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Fast and Efficient Purification of Synthetic Peptides by Preparative Chromatography Followed by Novel Fraction Minimizing Technique

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Abstract – Post-synthesis purification and concentration of purified peptides is often the most difficult part of the Purification procedure. In this study we describe the purification of synthetic peptides (Temporin A and calcitermin) by Reverse phase HPLC and removed aqueous buffer by Novel fraction minimizing technique before Freeze drying. Our results indicate that Reverse phase HPLC followed by fraction minimizing technique is a rapid, inexpensive, and relatively efficient method for post-synthetic purification of synthetic peptides.

Key Words: Synthetic Peptide, Peptide Purification, Fraction Distillation, Preparative Chromatography

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INTRODUCTION

Purification of peptides is often the most difficult part of the process used to obtain the pure compounds[1]. Several methods are used for isolation and subsequent purification of the post-synthesis mixture. Depending on the properties of the peptide and the types of impurity, commonly used techniques are ion-exchange chromatography and gel filtration

High-performance liquid chromatography (HPLC)[4-5]is noble method for isolation of small to medium sized synthetic peptides with m.wt less than 10Da. which enables separation of substances with negligible differences between their chemical structures. Unfortunately, Fractions obtained from preparative separations, typically in a large volume of aqueous buffer with volatile solvents, In this *Novel fraction minimizing technique(FMT)* collected pure fraction are Re-injected into the preparative HPLC and creating a new fraction in a volatile organic solvent. Minimal modification is required to existing instruments to use FMT and preparative HPLC technique, and unattended operation is possible. Mobile-phase additives, likely to form salts with the target compound are removed before evaporation. Recovery and purity are unaffected[6].

In this paper we describe the synthesis, subsequent Reverse phase purification and fraction minimizing of peptides. We show, that peptides can be purified by Reverse phase chromatography, one-step procedure furnishes products of >97%-99% purity. Moreover no need of sophisticated equipment and consumption of mobile phase is minimal.

We postulate that use of fraction minimizing technique could be especially beneficial in the isolation of peptides of the various post purification procedures, evaporation to dryness of aqueous/organic matrices is the most time consuming and represents the bottleneck of the whole purification operation (within our laboratories). During this procedure, the collected target material must be recovered intact from a solution containing low-volatile solvents (particularly water) and condiments. Taken precautions against sample decomposition, salt formation, because any errors in this step will affect the purity and/or concentration of the final product.

Our Previous experiences mirroring ,that the chromatography enhancing additives routinely used during preparative liquid chromatography separations (Formic acid, Trifluoroacetic acid (TFA), ammonium hydroxide, and others) get concentrated in the fractions during the evaporation process, potentially leading to salt forms, hydrolysis of some functional groups (e.g. Esters) or derivatization of others (e.g. formulation of amines)[7].

Reduce the time taken for solvent evaporation, avoid salt formation, and reduce exposure to heat (while still ensuring that no residual solvent remains), an investigation into the removal of water and HPLC additives from the fractions before evaporation step was initiated. Various criteria were defined, not least that the quality of the final product must be maintained or improved, little or no capital investment would be required, the existing capabilities of instruments would be maintained, and the overall integrity of the purification platform would not be compromised and the end result is *Fraction Minimizing Technique* (FMT).

MATERIAL AND METHODS:

HPLC-grade acetonitrile (Merck, Darmstadt, Germany), Water (Millipore, Bedford, MA), were used in the preparation of the mobile phases. The HPLC system was fully composed from Waters 4000 Series modules and controlled by Empower software. Kromasil C18 (5-µm particle size, 100-Å pore size) was purchased from Eka Chemicals AB (Bohus, Sweden).

