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Investigation of an on-line two-dimensional chromatographic approach for peptide analysis in plasma by LC–MS–MS

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ABSTRACT

Reversed phase and hydrophilic interaction chromatography (HILIC) were successfully coupled for the on-line extraction and quantitative analysis of peptides by ESI–LC–MS/MS. A total of 11 peptides were utilized to determine the conditions for proper focusing and separation on both dimensions. Minor modifications to the initial organic composition of the first reversed-phase dimension provided options between a comprehensive (generic) or more selective approach for peptide transfer to the second HILIC dimension. Ion-pairing with trifluoroacetic acid (TFA) provided adequate chromatographic retention and peak symmetry for the selected peptides on both C_{18} and HILIC. The resulting signal suppression from TFA was partially recovered by a post-column "TFA fix" using acetic acid yielding improvements in sensitivity. Minimal sample preparation aligned with standard on-line extraction procedures provided highly reproducible and robust results for over 300 sequential matrix injections. Final optimized conditions were successfully employed for the quantitation of peptide PTHrP (1–36) in rat K_3EDTA plasma from 25.0 to 10,000 ng/mL using PTHrP (1–34) as the analog internal standard. This highly orthogonal two-dimensional configuration was found to provide the unique selectivity required to overcome issues with interfering endogenous components and minimize electrospray ionization effects in biological samples.

1. Introduction

Advances in molecular biology and the completion of the human genome project have lead to the elucidation of new molecular targets resulting in opportunities to design new and innovative medicines [1]. Traditional small molecule drug development strategies focused on both rational and empirical design to identify candidates for predefined targets. Although small molecules will continue to provide a significant percentage of new drug entities, the emergence of protein-based therapies is rapidly becoming a key component of contemporary drug development [2]. Emerging macromolecular medicines such as proteins, peptides, oligonucleotides, monoclonal antibodies and many others are becoming a larger proportion of the development portfolios within the pharmaceutical industry. To meet this growing market, pharmaceutical and biotechnology companies are directing development resources to identify and generate reliable and robust analytical techniques.

Increased interest in proteome research for drug targeting and development has driven advancements in technologies for comprehensive proteomic analysis. Enzymatic digestion of complex protein mixtures has routinely been employed to analyze resultant peptide fragments. The separation of peptides in complex mixtures has centered around high performance liquid chromatography (HPLC) and capillary electrophoresis (CE). Reverse phase chromatography (RPC) has traditionally been used for peptide separation with HPLC applications [3,4]. More recently two-dimensional (2D) and multidimensional chromatographic techniques have been employed encompassing HPLC, capillary liquid chromatography (LC) and CE to improve the selectivity for peptide analysis [5,6]. Coupling these and related techniques with mass spectrometric detection increases the speed, sensitivity and reproducibility of the methods for qualitative or quantitative detection [7–10].

Implementing orthogonal (multimodal) chromatographic techniques can potentially reduce the amount of endogenous interfering components found in complex biological mixtures that contribute to selectivity issues and ionization/matrix effects. A comprehensive review by Issaq et al. [11] describes these multimodal strategies for proteome and peptide separation in detail. Peptide separations in two-dimensional liquid chromatography (2D-LC) have been performed both off-line [5–7,12] and on-line [8–10,13,14]. Off-line peptide separation procedures often require rigorous concentration and reconstitution procedures to attain an acceptable sample composition suitable for injection and analysis. Additionally, off-line extractions such as protein precipitation and

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Table 1
Sequences of 11 therapeutic peptides.

Analyte	MW	Sequence[a]	Retention coefficients	
			ODS[b]	Silica-60[b]
Neuromedin B	1132.3	GNLWATGHFM-NH2	6.8	9.4
Neurokinin A	1133.3	HKTDSFVGLM-NH2	5.3	11.7
Adrenocorticotropic hormone fragment 1–10	1299.4	SYSMEHFRWG	7.6	11.0
Ranakinin	1350.6	KPNPERFYGLM-NH2	6.4	13.2
Neurotensin	1672.9	pELYENKPRRPYIL	8.1	14.1
Dynorphin A	2147.5	YGGFLRRIRPKLKWDNQ	11.2	19.27
Galanin	3210.6	GWTLNSAGYLLGPHAIDNHRSFHDKYGLA-NH2	16.1	21.6
Vasoactive intestinal peptide	3325.8	HSDAVFTDNYTRLRKQMAVKKYLNSILN-NH2	12.3	25.6
PTHrP (1–34)	4016.6	AVSEHQLLHDKGKSIQDLRRRFFLHHLIAEIHTA-NH2	19.8	33.2
PTHrP (1–36)	4259.9	AVSEHQLLHDKGKSIQDLRRRFFLHHLIAEIHTAEI-NH2	21.4	32.6
Adrenocorticotropic hormone intact	4541.1	SYSMEHFRWGKPVGKKRRPVKVYPNGAEDESAEAFPLEF	19.1	29.3

[a] Each peptide was dictated by the sequence of amino acids in single-letter code.
[b] Calculated peptide net hydrophilicity (Silica-60) and hydrophobicity (ODS) retention coefficients developed by Yoshida et al. [22] are presented to demonstrate the general range of peptide affinity for a RPC and HILIC system. These coefficients can be useful in evaluating the applicability of a given peptide for RPC–HILIC coupling.

solid phase extraction (SPE) often lead to unacceptable recovery of the peptide due to challenges with maintaining adequate peptide solubility in the high aqueous or high organic conditions typically utilized for elution or reconstitution. The on-line extraction of peptides offers the potential to bypass more complex extraction issues and simplify the overall approach for analysis. Although the utility of an off-line versus on-line peptide separation is application dependent, on-line separations can offer efficiency and productivity advancements through simplified automated sample preparation as well as HPLC system multiplexing. An example of on-line two-dimensional extractions for proteome digests is MudPIT (multidimensional protein identification technology) [13]. This technology couples strong cation exchange (SCX) chromatography with reverse phase chromatography to leverage the advantages of orthogonal chromatography for peptide separation. Alternatives to this strategy have suggested that changing the SCX first dimension with hydrophilic interaction liquid chromatography (HILIC) may offer better peak capacity and improved orthogonality [15].

