



## PREP-RP-HPLC: ADDITIONAL / SURROGATE STATIONARY PHASES (ASP/ SSP) BOUND TO REVERSED PHASE COLUMNS RESULT IN 10-FOLD INCREASE IN SAMPLE LOADABILITY

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### Abstract

There are only two ways to increase the amount of sample that can be purified by preparative reversed phase high performance liquid chromatography (Prep-RP-HPLC) in a single run in spite of recent advances in the production of reversed phase derivatized silica stationary supports: (1) The traditional approach is to use a bigger column (greater amount of stationary phase); and (2) Use displacement chromatography which (while labor intensive to develop) uses the stationary phase more effectively. This invention describes a unique Prep-RP-HPLC technique that uses a C-18/ C-8 derivatized silica coated with a hydrophobic quaternary ammonium

salt or quaternary phosphonium salt to result in 7 to 12 fold increase in sample loading (of the crude mixture of organic compounds including synthetic crude peptides) in contrast to the conventional Prep-RP-HPLC technique. This increase in sample loading capacity and output is due to the additional surrogate stationary phase characteristic of the C-18/ C8 adsorbed (bound) quaternary salt. The quaternary surfactant is bound to the C-18/ C-8 chains of the stationary phase via Van der Waals forces (hydrophobic interactions) and ionic interactions with the residual silanols of the stationary phase.

### Introduction

Reversed phase high performance liquid chromatography (RP-HPLC) is used ubiquitously in academic institutions, forensic laboratories, fine chemicals, and pharmaceutical industries etc. for the analysis, characterization, separation, purification and/or isolation of small organic molecules, natural products, and biologically active molecules such as polypeptides, proteins, and nucleotides. In the

pharmaceutical industry, analytical RP-HPLC is used for the release and characterization of raw materials, intermediates, and active pharmaceutical ingredients (APIs). Preparative reversed phase high performance liquid chromatography (Prep-RP-HPLC) is used for the commercial production of Peptide APIs, and most other complex APIs that are not amenable to crystallization.

## Results and Discussion

Preparative RP-HPLC in the elution mode is limited by the loading capacity of the analyte. In the elution preparative RP-HPLC mode, the typical loading capacity of synthetic peptides is in the range of 1 to 2 mgs per ml of packed column volume (viz., 0.1% to 0.2% with respect to total column volume). Table 1 lists the performance of surrogate stationary phase aided Prep-RP-HPLC in contrast to standard Prep-RP-HPLC using crude Leuprolide as example, and tetra-n-butylammonium hydrogen sulphate (TBAHS) as ASP/ SSP.

Table 1

### Purification of Leuprolide: Comparison of the Surrogate Stationary Phase aided Prep-RP-HPLC with the Standard Prep-RP-HPLC

Entry #	Prep RP-HPLC Method	Column Dimensions (ID x L)	Total Column Volume (mL)	Input Crude API (g)	Output Pure API (g)	% Yield	% Purity by HPLC (USP Method)	Relative Loading Capacity
1	Standard RP-HPLC [Comparitive Example]	YMC, ODS-AQ (50 mm x 250 mm, C18, 10 u, 120 Å pore diameter)	491.0	4.0 g	1.20 g	30.0 %	99.86 %	1.0
2	SSP-Purification Method [TBAHS-SSP]	Waters Symmetry (19 mm x 50 mm, C8, 5 u, 100 Å pore diameter)	14.2	1.4 g	0.42 g	30.0 %	99.79 %	12.1
3	SSP-Purification Method [TBAHS-SSP]	Discovery Bio Wide Pore (10 mm x 250 mm, C8, 5 u, 300 Å pore diameter)	19.6	1.2 g	0.32 g	26.7 %	99.73 %	6.7

The purified product (Leuprolide) output of the standard Prep-RP-HPLC is 2.45 mg/ mL of column volume: In contrast the purified product output of the surrogate stationary phase aided Prep-RP-HPLC is 29.6 mg/ mL of column volume (table 1, entry 2) and 16.3 mg/ mL of column volume (table 1, entry 3). These results suggest that loadings of 7 to 12 times capacity of conventional prep-RP-HPLC are achievable with the processes described in the present invention.

