Choosing the Appropriate Solid Phase Extraction Sorbent for Peptide Library Clean Up

Elizabeth Denton[1]
[1]Biotage, 10430 Harris Oaks Blvd., Suite C, Charlotte North Carolina 28269, USA

Introduction

Peptides have returned to the forefront of drug discovery efforts as unbiased screening technologies have improved and the rules defining passive cell permeability have been clarified. As a result, the demand for synthetic peptide libraries has increased significantly, particularly during secondary screening as lead compound programs progress. At these early discovery phases though, highly pure peptide samples may not be necessary when evaluating these compound libraries in assays. With this in mind and given the improvements in automated peptide library synthesis strategies, a solid phase extraction methodology may be appropriate to parallelize peptide library cleanup and significantly reduce library purification time.

Herein we present data demonstrating that sorbent selection, and specifically the sorbent parameters themselves, impacts the predictability of elution profile when using solid phase extraction to improve the purity of synthetic peptide libraries across a wide range of therapeutically relevant peptides.

Experimental Protocol

Peptide Synthesis and Analysis

Peptides under investigation were synthesized automatically with a Biotage® Initiator+ Alstra™ peptide using ChemMatrix® Rink Amide resin, default methods, DIC/Oxyma as coupling reagents, and Fmoc-protected amino acids.

Peptide cleavage occurred in a cocktail of 95% TFA, 2.5% TIS and 2.5% H_2O for 2 hours at room temperature. The cleavage cocktail was evaporated using the Biotage® V-10 Touch evaporation system and the resulting crude sample residue was dissolved in DMSO for purification. Crude peptide samples were purified using a Isolera™ Dalton 2000 equipped with a 25-gram Biotage Sfär Bio C18 column. Crude and purified peptides were analyzed for purity with an Agilent 1260 Infinity series HPLC

Purified peptide samples were subjected to solid phase extraction (SPE) procedures using a Biotage® Extrahera™ sample preparation automation system equipped with 96-well plates packed with 500 mg per well of C18-functionalized silica. Purified peptide samples were dissolved in DMSO and prepared such that 2 mg, 5 mg, or 10 mg peptide was loaded into each of 2 sorbent containing wells in 300 µL of total volume. The sorbent plate was conditioned with 1500 µL of 100% Acn, then equilibrated with 1500 µL 10% $Acn_{(aq)}$ prior to sample loading. After sample loading the sorbent was treated with 1500 µL 10% $Acn_{(aq)}$ to remove DMSO, washed with 65% $Acn_{(aq)}$, then 70% $Acn_{(aq)}$, and cleaned with 100% Acn. All solvents utilized were modified with 0.1% TFA and solvent concentrations were selected based on calculated elution point using analytical HPLC.

Results and Discussion

Sorbent Selection

Media utilized for traditional sample preparation methodologies are inherently designed to retain small molecules, like drugs of abuse, while excluding other biological matrix components like proteins, peptides, and lipids. This design feature however, could become a liability when applied to alternative chemical systems. Two reversed phase media with physiological properties more amenable to peptide purification are compared in this investigation for applications as a solid phase extraction sorbent, Table 1.

	UltraC18	BioC18	"standard" SPE C18
Particle size (µm)	30	20	50
Pore size (Å)	100	300	60

Table 1. *Comparison of sorbents used for potential peptide cleanup.*

Comparing these two more appropriate media will allow a more direct comparison of how size, both compound and particle pore, impact SPE predictability and sample recovery.

Peptide Selection

This work aims to expand the available dataset and formulate guidelines toward sorbent selection when considering (SPE) for peptide library cleanup. The two peptides selected (Table 2), primarily for comparison to clinical relevance, but also for chromatographic similarity. Chromatographic behavior similarities (hydrophobicity, retention time) enable assignment of observed media interaction differences to generic properties – size and solution structure being the most important here.

	18 Amino Acids	**37 Amino Acids**
Sequence	DWLKAFYDKVAE KLQEAF-NH_2	HDEFERHAEGTFTSDVSSYLE GQAAKEFIAWLVKGRG-NH_2
Molecular Weight	2200.49	4168.49
Net Charge (pH 7, 2)	0, +4	-1.8, +6
Isoelectric Point	7.01	5.44
GrAvy	0.22	0.24
HPLC Retention Time	6.01 min	5.65 min
Calculated Acetonitrile Elution	60.1%	56.5

Table 2. *Comparison of selected peptide characteristics.*

Linear 18 Amino acid Peptide Sorbent Comparison

A medium sized peptide was selected for the first comparison as this is similar to peptides currently under evaluation for therapeutics. To compare the sorbents initially, the pre-purified 18 amino acid peptide was loaded into pre-conditioned and -equilibrated wells of a 96-well plate containing 500 mg of either UltraC18 or BioC18 media, in 2 mg, 5 mg or 10 mg quantities per well, and elution was attempted using a mobile phase containing 65% Acn + 0.1% TFA, slightly above the calculated elution concentration as determined by analytical HPLC, Figure 1.