HILIC, first introduced by Alpert in 1990, is a pseudo-normal phase chromatographic technique [16]. A comprehensive review of HILIC detailing the history, mechanisms and applications is described by Hemstrom and Irgum [17]. In addition, Yoshida has reviewed the application of HILIC for peptide separations [18]. As previously described, recent reports have detailed the application of HILIC in two-dimensional chromatography for peptide separations [15,19–21]. To facilitate on-line extraction of peptides with greater selectivity, more highly orthogonal approaches have been used to attain more robust analytical methods [19–21].

The degree of orthogonality and the order in which the dimensions are used play an important role in the peak capacity and selectivity of the 2D separation. Using HILIC as one of the dimensions in 2D-LC offers an attractive option for peptides due to the increased hydrophilicity the functional groups carry as a result of charge [19]. It has been previously proposed that due to the high organic solvent composition required for HILIC separation, direct coupling of HILIC to RPC could not be obtained [15]. However, published reports reveal that direct HILIC–RPC coupling is possible and offer a suitable complement to other 2D-LC methods for peptide analysis [19,20]. Gilar et al. [19] has shown suitable orthogonality for 2D-LC using SCX–RPC, HILIC–RPC and RPC–RPC. The coupling of RPC in the first dimension with HILIC as the second dimension can provide unique advantages with selectivity while maintaining a highly orthogonal system. To the best of our knowledge, there have been no reports of coupling RPC–HILIC in this specific order.

The goal of this work was to develop an on-line extraction procedure for the quantitative analysis of peptides using a highly orthogonal two-dimensional liquid chromatographic system. Some of the key factors involved in this process included (1) the evaluation of analyte solubility in the injection sample, (2) selection of mobile phase composition for proper focusing and separation on both dimensions, (3) separation of interferences and potential ionization effects, (4) optimization of supplemental organic required for transfer between dimensions and (5) utilization of tools for recovering signal intensity. The feasibility of both comprehensive (generic) and more selective peptide transfer to the second HILIC dimension was also explored to provide greater flexibility and a broader scope of potential applications. Final optimized conditions were successfully employed for the quantitation of peptide PTHrP (1–36) in rat K_3EDTA plasma from 25.0 to 10,000 ng/mL using PTHrP (1–34) as the analog internal standard.

2. Experimental

2.1. Materials and reagents

The initial phase of this project utilized 11 therapeutic peptides spanning a relatively wide range of molecular weight and hydrophilicity/hydrophobicity (Table 1). Peptide PTHrP (1–36) was synthesized by the William Keck Peptide Synthesis Facility at Yale University (New Haven, CT). All other peptides were purchased from Sigma–Aldrich Inc. (St. Louis, MO). HPLC grade acetonitrile, water, trifluoroacetic acid (TFA), and acetic acid were from J.T. Baker Inc. (Philipsburg, NJ). K_3EDTA rat plasma was obtained from BioChemed Services (Winchester, VA) and used for the preparation of calibration standards, quality control (QC) samples and blanks. Polypropylene 96-well plates for performing the initial sample dilution step were from Waters Inc. (Milford, MA).

2.2. HPLC conditions

A schematic of the system set-up is shown in Fig. 1. The two-dimensional LC system was composed of a Leap Technologies HTS PAL autosampler (Carrboro, NC), six HPLC pumps, and two chromatographic columns separated by a Valco VICI EHMA 6-port, 2-position switching valve (VICI Valco Instruments Co., Inc. Houston, TX). Two Shimadzu LC-10AD HPLC pumps (Columbia, MD) delivered the mobile phase to the first dimension on a RPC column (ACE C_{18}; 2.1 mm × 50 mm; 5 μm; 100 Å; MAC-MOD Analytical, Inc., Chadds Ford, PA). Mobile phase for the first dimension was water with 0.02% TFA (mobile phase A) and 95:5 acetonitrile/water (v/v) with 0.02% TFA (mobile phase B). A TFC column (TurboFlow HTLC C_{18}; 0.5 mm × 50 mm; 50 μm; 100 Å; Thermo Scientific) was also investigated as a more traditional extraction column on the first dimension. An Agilent 1100 binary pump (Hewlett-Packard,

