The use of a peptide retention-time calibration mixture as a tool in scheduled SRM method construction

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Overview

Purpose: Develop and apply a peptide retention-time calibration (PRTC) mixture to evaluate LC performance and to ease creation of scheduled SRM methods.

Methods: An equimolar mixture of 15 heavy peptides that elutes across the full C_{18} chromatographic gradient (5-40% acetonitrile) was developed on multiple LC-MS platforms.

Results: A PRTC mixture can accurately predict tryptic peptide retention times with high correlation to experimentally determined times ($R^2 > 0.9$). The PRTC mixture enabled reliable peptide retention time prediction and SRM scheduling for peptides of unknown retention time.

Introduction

Mass spectrometry (MS)-based targeted protein quantification methods offer high multiplexing capability, excellent reproducibility, short analysis time, and automation for replicate analysis. The most common targeted MS strategy is selective reaction monitoring (SRM). To achieve sufficient dwell times required for accurate multiplexed quantitation by SRM, the number of monitored SRM transitions per cycle must be limited. This is accomplished by proper gradient choice and scheduling of SRM transitions based on peptide retention. Scheduling limits the detection of SRM transitions only to those peptides eluting within a specific time window.

The Thermo Scientific Pierce Peptide Retention Time Calibration (PRTC) Mixture (Part No. 88321) enables assessment of chromatographic performance and chromatography column calibration for retention time prediction. When used with Thermo Scientific Pinpoint 1.1™ Software, the PRTC mixture can predict peptide relative retention times across platforms and can predict retention times of theoretical peptides sequences using a hydrophobicity factor (HF) calculation algorithm.[1]

In addition to assisting with SRM method building, the PRTC mixture of 15 heavy peptides is also an excellent tool for verifying routine instrument performance. Because the mass and sequence of all peptides are known, they can be used to optimize peak width and peak resolution or benchmark C_{18}-based packing materials and chromatography columns of varying lengths and diameters. The PRTC mixture can also be used to normalize for autosampler variability and retention time shifts.

Methods

Twelve protein mix sample preparation: A 12 protein mixture consisting of cytochrome c (horse), α-lactalbumin (bovine), serum albumin (bovine), carbonic anhydrase (bovine), lactoperoxidase (bovine), glutamate dehydrogenase (bovine), ovalbumin (chicken), α-casein (bovine), β-casein (bovine), ribonuclease (bovine), transferrin (bovine) and alcohol dehydrogenase (yeast) was prepared at equimolar ratios. Cysteines were alkylated with iodoacetic acid. The equimolar mixture was digested with trypsin for exactly 2 hours in 50 mM ammonium bicarbonate (pH = 7.8) at 37 °C. In all experiments, exactly 1.0pmol was analyzed on the 1.0mm columns and 250fmol were analyzed on 75µm columns.

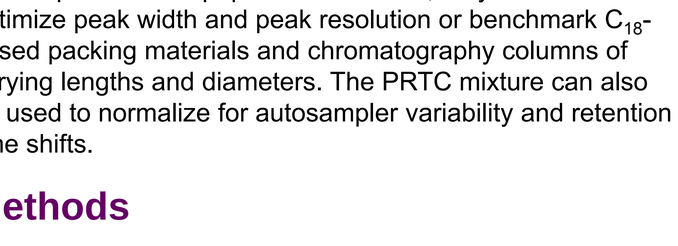
Preparation of peptide digests from insulin stimulated 293T cells: 293T cells ($2.0x10^8$ cells) were treated with insulin (5µg/mL) for 30 minutes. Cells were harvested and lysed in 8M urea containing Thermo Scientific Halt Protease and Phosphatase Inhibitor Cocktail (Part No. 78440). The lysates were sonicated briefly and protein concentrations were determined with the Thermo Scientific Pierce 660nm Protein Assay (Part No. 28660). Ten milligrams of protein samples were digested overnight with trypsin (1:250 enzyme/substrate) at 37°C and frozen in aliquots at – 80 °C or desalted offline using C_{18} cartridges. Lyophilized peptides were dissolved at 5 mg/mL in 25 mM ammonium bicarbonate and then analysed (10 mg). The frozen samples were desalted online by diverting the first 5.0 minutes of the injected material to waste before initiating the LC gradient.

LC-MS/MS analysis: LC-MS/MS analysis was performed on a Thermo Scientific LTQ-XL Orbitrap Mass Spectrometer using a self-packed column (75□m x 20cm) containing Magic C_{18} (Michrom Bioresources). Gradients ranging from 0.25-5.0% per minute were evaluated using Buffer A (0.1% formic acid) and Buffer B (0.1% formic acid/99.9 % acetonitrile) at 300nL per minute. SRM experiments were performed on a Thermo Scientific TSQ Vantage mass spectrometer using a Hypersil Gold C18 column (1.0 x 150mm; Part No. 25005-150165) at flow rates of 120 µL per minute. All SRM methods were constructed using Pinpoint 1.1.

