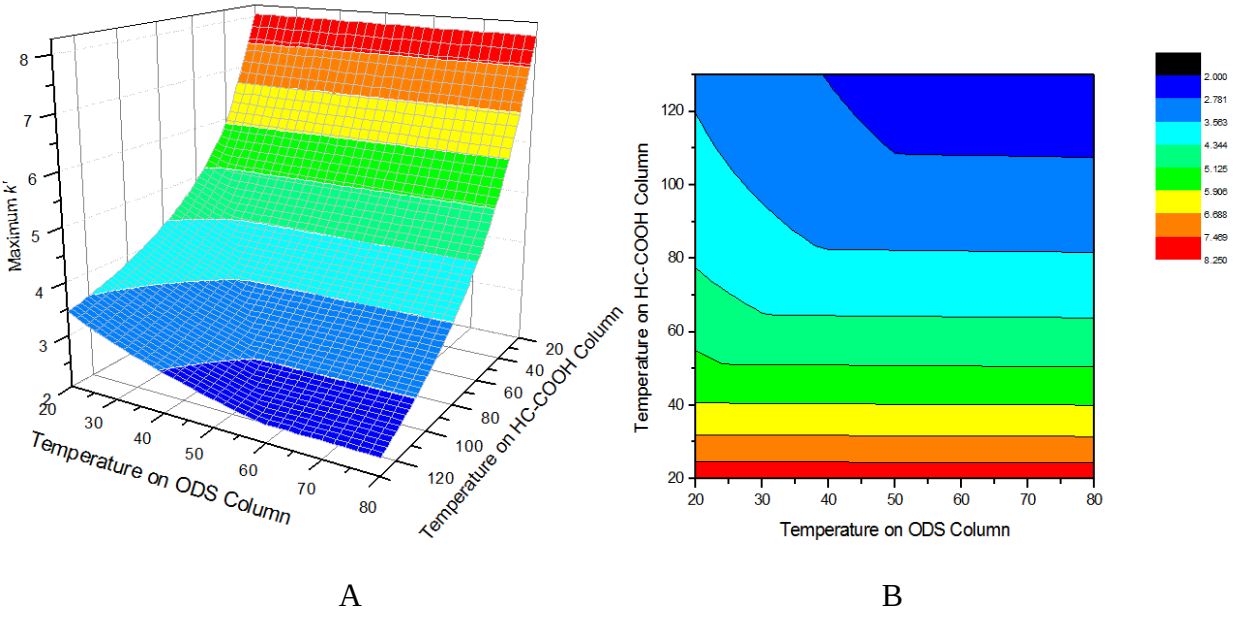


Figure 13. *Chromatogram of the separation of neuropeptide mixture on T^3C with ODS at 35 ℃ and HC-COOH at 60 ℃ (A) ODS at 45 ℃ and HC-COOH at 80 ℃ (B). Mobile phase is same as Fig. 8, flow rate 0.5 μL/min. Solutes: 1, Bj-PRO-5a (pEKWAP); 2, [Des-Tyr1] Leu-Enkephalin (GGFL); 3, Leu-enkephalin (YGGFL); 4, Pentagastrin (βAWMDF); 5, Antho-RW-amide I (pESLRW); 6, Dynorphin A 1-6 (YGGFLR); 7, Angiotensin II (DRVYIHPF).*

Every solute was successfully separated with a minimum R_s of 1.23. Compared to the calculated t_r values based on the model in Fig. 12, the average relative error was below 4% which was comparable to that obtained in T^3C study on basic pharmaceuticals[85]. Considering the solute coelution on single columns (Fig. 8) and the fact that isocratic condition seldom works well on peptide separation due to adsorption/desorption mechanism, this result shows significant improvement of the overall separation for the seven neuropeptides sample mixture. Moreover, hit-or-miss experience was avoided by computer programming that generates window diagram based on only four initial runs (we did three to confirm linear correlation but only two on each phase is necessary).