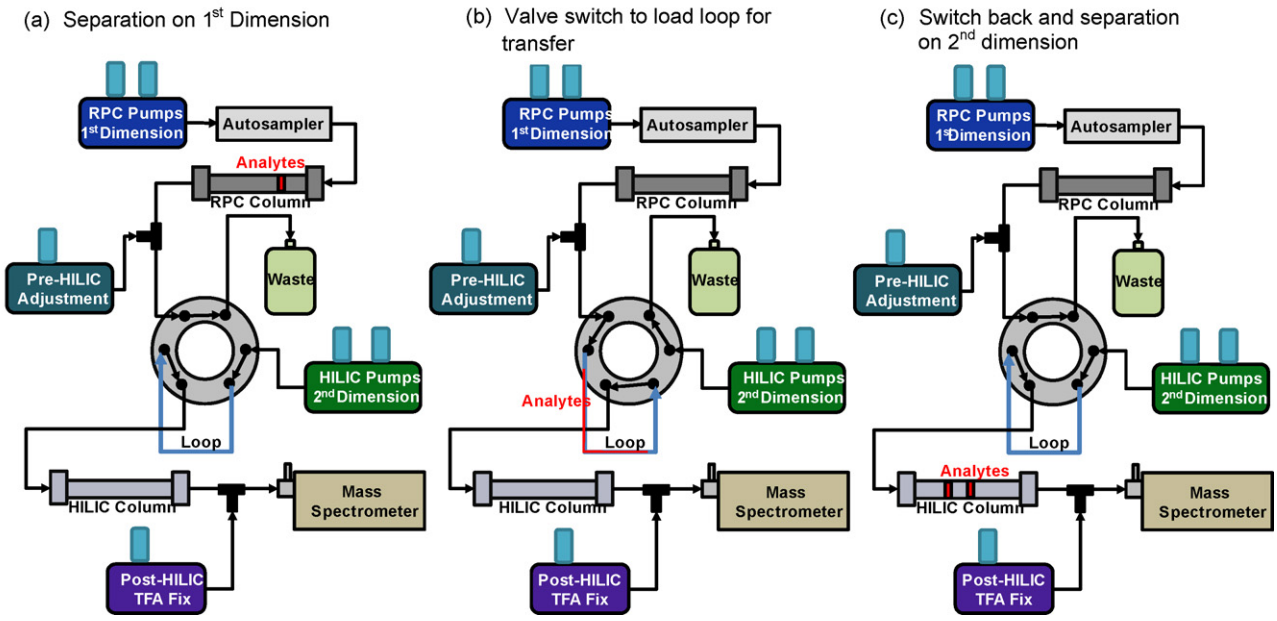

Fig. 1. Schematic diagrams of the two-dimensional separation system. Extraction and chromatography (a) take place on the first dimension (RPC) while the second dimension (HILIC) is being equilibrated for the transfer. The valve switch (b) transfers the desired plug of eluent from the first dimension onto the sample loop. The valve is then returned to the original position (c) and the contents of the loop flushed back onto the HILIC column where separation on the second dimension takes place. (a) Separation on 1st dimension, (b) valve switch to load loop for transfer, and (c) switch back and separation on 2nd dimension.

Waldbronn, Germany) delivered mobile phase to the second dimension HILIC column (Atlantis™ HILIC Silica; 2.1 mm × 50 mm; 5 μm; 100 Å; P/N 186002012; Waters Corporation, Milford, MA). Mobile phase for second dimension matched the first dimension using water with 0.02% TFA (mobile phase C) and 95:5 acetonitrile/water (v/v) with 0.02% TFA (mobile phase D). An additional two Shimadzu LC-10AD HPLC pumps were used for pre-HILIC composition adjustment and a post-HILIC suppression fix. The pre-HILIC composition adjustment decreased the net aqueous percentage transferred onto the HILIC column by delivering 100% acetonitrile. The post-HILIC suppression fix added 2% acetic acid in acetonitrile/water (95:5, v/v) before MS detection to recover signal intensity.

A 300 μL peek loop was placed on the switching valve between the first and second dimensions to assist in further adjusting the solvent composition being transferred to HILIC. The high organic content mobile phase equilibrating the HILIC column passed through and filled this loop. As the eluent from the first dimension leaves the column it is diluted with acetonitrile from the "pre-HILIC" adjustment pump. A plug of this diluted eluent is then transferred to the sample loop using the switching valve, where it is further diluted by mixing with the high organic content mobile phase. Once this plug is within the loop the valve is switched back to the initial setting and eluted back onto the equilibrated HILIC system. The autosampler syringe (Leap Technologies L-Mark Syringe; P/N LMK.2620719), and injection valve were washed four times with acetonitrile/water (50:50, v/v) containing 1% TFA, followed by two additional rinses with water containing 1% TFA to minimize carryover. The entire LC system was operated at room temperature for all experiments.

2.3. Column switching

Selected regions of eluent containing the target analytes from the first dimension (RPC column) were transferred to the second dimension (HILIC column) using the six-port, two-position switching valve. Diluted plasma samples were loaded onto the first dimension while the second dimension analytical column was equilibrated. In extraction mode as shown in Fig. 1a, the target peptides were selectively separated from endogenous matrix components and focused in a narrow band. When the valve was switched as shown in Fig. 1b, the analytes extracted from the first dimension were mixed and further diluted by teeing in acetonitrile. Additionally, this transferred eluent was further mixed with the high organic solvent in the sample loop (initial HILIC mobile phase composition) prior to transfer on the HILIC column. This process increased the net organic composition of the eluent being transferred from the extraction column and allowed for adequate focusing on the HILIC system to achieve more detailed separation. During chromatographic focusing and separation on the second dimension, the extraction column was washed and re-equilibrated. Several LC gradients and starting conditions were evaluated for both dimensions during the investigation.

2.4. Mass spectrometric conditions

An Applied Biosystems API 4000 mass spectrometer (Concord, Ontario, Canada) equipped with a TurboIonspray™ source was employed for peptide detection. All evaluations were performed in the positive-ion electrospray mode. The source temperature was maintained at 650 °C with nebulizer, desolvation gas and curtain gas settings at 50, 60 and 15, respectively. Ion spray voltage and entrance potential (EP) were maintained at 3500 and 10 V, respectively. Analytes were detected using multiple reaction monitoring (MRM) at a dwell time of 40 ms per transition. Each peptide was optimized for Q1 selection, fragmentation, and Q3 selection using declustering potential (DP) and collision energy (CE) as shown in Table 2.

2.5. Sample preparation

Individual stock solutions of peptides were prepared in acetonitrile/water (50:50, v/v) with 1% acetic acid at the concentration of 1 mg/mL and stored at −20 °C. Working solutions containing all 11 peptides were prepared by combining and diluting the latter stock solutions in different compositions of acetonitrile, water and TFA. These solutions were then used for the optimization of peptide

Table 2
Selected LC–MS/MS parameters for the 11 investigated peptides.

Analyte ID	[M+XH]+X	MRM	CE (V)	DP (V)
Neuromedin B	2	567.1/172.2	71	46
Neurokinin A	2	567.5/493.6	83	61
Adrenocorticotropic hormone fragment 1–10	2	650.6/223.2	45	76
Ranakinin	2	676.0/104.0	59	86
Neurotensin	2	837.4/136.2	95	121
Dynorphin A	4	537.9/629.8	29	41
Galanin	4	803.6/244.2	117	71
Vasoactive intestinal peptide	5	666.1/110.2	79	61
PTHrP (1–34)	4	1005.0/110.2	129	106
PTHrP (1–36)	4	1065.8/110.1	129	81
Adrenocorticotropic hormone intact	4	1136.1/120.2	129	111

injection composition, chromatography on the first dimension and experiments for coupling both dimensions together. Calibration standards for quantitating PTHrP (1–36) in K_3EDTA rat plasma were prepared using a series of dilutions in plasma to reach final concentrations of 10,000, 5000, 2500, 1000, 500, 250, 100, 75, 50 and 25 ng/mL. Control (blank with internal standard) and double blanks were prepared using the same matrix. PTHrP (1–34) was used as the analog internal standard (IS) for PTHrP (1–36) measurements. A working IS solution of PTHrP (1–34) was prepared by diluting the initial PTHrP (1–34) stock solution to 550 ng/mL with 33:67 acetonitrile/water (v/v) with 0.55% TFA. Plasma samples were minimally processed by aliquotting into a 96-well plate and mixing at a ratio of 1:10 with the PTHrP (1–34) working IS solution.