Interestingly, the peptide behaved more predictably when using UltraC18, with 100Å pores, than when subjected to BioC18 media

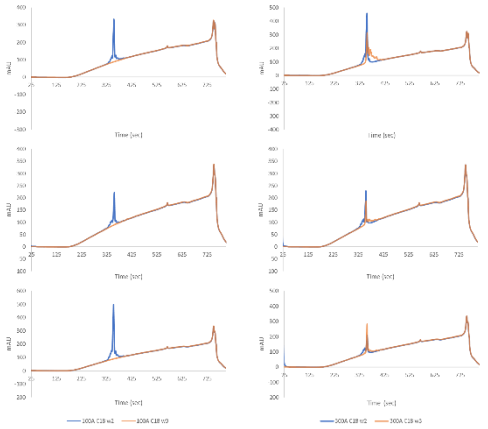

Figure 2. *Behavior of an 18 amino acid peptide when subjected to SPE procedures with two different C18 sorbents. Sample loads of 2 mg (top), 5 mg (middle) or 10 mg (bottom) were tested for retention behavior using either UltraC18 (left) or BioC18 (right) media.*

with 300Å pores. Even at the lowest sample load (0.5% m/m sorbent), incomplete elution of the sample from the BioC18 stationary phase is observed. As the sample load increases, the degree of failed elution, or lost sample, increases as well.

These observations are contrary to the behavior of this same peptide subjected to medium pressure reversed phase purification using a linear gradient. The larger pores, when using gradient-based purification, enable greater access to the stationary phase alkyl chains, ultimately improving resolution and final purity.

Linear 37 Amino Acid Peptide Sorbent Comparison

Given the differences in resolution observed for these two medias when used for flash purification, a larger peptide, expected to behave similarly chromatographically, was also used to compare the two sorbents in an SPE application, Figure 2.

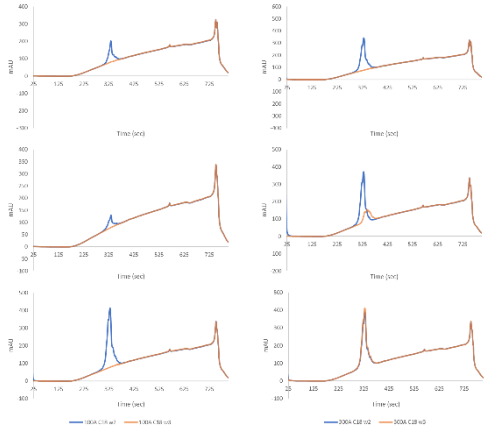

Figure 1. *Behavior of a 37 amino acid peptide when subjected to SPE procedures with two different C18 sorbents. Sample loads of 2 mg (top), 5 mg (middle) or 10 mg (bottom) were tested for retention behavior using either UltraC18 (left) or BioC18 (right) media.*

Surprisingly, the larger peptide, postulated to be somewhat extended in solution, also behaved more predictably using the UltraC18 sorbent. As with the smaller peptide, when subjected to SPE with BioC18 media, the desired peptide is not fully eluted in the desired fraction. This is somewhat surprising given the difference in elution solvent strength (65% Acn) when compared to expected elution concentration (56% Acn). Typically a 10% difference would cause a compound to immediately elute from C18, but that was not the case for samples subjected to SPE with BioC18 media. In a similar manner as the previous sample, increasing sample load also corresponded to increased sample loss due to over-retention.

Conclusion

Herein we compared how sorbent parameters can impact chromatographic behavior under SPE-like conditions for two different peptides. It was hypothesized that increased peptide size would effectively create a sizing effect for the sorbent with smaller pores. This was not observed. In fact, both a moderate and relatively long peptide behaved with high fidelity relative to analytical HPLC information with high recovery using UltraC18 as the sorbent. Future work to further define criteria for sorbent selection while maximizing recovery will include a comparison of linear and cyclic peptides, evaluating the impact of compound shape with behavior. As the rules are further defined, SPE-based cleanup becomes a more attractive library for synthetic peptide libraries – increasing the crude library purity with significantly less time and solvent investment.

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