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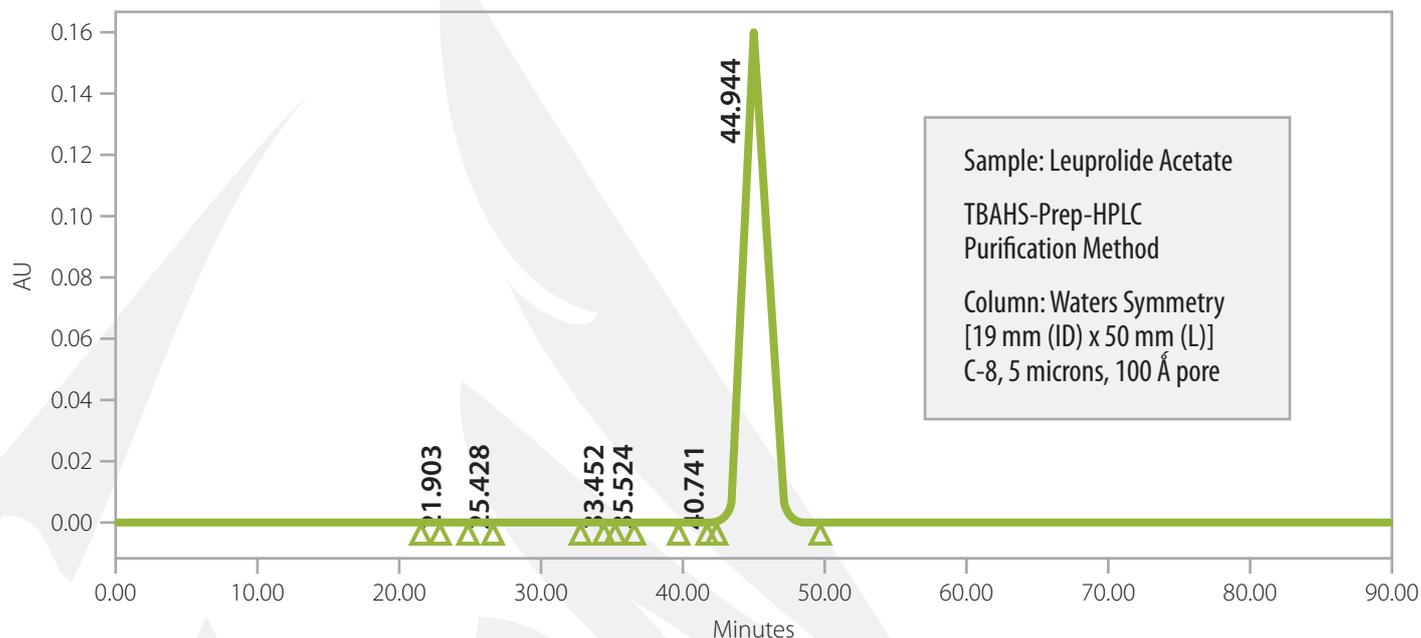
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## Analytical RP-HPLC Profile of Leuprolide acetate obtained using the Additional Stationary Phase aided Prep-RP-HPLC



### Peak Results

	RT	Area	% Area	RT Ratio	USP Plate Count
1	21.903	9593	0.05	0.49	5879
2	25.428	5475	0.03	0.57	6217
3	33.452	6803	0.04	0.74	2938
4	35.524	2789	0.02	0.79	6935
5	40.741	12661	0.07	0.91	10954
6	44.944	17743608	99.79	1.00	3829

### Illustrative Example

The C-18/ C-8 reversed phase column is equilibrated with 5 to 10 column volumes ( $V_c$ s) of 5 to 10% aqueous acetonitrile containing 10 mM TBAHS. The pH of the starting buffer was not adjusted, and was about 1.95 (It is important to keep the concentration of acetonitrile lower than the concentration needed to elute the product on an analytical HPLC column). The crude compound to be purified was dissolved in starting buffer A or aqueous TFA or aqueous HOAc and loaded

on to the column. After the loading is complete, the column is equilibrated with 2  $V_c$ s of Buffer A. Next, the gradient elution process is started. The buffer B is usually 300 mM to 500 mM TBAHS in 5 to 10% aqueous acetonitrile. A linear gradient of 0%B to 100% Buffer B over 10  $V_c$ s is applied. When the product of interest (API) is about to elute, a gradient hold may be applied until all the API has eluted from the column.

## Illustrative Example (continued)

Alternately if it is desired to elute the product in a concentrated form the gradient may be allowed to run its course. The fractions containing the pure API product are combined after confirming that the pooled fraction meets the purification criteria. The approximate quantity of the associated TBAHS is calculated. This is then treated with 1.5 to 2 equivalents of sodium tetrafluoroborate ( $\text{NaBF}_4$ ) and extracted 3 times with chloroform to remove the TBA cation as its tetrafluoroborate salt. The aqueous residue is then loaded on to a C-18/ C-8 column from which all the TBAHS (quaternary ammonium/ phosphonium salt) has been removed. Removal of TBAHS from the C-18/ C-8 column is accomplished by the following steps: The column is first washed with at least 3  $V_c$ s of 80% Acetonitrile-20% Water. Next, the column is washed with 3  $V_c$ s of 100 mM  $\text{NaBF}_4$  in 80% Acetonitrile-20% water. The column is equilibrated with 1M Acetic Acid in 1% Aqueous Acetonitrile

(10  $V_c$ s). The aqueous phase containing "pure API" and excess  $\text{NaBF}_4$  is diluted with water (5X its volume) and loaded on to the C-18/ C-8 column on to the column. The column is washed with 5 to 10  $V_c$ s of 1% phosphoric acid – 1% Acetonitrile – 98% Water to exchange the  $\text{BF}_4$  anions for phosphate anions. The column is then washed with 5 to 10  $V_c$ s of 100 mM aqueous Guanidine. HCl to remove the phosphate anions and to exchange the phosphate anions to chloride anions. Finally the chloride anions are exchanged for acetate anions. The fractions containing the "pure product acetate salt" are combined, and the organic volatiles are removed under reduced pressure. The aqueous residue is lyophilized or precipitated after removal of water. The final API is analysed according to the USP/ EP Methods of Analysis. The overall purification yield was about 30%.

## Summary / Conclusions

1. We have developed a novel Prep-RP-HPLC method that uses a quaternary ammonium salt as an additional/ surrogate stationary phase.
2. The SSP/ ASP-Prep-RP-HPLC has 7X to 12X loading capacity of conventional Prep RP-HPLC Column.
3. We have used this Prep-RP-HPLC method in the solution phase cGMP production of 32 Kgs of a decapeptide NCE.
4. Please refer to Neuland Patents WO 2014/118797 A1 and WO 2015/040635 A2 for additional details.