The plate was then centrifuged at 1750 × *g* for 10 min at 4 °C and 10 μL aliquots of the supernatant were injected for two-dimensional LC–MS/MS analysis. The final injection composition was acetonitrile/water (30:70, v/v) with 0.5% TFA. The dilution step did not result in any visible protein precipitation in the samples with 1:10 dilution. Data acquisition was performed using Sciex Analyst, version 1.4.1 software. The peak area ratios (analyte/IS) were plotted versus the concentration using a linear least-squares regression with a weighting factor of $1/x^2$.

3. Results and discussion

3.1. Optimization of the first dimension

3.1.1. Column selection

Both traditional C_{18} RPC and turbulent flow chromatography (TFC) were evaluated as the first dimension for peptide extraction and analysis. TFC columns marketed by Cohesive Technologies are widely used for on-line extraction of small molecules in biological matrix [23–27]. A TurboFlow HTLC C_{18} column (0.5 mm × 50 mm; 50 μm; 100 Å) was evaluated for its effectiveness and feasibility for use as the extraction column for the selected 11 therapeutic peptides. Numerous efforts and system modifications (*i.e.*, additional six-port, two-position switching valve for backflushing) mimicking those described in the latter TFC column publications were investigated. Backflush mobile phase composition was optimized to better focus peptides in a narrow band. These efforts produced marginally adequate focusing with significant column carryover that required numerous wash cycles to remediate. Carryover under these "trap and elute" type conditions is believed to be due to challenges with peptide solubility that can result in re-trapping of target peptides during the elution phase. This was evidenced by elution of focused peaks during the high and low organic cycles of subsequent wash gradients. Due to the issues with column carryover resulting from the use of backflushing techniques to elute peptides trapped on the head of the column, the TFC and other more common on-line extraction approaches were not used for further investigations. More standardized two-dimensional chromatographic approaches were pursued in an attempt to bypass these issues.

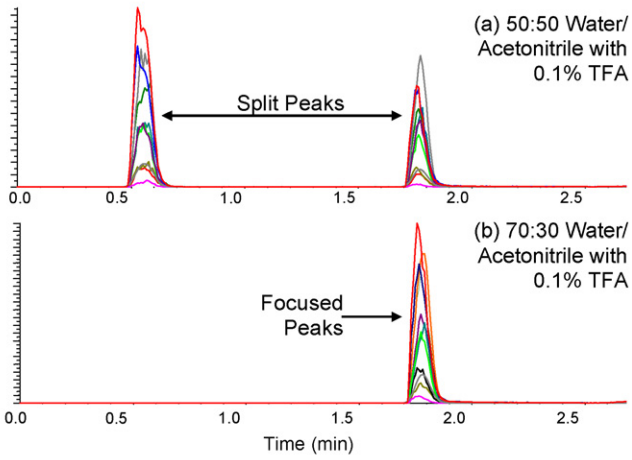

Fig. 2. Optimization of peptide injection composition on the first dimension RPC column using the 11 investigated peptides. (a) 50:50 water/acetonitrile with 0.1% TFA and (b) 70:30 water/acetonitrile with 0.1% TFA.

In addition to effectively reducing column carryover, using chromatographic separation for the first dimension in lieu of trap and elute methodologies provides the possibility to further separate out endogenous components that impact detection selectivity and ionization effects (*i.e.*, suppression). The employment of the ACE C_{18} RPC column was found to be effective for this purpose with no significant carryover issues observed for all 11 peptides. As a result, this RPC column was used for subsequent experiments with coupling RPC to HILIC and the direct injection of diluted matrix samples.

3.1.2. Sample injection composition

In RPC, the sample injection composition often requires a relatively low organic content to achieve adequate focusing, especially when trying to maximize injection volume and avoid peak symmetry issues. HILIC has been found to be relatively sensitive to aqueous content, the stronger solvent in this case, often requiring levels that are equivalent to or less than the initial mobile phase composition. This consequentially yields organic (*i.e.*, acetonitrile) concentrations from 85% to 95% and reduced solubility for peptides, which like their larger counterparts in biological matrices, may "co-precipitate" from solution. RPC techniques offer more flexibility in injection composition with moderate levels of organic without significantly compromising peak symmetry and retaining adequate solubility in more hydrophobic polypropylene containers. Additionally, a certain volume of organic solvent is necessary for disrupting the interactions between matrix components and the analytes of interest in biological samples. Peptide injection composition was evaluated from 0% to 50% acetonitrile for the ACE C_{18} RPC column to determine the conditions that provide adequate peptide solubility and chromatographically acceptable peaks. These experiments tested 10 μL injections which approximated 10% of the total column void volume. Peak splitting for all 11 peptides was observed in samples injected with 50% organic (Fig. 2a). Under these conditions the composition of organic solvent was too high to fully focus the peptides with a significant fraction being carried through the column in the injection plug without interacting with the stationary phase. Organic levels at 30% provided a good balance between peak shape and peptide solubility (Fig. 2b). Solubility of the peptides was qualitatively assessed by comparing relative peak areas. Additionally, the assessment of peak areas derived at

Table 3
Final PTHrP (1–36) analytical conditions.