Figure 1. Comparison of Peptide Retention Time Calibration Mixture on Thermo Scientific Orbitrap (A) and TSQ Vantage (B) Instruments.

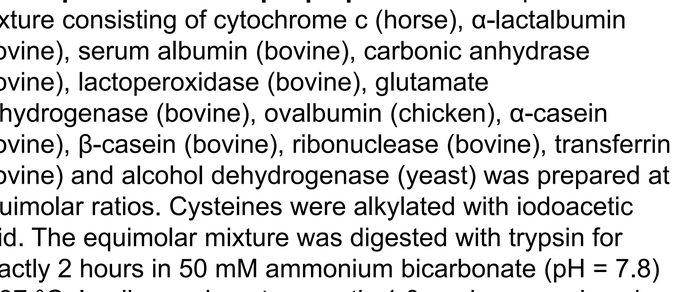
A

Gradient = 0.25 % per minute



Gradient = 1.0 % per minute
SRM mode

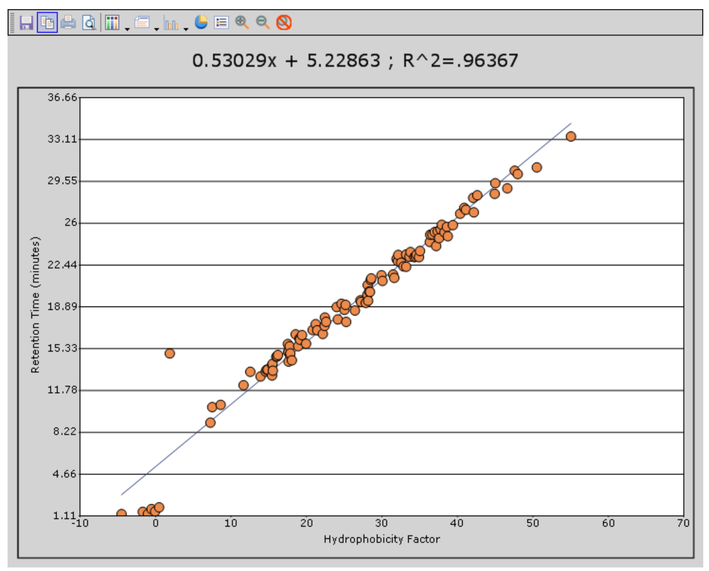
B



SRM method construction with hydrophobicity calculations: The sequences of the PRTC peptides (Table 1) and the raw MS files from the PRTC mixture were analyzed in triplicate using a gradient of 1.0% per minute on the TSQ Vantage and imported into Pinpoint 1.1™ Software. A linear relationship between observed retention time and peptide hydrophobicity was computed based on hydrophobicity calculations.[1] FASTA-formatted lists of target proteins were then imported and digested *in silico*, modifications were added, and the transitions and retention times were calculated. Only y type ions (y3 – yN) were used with a molecular mass of 300-1500Da. Methods were then exported so that < 200 transitions per scan cycle were monitored on a TSQ Vantage. The initial results were then refined in Pinpoint 1.1 Software to only include peptides with at least three strong transitions.

SRM method construction by instrument interpolation: The PRTC mixture was analyzed on the Orbitrap-XL (100 minutes, 0.25% per minute gradient) and the TSQ Mass Spectrometers (35 minutes, 1.0% per minute gradient). Digested peptides from a 12 mix or 293T digest were then analyzed on the Orbitrap Mass Spectrometer with the same gradient with the PRTC mixture spiked in at 100fmol/µL (500fmol on column). The data from the neat PRTC mixture and the digested samples were processed using Thermo Scientific Proteome Discoverer 1.2 and the resulting search (.msf) files were saved. The search file from the neat trainer set and the raw PRTC data from the TSQ were used in Pinpoint Software for the column calibration between instruments. Finally, the search files for the digested samples were imported into the Pinpoint Software as spectral libraries, retention times were predicted, and SRM methods were constructed as described above.

Figure 2. Validation of scheduled SRM analysis of 12 protein mix peptide retention times on a TSQ with hydrophobicities calculated from peptide sequence alone.



Results

Analysis of the PRTC mixture on Orbitrap and TSQ Vantage Instruments configured for discovery or targeted quantitation, respectively, demonstrated high resolution separation of the PRTC mixture across column stationary phases, formats and flow rates (Figure 1). The elution of peptides across the entire gradient also permitted optimization of chromatographic conditions and assessment of performance for multiple runs.

The peptide sequences and the wide distribution of hydrophobicities for the PRTC mixture were used to predict retention times for peptides based on sequence alone. A comparison of the experimentally validated retention times (based on prediction) for peptides from a mixture of 12 standard proteins had strong correlation (R^2=0.96, Figure 2).

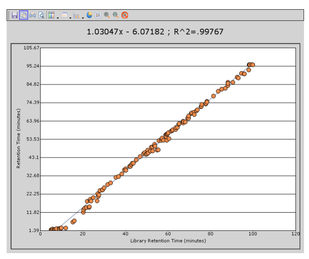
Table 1. Heavy isotope-labeled peptides in the peptide retention time calibration (PRTC) mixture.