We further plotted the *k'* values for the most retained solute versus the temperatures on both columns (Fig. 14). It is clearly shown that maximum *k'* decrease significantly with increasing temperature on HC-COOH, while this effect is not that evident on ODS column. Therefore T^3C separation was further operated under temperatures indicated by point (b) in Fig.

12 (80 ℃ on HC-COOH column and 45 ℃ on ODS column) and chromatogram (B) in Fig. 13 was obtained. Total analysis time was shortened to within 25 mins without sacrificing resolution (minimum R_s 1.33). In addition, the peak shapes for solute dynorphin1-6 and angiotensin II were considerably improved at higher temperature. Of course, T^3C total analysis time could also be shortened by enhancing flow rate.



A B

Figure 14. *Plot of maximum k' vs. temperatures on ODS and HC-COOH columns: (A) three-dimensional plot; (B) contour plot.*

In Fig. 13, The R_s for the critical pair is smaller than the predicted value in both chromatogram A and B. The extra column volumes and probable inaccuracy of temperature control in the connection area (from injector to column, between two columns and from column to detector) might be the reasons. Overall, compared with single column separations, T^3C system dramatically improved sample selectivity based on only four initial chromatographic runs without a substantial increase in analysis time. If we had tried to improve the separation on the ODS alone by using a longer column or by lowering the amount of organic modifier, a much longer analysis time would have resulted and the elution order cannot be easily and

systematically optimized. T^3C added another dimension for the selectivity tuning so that peptide separation was largely enhanced.

5.0 CONCLUSION

To summarize, capillary RPLC has been well established as an effective technique for peptide separation. It not only possesses all the features from traditional RPLC that are essential for biomolecule separation, but further downsizes instrumental dimensions to enhance sensitivity and minimize waste. Capillary columns are readily compatible with other HPLC parts (pump, injector, detector and etc.) and have excellent flexibility in column dimensions and experimental conditions (especially temperature). Structure related neuropeptides Gal/Gal1-16, DynA/DynB and YGGFL/GGFL were very well separated on RP capillary columns with both isocratic and gradient elution. (See section 4.1) Due to adsorption/desorption mechanism, it is difficult to find one system condition that universally suitable for a variety of peptide samples.

Based on single capillary column experiments, thermally tuned tandem column (T^3C) concept was introduced for the first time to capillary column separation of peptides. The combination of an ODS phase and a polymeric coated HC-COOH phase operated in the mobile phase buffered by ammonia acetate was proved to be very useful in peptide separation, although neither phase can give adequate resolution. A sample mixture of seven neuropeptides was successfully separated on the T^3C system with only four necessarily initial runs. Instead of hit-or-miss experiments, accurate prediction of retention time and resolution by computer program was realized in T^3C system on the basis of linear correlations obtained from individual column trials. In order to provide enough tunable range, radical different selectivities are required for the

two phases which can be quantitatively measured by κ-κ plots and elution order plots. Stationary phase type has been shown to have largest effect on selectivity. The T^3C system realized adjusting stationary phase continuously via controlling the contribution of each phase by temperature.

In T^3C system, temperature largely determines the overall selectivity without significantly increasing total analysis time, in contrast to very small influence that temperature have on α in single column separation. The final separation by T^3C revealed its powerful potential in selectivity tuning for analytes that are impossible or extremely difficult to be separated. Computer programming saves time and labor while provides acceptable prediction. With the advancement of instrumentation, more complex peptides and protein digests are expected to be separated with the aid of T^3C. Peptide concentration in real sample can be determined quantitatively by peak height or area ratio versus standard. Ion exchange phase imparts one more variable—ion displacer concentration. Future work may include temperature programming, counter ion concentration gradient, pH gradient as well as new phase discovery.