Step	Start	Event time (min)	First dimension RPC column				Pre-HILIC adjustment	Switching valve	Second dimension HILIC column				Post-HILIC
			Function	Flow (μL/min)	%A	%B	Flow (μL/min)	Position	Function	Flow (μL/min)	%C	%D	Flow (μL/min)
1	0.0	1.0	Isocratic Hold	200	80	20	600	Waste	Isocratic hold	700	5	95	150
2	1.0	2.5	↑ Flow	800	80	20	–	–	–	–	–	–	–
3	3.5	0.1	↓ Flow	200	80	20	–	–	–	–	–	–	–
4	3.6	1.4	Elution gradient	200	69	31	–	–	–	–	–	–	–
5[a]	4.3	0.3	–	–	–	–	–	HILIC	–	–	–	–	–
6[a]	4.6	–	–	–	–	–	–	Waste	–	–	–	–	–
7	5.0	0.1	↑ Flow	800	25	75	–	–	End isocratic	700	5	95	–
8	5.1	2.0	Isocratic wash	800	25	75	–	–	Start gradient	700	5	95	–
9	7.0	0.1	Step	800	80	20	–	–	–	–	–	–	–
10	7.1	1.9	Isocratic hold	800	80	20	–	–	End gradient	700	50	50	–
11	7.9	0.1	–	–	–	–	–	–	Isocratic hold	700	50	50	–
12	8.0	1.0	–	–	–	–	–	–	Step	700	5	95	–
13	9.0	0.1	↓ Flow	200	80	20	–	–	Re-equilibrate	700	5	95	–
14	9.1	1.0	Equilibrate flow	200	80	20	–	–	–	–	–	–	–
15	10.0	End	–	–	–	–	–	–	–	–	–	–	–

[a] Transfer of 0.3 min window of eluent containing PTHrP (1–36) and PTHrP (1–34) to the second dimension.

Fig. 3. Chromatograms of PTHrP (1–36) on the first dimension RPC column using simple stepwise gradient (black straight lines). Both neat (non-matrix; red) and diluted plasma matrix (blue) were loaded on the ACE C_{18} column (2.1 mm × 50 mm; 5 μm; 100 Å) and detected using MRM mode. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

various organic-to-aqueous ratios from TFC backflush experiments indicated compositions between 25% and 50% acetonitrile in water modified with TFA released similarly high levels trapped on the stationary phase. This indicates that these compositions are reasonable for solubilization.

3.1.3. Chromatography within the first dimension

Chromatography on the RPC column was evaluated using neat and fortified matrix samples. Initial chromatographic conditions designed to simply trap and elute the target peptides yielded focused and symmetrical peaks in neat solution. Peak tailing, broadening and decreased peak intensity was observed with diluted plasma samples. Fig. 3 shows chromatograms of PTHrP (1–36) in neat solution and plasma matrix along with the mobile phase conditions. Part of the reason for these differences is attributed to the co-elution of endogenous components in the plasma matrix. The presence of ionization suppression was confirmed while running a blank plasma sample along with post-column infusion of the analytes (RPC only). This finding confirmed our expectation that along with our target peptides other potential suppressing matrix components (*e.g.*, phospholipids) would co-elute and be transferred to the second dimension. Once transferred to and separated on the highly orthogonal HILIC dimension these endogenous components were expected to be resolved from the target peptides and ionization effects would no longer be an issue.

The potential for selectivity issues between certain analytes and matrix components were evaluated by changing the chromatographic conditions in the first dimension to a more selective gradient (instead of step) and by the use of additional MRM transitions for the same peptide. Baseline separation of analytes from the majority of endogenous interference in matrix could be achieved using a relatively shallow gradient (Fig. 4, blue line). Moreover, total run time could be reduced to less than 10 min (Table 3) while maintaining resolution between peptides and interfering matrix components (Fig. 4, red line). The selectivity of different MRM transitions for a given peptide was observed in plasma matrix and compared with neat solution as shown for PTHrP (1–36) (Fig. 5). While the MRM transition of m/z 1066/110 was identified as the most sensitive transition for PTHrP (1–36), it was found to be relatively less selective in the presence of endogenous interference, compared to other transitions of m/z 853/110 and 711/110. This is evidenced by the peak eluting at the column void (approximately 0.3 min) and the peak fronting at a retention time of 4.4 min for the 1066/110 m/z transition in Fig. 5b (blue line). While the use of the 110 m/z product ion (immonium ion of histidine) [28] is not preferred for optimizing selectivity, it may be the only reasonably

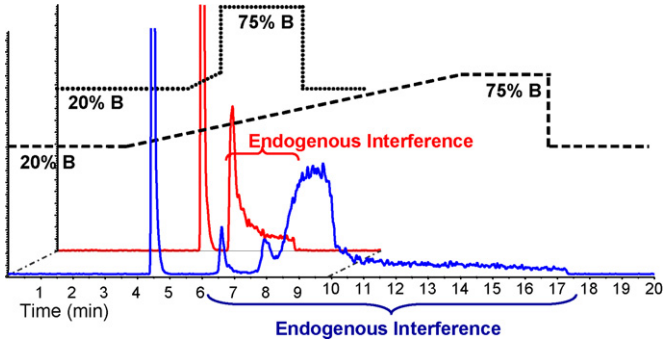

Fig. 4. Chromatograms of PTHrP (1–36) in plasma using the extraction RPC column with a shallow gradient. Chromatograms in blue and red correspond to the longer and truncated LC gradients, respectively. Endogenous interferences could be adequately resolved from the target analytes using more selective gradients allowing for a cleaner transfer to the second dimension. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

high fragment ion that can be attained for certain peptides under the CID conditions of a given instrument. This was found to be the case for both PTHrP (1–36) and (1–34), which did not produce any other more selective higher MW product ions that could reasonably be used for quantitation. Additionally, moderate mass resolution at the higher precursor ion *m/z* ranges can allow for more endogenous components to be detected than on a higher resolution system. These issues would certainly be a concern if analyses were only conducted using a single RPC dimension for analysis. The selectivity of HILIC and its use for the second chromatographic dimension can provide the ability to further resolve co-eluting components that are not separated in the first dimension. Overall, the selectivity of several MRM transitions should be carefully compared for the analysis of peptides in biological matrix, as interferences may also be misleading when optimizing chromatography for a given analyte.

3.2. Comprehensive and selective approaches—first dimension

As described above, the RPC first dimension can be used to effectively trap target peptides and selectively remove the majority of endogenous matrix components for transfer to the second dimension. Additionally, through slight variation of the aqueous-to-organic ratio for initial LC starting conditions, peptides can be further separated during the isocratic focusing period. The ability to focus peptides, keeping them together for combined elution, or to move certain other peptides away from those of interest provides additional strategies for peptide analysis.