Wang resin for peptide A* synthesis (1% DVB, 1.0 mmol g−1) was obtained from Fluka. Polystyrene AM RAM resin for Peptide B** synthesis (1% DVB, 0.7 mmol g−1) was from Rapp Polymere (Germany) Nα-9-fluorenylmethyl oxycarbonylaminoacids, Trifluoroacetic acid(TFA),1,3-diisopropylcarbodiimide(DIC), diisopropylethylamine (DIEA),4-dimethylaminopyridine (DMAP), piperidine, N-hydroxybenzotriazole (HOBt), Triton, triisopropylsilane (TIS),O-(benzotriazol-1yl) N,N,N′,N′-tetramethyluronium, tetrafluoroborate TBTU) were obtained from Fluka (Switzerland),and dimethyl formamide (DMF), N-methyl-2-pyrrolidone (NMP), dichloromethane (DCM) from Lancaster (Germany). All solvents and reagents used for solid-phase synthesis were of analytical grade.

INSTRUMENTATION:

Waters Delta Prep 4000 preparative chromatography system with waters prep LC Controller, Waters 2487 Dual λ Absorbance Detector, water fraction collector, Waters Prep Pump. The instrument was controlled using Empower 3 (Waters, Milford, MA).To reload fractions to the column added six port valve before the gradient pump (Figure 1) this valve operated by manually.

SYNTHESIS OF THE PEPTIDES:

The peptides were synthesized manually by a solid-phase method using the Fmoc/But procedure[8]. The side-chain-protecting groups of the amino acids were trityl for His; tert-butyl ether for Tyr, Ser, and Thr; tert-butyl ester for Glu; Pbf for Arg; and Boc for Lys. Attachment of the first residue to the Wang resin was achieved by DMAP-catalysed esterification[9]. Both peptides were synthesized using the Fmoc/But procedure method, according to a two-step procedure:

1. 5- and 15-min de protection steps using 20% piperidine in a 1:1 (v/v) mixture of DMF and NMP (in the presence of 1% Triton); and

2. Coupling reactions performed with the protected amino acid diluted in a 1:1 (v/v) mixture of DMF and NMP in the presence of 1% Triton, using DIC as the coupling reagent in the presence of HOBt (Fmoc-AA:DIC:HOBt 1:1:1) for 1 h.

Completeness of each coupling reaction was monitored by means of the chloranil test. If the test was positive, the coupling reaction was repeated using TBTU and HOBt in the presence of DIEA, with mixing for 2 h.

The protected resins were treated with a mixture of 95% Trifluoroacetic acid (TFA), 2.5% water, and 2.5% TIS for 1 h. After cleavage the solid support was removed by filtration and the filtrate was concentrated under reduced pressure. The cleaved peptide was precipitated with diethyl ether and lyophilized.

PURIFICATION OF CRUDE PEPTIDE:

Crude peptides were purified by preparative RP-HPLC using a Kromosil C18 column (21.2 × 250 mm) with 0.2%Trifluoroacetic acid in water (Mobile phase A), Acetonitrile (Mobile phase B) at a flow rate of 18 ml/min with HPLC gradient 90% mobile phase A to 90%mobile phase B over 25minutes.

Preparative system is stabilized with Protocol A (Figure2) by changing solvent selection value in to mobile phase mode. A known accurate weight of peptide (approximately 5.0 g) was dissolved in 10.0ml of DMSO: Acetonitrile (50:50). Preparative system is 5min stabilized with initial gradient conditions and 1mL crude solution was injected into the system and peptide is eluted with gradient program and collected desired peptide in to tantamount fractions in a 30sec time interval with Combination of Trifluoroacetic acid, water and acetonitrile.

Purity of collected peptides fractions was confirmed by ESI-MS and analytical RP-HPLC using a Kromosil C18 column (5µm, 4.6 × 250 mm) and a gradient of 100% A to 100% B over 20 minutes at a flow rate of 1 ml/min. The total crude solution is completed in continues repeated cycles and all the fractions containing >97% ~200ml pure fraction solution were combined, eliminated water and Trifluoroacetic acid by using fraction minimizing technique and produced new fraction, Concentrated this new fraction at 25°C temp and Freeze dried to get final pure peptide.

ELIMINATING ADDITIVES AND AQUEOUS MOBILE PHASE:

After purification pure fractions reloaded on column to eliminate water, Trifluoroacetic acid which is difficult

to remove by concentration**,** pure fraction was diluted with water to retain on column.