#	*Sequence*	*$[M+2H]^{2+}$*	*Hydrophobicity Factor (HF)*
1	SSAAPPPPP**R**	493.77	7.56
2	GISNEGQNASI**K**	613.32	15.50
3	HVLTSIGE**K**	499.28	15.52
4	DIPVPKP**K**	451.28	17.65
5	IGDYAGI**K**	422.73	19.15
6	TASEFDSAIAQD**K**	695.83	25.88
7	SAAGAFGPELS**R**	586.80	25.24
8	ELGQSGVDTYLQT**K**	773.89	28.37
9	GLILVGGYGT**R**	558.32	32.18
10	GILFVGSGVSGGEEGA**R**	801.41	34.50
11	SFANQPLEVVYS**K**	745.39	34.96
12	LTILEEL**R**	498.80	37.30
13	NGFILDGFP**R**	573.30	40.42
14	ELASGLSFPVGF**K**	679.37	41.18
15	LSSEAPALFQFDL**K**	787.42	46.66

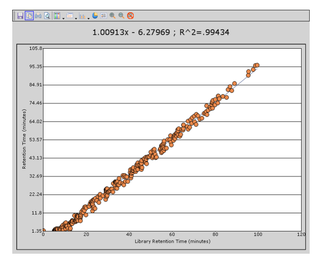
Heavy amino acid in bold

Figure 3. Prediction of TSQ peptide retention times for a 12 protein mix (a) and 35 proteins from insulin stimulated 293T lysate (B,C) using elution interpolation from an Orbitrap Instrument.

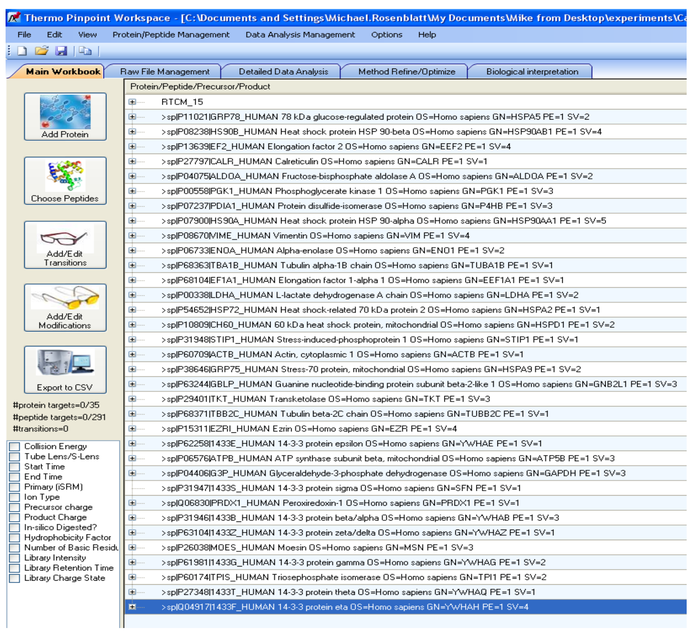
A



B



C



In addition, the PRTC mixture was used to calibrate chromatographic performance between instruments and to ease the migration from protein discovery to targeted quantitation methods. By running the PRTC mixture on both instrument platforms, retention times and spectral libraries from proteins discovered on an Orbitrap Instrument could be readily used by Pinpoint Software to build scheduled, targeted SRM assays on the TSQ. This was assessed using peptides from a 12 protein mixture and from a 293T cell lysate (Figure 3). Again, the observed retention times on the TSQ correlated well with the library retention times observed on the Orbitrap Instrument. This discovery-to-targeted strategy also provided reference CID or HCD spectra to assist in the building and assessment of SRM transitions. This interpolation method also eased the building of targeted SRM methods for hydrophilic peptides where retention can be difficult to predict, such as phosphopeptides (data not shown).

Conclusions

The PRTC mixture is a powerful retention time calibration tool. Using this mixture, scheduled SRM methods can be created for the multiplexed analysis of peptide targets in a single short run. Robust, multiplexed SRM methods for targeted quantitation can be built using peptide sequence alone, using data from discovery MS experiments, or using public mass spectral libraries.

- Chromatography and MS instrument performance can be quickly assessed and optimized
- Peptide retention times can be predicted with sequence using calculated hydrophobicity factors
- Peptide elution for multiple instrument platforms can be predicted using sequence or instrument interpolation
- Prediction of elution simplifies the optimization of scheduled SRM windows for improved quantitation and increased multiplexing
- The PRTC mixture provides an internal standard to normalize retention times and peak intensities between runs

References

1. Spicer, V., *et al*. (2007). Sequence-specific retention calculator. A family of peptide retention time prediction algorithms in reversed-phase HPLC: applicability to various chromatographic conditions and columns. *Anal Chem* **79(22):**8762-8.

Acknowledgements

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