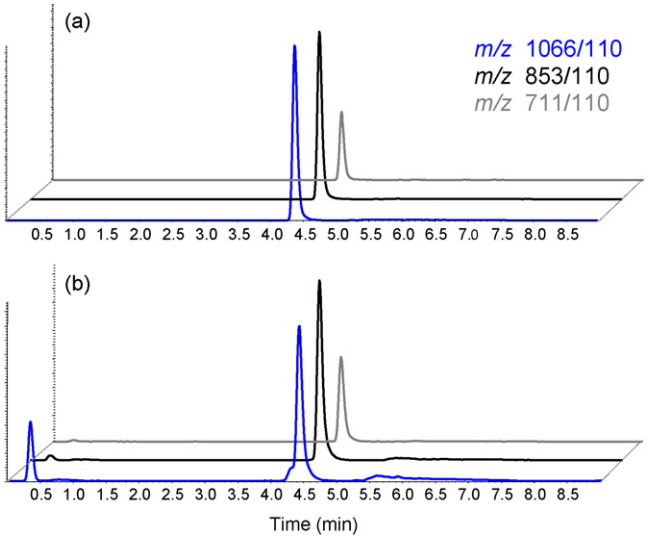

Fig. 5. Comparison of different MRM transitions for PTHrP (1–36) in neat peptide mixture (a) and plasma matrix (b). PTHrP (1–36) was extracted using the first dimension RPC column and detected using different MRM transitions of *m/z* 1066/110 (blue), *m/z* 853/110 (black) and *m/z* 711/110 (gray). Results indicate the presence of co-eluting endogenous materials and issues with the selectivity of certain MRM transitions. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

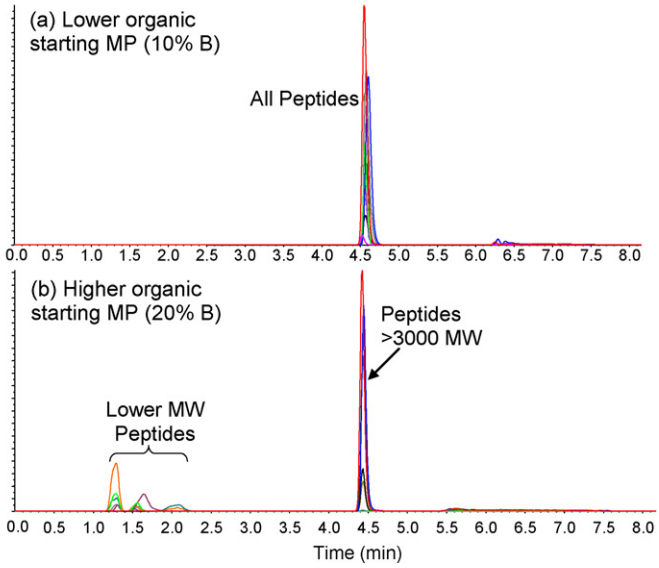

Fig. 6. Different chromatographic strategies achieved on the first dimension RPC column using various LC starting conditions. Lower organic starting mobile phase (10% B) (a) offered a comprehensive approach by focusing all 11 peptides. Higher organic starting mobile phase (20% B) (b) provided additional selectivity for peptide analysis. Mobile phase A consisted of water with 0.02% TFA while mobile phase B consisted of acetonitrile/water (95:5, v/v) with 0.02% TFA.

An initial delivery of 10% mobile phase B (95:5 acetonitrile:water with 0.02% TFA) provided the conditions to focus all 11 peptides in a narrow band on the first RPC column (Fig. 6a). During the appropriate phase of the elution gradient these peptides were transferred together to the second dimension using the intermediate switching valve. All 11 peptides were well resolved on the second HILIC column after supplemental acetonitrile was added following the first dimension. Fig. 7 shows chromatograms for these peptides transferred onto HILIC using different ratios of acetonitrile-to-eluent (teed-in) between the two dimensions. Peak broadening and fronting of less hydrophilic peptides were observed on HILIC when teeing in 600 μL/min acetonitrile (1:3 eluent-to-acetonitrile), whereas 1000 μL/min acetonitrile (1:5 ratio) provided good peak symmetry for all 11 peptides. These results indicate the need to adjust the loading composition on HILIC and reduce the net aqueous content to ensure proper focusing of less hydrophilic peptides. Other peptides like galanin, adrenocortitrophic peptide, vasoactive intestinal peptide and both PTHrP fragments investigated that are better retained by HILIC showed acceptable peak shape even at lower organic ratios. This can allow for net reductions in the flow rate required for HILIC and any post-column "TFA fix" which will significantly improve sensitivity for quantitative purposes.

The use of higher organic starting mobile phase (20% B) on the first dimension offered additional selectivity by separating the 11 peptides into two groups (Fig. 6b). This demonstrates the utility of the first dimension to serve as more than an "extraction column" by potentially separating certain matrix components or metabolites away from desired targets. Although the mechanism of this selective approach was not clear, the molecular weight cut-off of 3000 Da was observed on the first column. This may indicate that