6.0 FUTURE PLANS

As mentioned in section 4.2.4, elevating column pressure, minimizing extra column volume and decreasing HC-COOH particle size can be done to improve separation. Two stationary phases can be packed into one capillary column serially to eliminate dead volume in column connector. This technique was utilized in proteomic study by LC-MS/MS[101-103], which also avoided sudden i.d. change within the union. HC-COOH particle smaller than 5 μm is not commercially available. Fortunately we have its synthesis scheme and optimized synthetic conditions for hyper-crosslinked stationary phase from Dr. Peter Carr's group[104]. The polymeric crosslinking for the 5 μm HC-COOH particle was based on a 5 μm type-B HiChrom silica particle which can be replaced by 1.8 μm Zorbax silica to obtain HC-COOH particles around 1.8 μm. The amount of reagents can be scaled down proportionally to maintain the same effective concentration.

One of the major advantages of capillary system is its rapid response to temperature variation. It has very fast equilibrium and little radial temperature gradient because of excellent heat transfer in small i.d. column. Besides speeding up separation, T^3C emphasizes the effect of temperature on selectivity and column efficiency. As we can see from Knox equation[105], both *B* and *C* terms increase with increasing *k'* where *B* refers to the longitudinal diffusion and *C* relates to mass transfer. This illustrates the effect of temperature with respect to plate height and N. Studies showed that elevating temperature generally benefits column efficiency and in turn peak

shape, especially for macromolecule with low diffusivities[106-108]. In practice, temperature gradient can start at t_r=15min after the elution of the third peak because we don't want to shorten the t_r for the first three species. Also, with the introduction of the IEC phase, pH gradient and ion displacer gradient serve to be alternative ways to assist elution. Simultaneously varying temperature and B% in a T^3C set is a natural extension of the present work. As denoted by Snyder and co-workers[109-115], retention can be expressed as a function of T and B% as follows:

$$\log k' = A + \frac{B}{T} + C\phi + D\phi/T \qquad (14)$$

where T is absolute temperature and ϕ is the same as in Eq (3). Hence the retention can be estimated based on Eq. (14) with only four initial experiments. The dwell volume issue is expected to be solved by predetermining the pump dwell time and beginning the gradient profile at a certain time period beforehand, or using a pump system specifically designed for capillary experiments with significantly small inside volume.

Our ultimate goal is to measure the enzymatic activities of the ectopeptidases in hippocampal tissue quantitatively. The T^3C is anticipated to be used to analyze the relative amount of peptides and their hydrolysis fragments, in order to determine the K_m[40]. Both tryptic digested standard polypeptides and real sample from animal can be examined. When facing complicated samples, peak identification might be an issue. The coupling of the T^3C with MS can be a further direction.

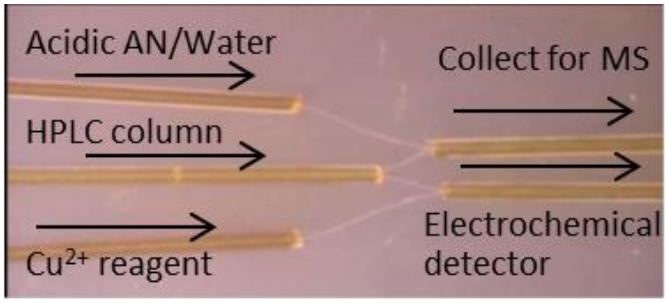


Figure 15. *Splitter with two reagents. HPLC column center left. Flow from left to right.*

I have previously used ESI mass spectrometry to confirm the peak identity. To me, an optimum strategy is to have a quantitative and sensitive detector for measurements on a routine basis with the possibility of fraction collection for offline MS confirmation of peak identity, or determining the identity of an unknown peak. The fraction collection can be achieved by a splitter shown in Fig. 15. Half of the effluent goes to the detector while another half is collected for MS analysis. We hope all the efforts above will allow us to gain insight into the role of peptides and peptidases in maintaining neuronal health. This interest motivates our development and application of new analytical approaches.

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