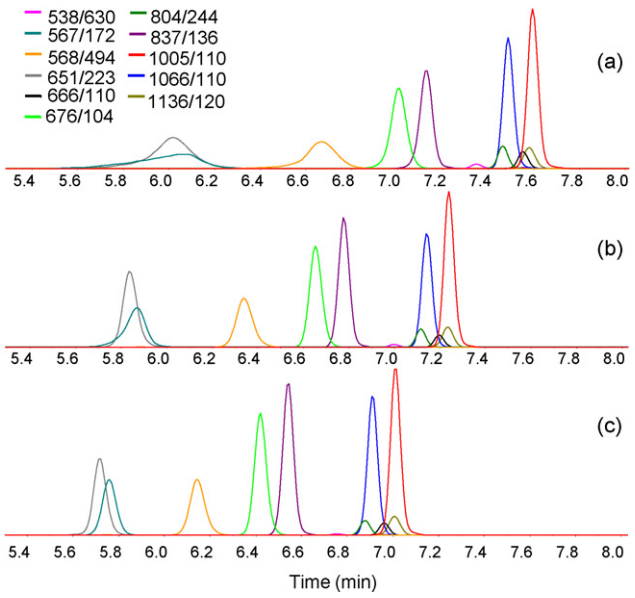

Fig. 7. Chromatograms of the 11 investigated peptides on the HILIC column coupled with the first dimension RPC column using a comprehensive strategy with lower organic starting mobile phase. Supplemental acetonitrile was introduced between RPC and HILIC at different flow rates (a: 600 μL/min; b: 800 μL/min; c: 1000 μL/min). The RPC column flow was kept constant at 200 μL/min during transfer. HILIC mobile phase flow rates were set to match the total flow from the RPC column and supplemental acetonitrile (*i.e.*, a: 800 μL/min, b: 1000 μL/min and c: 1200 μL/min). Peptides eluted earlier from the HILIC column due to the increase in flow rate. Results indicate that less hydrophilic peptides require a higher level of organic to provide adequate focusing on the second dimension.

better retention is possible for larger peptides containing greater numbers of protonated amine functional groups that can pair with TFA and increase the peptides net affinity for the stationary phase. This coupled with the presence of hydrophobic residues and character serve as the main contributors to retention on reversed phase systems. The 0.3-min retention window containing the higher molecular weight target peptides (Fig. 8a) was transferred using the switching valve for focusing and further resolution by HILIC (Fig. 8b).

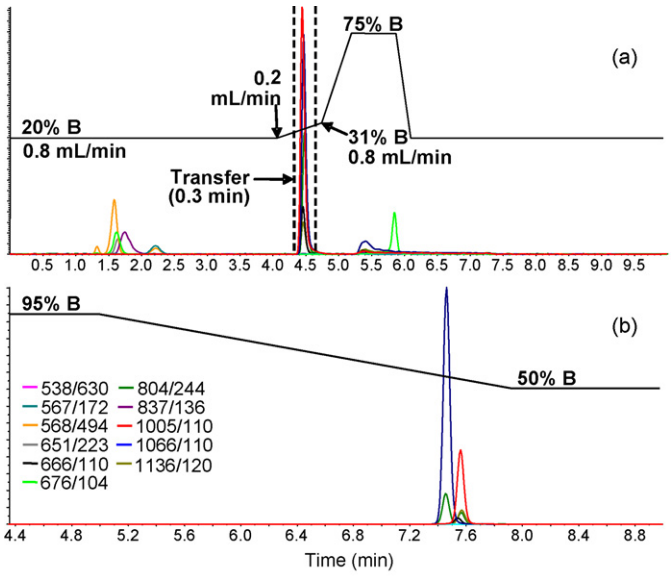

Fig. 8. Chromatograms from two-dimensional LC system coupling RPC (a) with HILIC (b) using the selective strategy. LC gradients on both columns are detailed in Table 3.

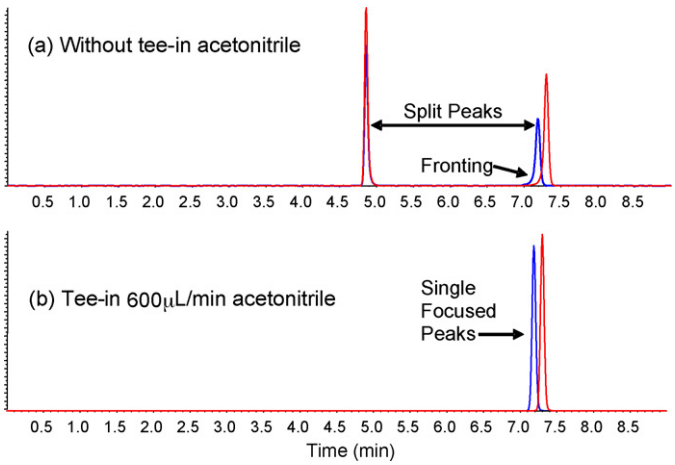

Fig. 9. Pre-HILIC composition adjustment. PTHrP (1–36) (blue) and PTHrP (1–34) (red) were eluted from RPC column and transferred to HILIC column without teeing in organic solvent (acetonitrile) (a) or by teeing in organic solvent (acetonitrile) at 600 μL/min (b). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

3.3. Coupling RPC to HILIC

Modification of the eluent being transferred from the first RPC dimension was required in order to resolve chromatographic issues on the second HILIC dimension. Fig. 9 shows the optimization of isocratic focusing upon the HILIC column during eluent transfer. Since the analytes were eluted from the RPC column with a relatively high aqueous content, which was found to be too strong for direct transfer to HILIC column, peak splitting and fronting were observed for both PTHrP (1–36) and PTHrP (1–34). Single focused peaks were obtained by adjusting the organic composition before loading on HILIC, where the higher aqueous mobile phase composition within the transfer window (approximately 75% water, 200 μL/min) was diluted 4-fold by teeing in 100% acetonitrile at 600 μL/min. The results further indicated that a minimum level of acetonitrile was required between the first and second dimensions to attain proper focusing of peptides with even a strong affinity for the HILIC stationary phase.

Additional benefits were observed with peak symmetry and focusing through the use of a 300 μL loop positioned on the switching valve immediately before of the HILIC column. Prior to the transfer of the acetonitrile-supplemented eluent from the first dimension, this loop was continuously flushed and filled with the equilibrating HILIC mobile phase. Eluent entering the loop from the first dimension was mixed with this higher organic content HILIC mobile phase further diluting the aqueous content and enabling better focusing on the HILIC system. The final adjusted flow reaching the HILIC column had a low aqueous content (less than 15%) which was sufficient to focus and adequately retain the peptides in the mixture. Without the use of this loop, broad peaks and substantial fronting (especially for the less hydrophilic peptides) were observed.

The width of the transfer window was optimized to 0.3 min for both the comprehensive and selective approaches. It was observed that the majority of the peptide peak area (95+%) eluting from the acetonitrile adjusted first dimension fell within a band of approximately 0.2 min. This band was centered within the 0.3 min window to minimize the potential for cutting off a portion of the eluted peaks that may result from slight changes in retention over time. Further opening of the transfer window requires a higher proportion of acetonitrile to adjust the composition after the RPC column as well as a larger transfer loop. Keeping the transfer band as nar-

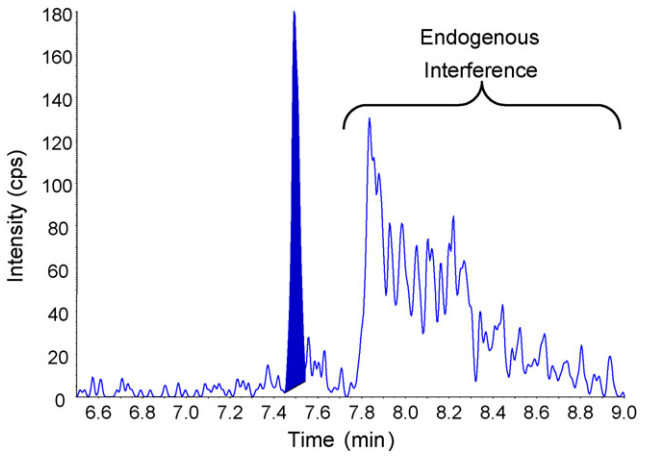

Fig. 10. Chromatogram of LLOQ sample for quantitative analysis of PTHrP (1–36) in plasma using on-line two-dimensional RPC–HILIC LC–MS–MS. Separation of residual interfering endogenous components allows for accurate quantification at low levels.

row as possible allows for the less hydrophilic peptides to be better focused on HILIC.

3.4. Post-column infusion for signal recovery

Ion-pairing reagents such as TFA are commonly used and required as mobile phase additives for the analysis of peptides. Our goal with TFA for the development of this on-line two-dimensional method for PTHrP (1–36) was to use the minimal amount necessary to attain acceptable chromatography and reduce carryover throughout the system. The use of 0.02% TFA was found to provide good chromatography and retention in both dimensions, but suppressed the electrospray signal of analytes due to its ability to form gas-phase ion pairs with positively charged analyte ions [29]. Partial recovery of signal suppression can be achieved using a post-column infusion of acetic or propionic acid in a procedure commonly referred to as the "TFA fix" [29]. For these experiments a 2% solution of acetic acid in 95:5 acetonitrile/water was used for signal recovery optimization. The flow rate for the infusion of this solution was varied between 50 and 250 μL/min (relative to the eluent flow from the HILIC column at 700 μL/min) to identify the most beneficial final composition. Results indicated an approximate improvement in signal-to-noise ratio from 2- to 30-fold for PTHrP (1–36) and (1–34) depending on flow with the most favorable conditions seen at 150 μL/min. Additionally, by keeping the acetonitrile percentage of the post-infusion solution at higher levels we could enhance desolvation to help compensate for losses in ionization efficiency experienced at higher overall flow rates.

3.5. On-line two-dimensional method for PTHrP (1–36)

The optimized on-line two-dimensional LC–MS/MS methodology using the more selective chromatographic conditions for the first dimension has been successfully applied to the analysis of PTHrP (1–36) in K_3EDTA rat plasma, using PTHrP (1–34) as the internal standard. Plasma samples were subjected to simple dilution with the working IS solution and then directly injected onto LC system. No significant carryover (<20%) was observed after more than 300 injections. Carryover was based on the integrated analyte area for a double blank sample immediately following injection of the highest concentration calibration standard. This area was then compared to the total area of the LLOQ standard. The calibration curves were linear over the range of 25–10,000 ng/mL with an average correlation coefficient of 0.996 using weighted ($1/x^2$) linear regression. The lower limit of quantification (LLOQ) was 25 ng/mL. The chromatogram of the LLOQ sample (Fig. 10) indicated that potentially interfering endogenous components were further removed in the second dimension. Injection of double blank selectivity samples showed no significant response (<20%) within the retention region of the analyte and IS. Additional ionization suppression experiments involving the injection of a double blank matrix sample and post-column infusion of analytes indicated that any ionization effects that could potentially impact the analyte and IS were adequately resolved. These findings show the benefit of using two-dimensional analysis for this application since separation conducted using the first dimension alone would have still resulted in co-elution of the endogenous interference, significantly limiting the low end of the calibration curve and substantially increasing variability and decreasing accuracy.

4. Conclusions

The highly orthogonal coupling of C_{18} to HILIC was achieved for the two-dimensional analysis and on-line extraction of peptides in biological samples. Fortification of mobile phase with TFA allowed for chromatographic focusing and separation in both dimensions. The balance of aqueous-to-organic in the initial sample composition was required for adequate peptide solubilization, disruption of the interactions between endogenous protein/peptides from biological matrix, and proper focusing and retention of peptides in the first dimension. The use of a higher starting aqueous mobile phase percentage for the first dimension provided adequate trapping of all 11 peptides in a narrow band for comprehensive transfer to the second HILIC dimension. Slight reductions in water levels for this composition initiated the chromatographic separation of peptides for more selective transferring to HILIC. This higher degree of chromatographic selectivity was found to be essential in the analysis of PTHrP (1–36) which was challenged by issues with detection selectivity from endogenous matrix components.

The addition of supplemental acetonitrile to the eluent being transferred from the first RPC dimension to the second HILIC dimension was essential for diluting excess water and allowing for adequate focusing and retention of peptides on the equilibrated HILIC system. A sample loop located prior to the HILIC dimension provided additional dilution of the aqueous content by mixing and diluting the incoming eluent with the higher organic content HILIC mobile phase. The combination of the supplemental acetonitrile and loop were identified as critical components in ensuring proper focusing on HILIC.

The application of this technology was successfully employed for the quantitation of peptide PTHrP (1–36) in rat K_3EDTA plasma from 25.0 to 10,000 ng/mL using PTHrP (1–34) as the analog internal standard. The direct injection of diluted matrix samples onto the first dimension was achieved for over 300 injections with no observed loss in system performance. Recovery of some signal suppression from TFA was achieved by an in-line post-column infusion of an acetic acid solution, allowing for improvements in the LLOQ for this analysis. To the best of our knowledge, the work presented in this paper is the first-reported successful direct two-dimensional coupling of RPC to HILIC (in this order). Additionally it is the first-reported application for on-line extraction of peptides.

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