Preparative system mobile phase A is replaced with mille-Q water and mobile phase B is kept constant, Kromosil C_{18} (21X250mm) column was washed with 18ml/min flow and stabilized with Mobile phase A for 5min, Solvent selection valve is switched to protocol B (Figure3) to load diluted pure fractions directly to column with same flow, solvent selection valve turn back to Protocol A after total fraction loaded on the column.

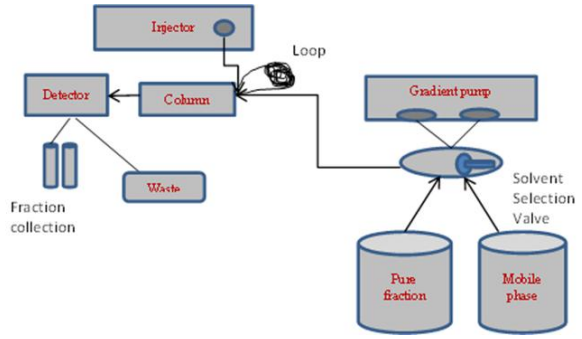


Figure1. Schematic of the modified Waters Instrument configured to perform both preparative separations Fraction minimizing technique using direct-injection and at column-dilution injection protocols

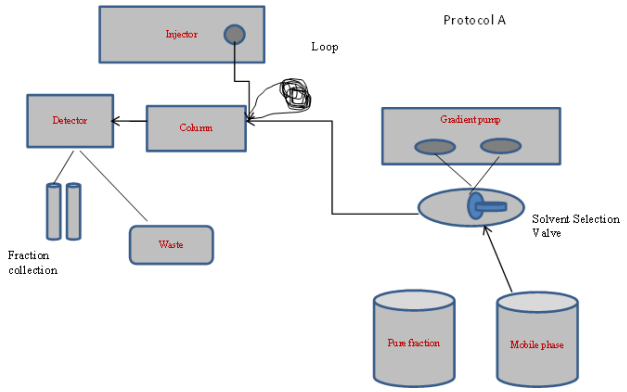


Figure2. In protocol A the modified system performs as regular system the mobile phase is pumped through the solvent section valve.

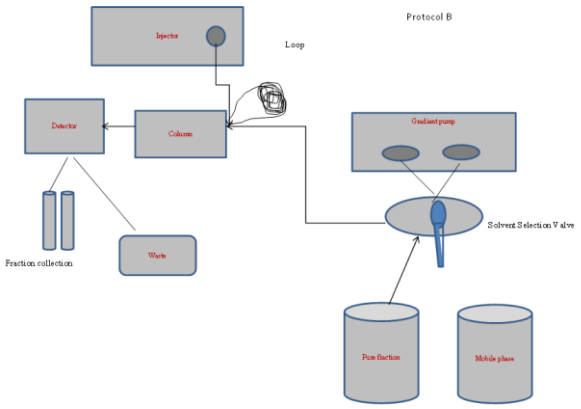
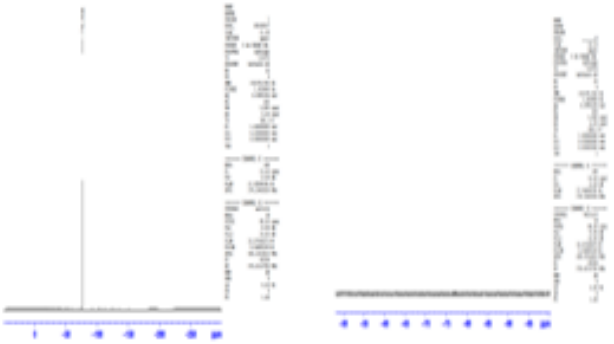


Figure3. In protocol B the modified system used for **Fraction Minimizing Process** the pure fraction was pumped through the solvent section valve.

An isocratic hold in Mobile phase A for 4 min was followed by an isocratic hold in Mobile phase B for 3min to elute pure peptide in acetonitrile and eluted fraction was collected, in total cycle flow was kept constant.

For a typical fraction volume of 15 mL, the overall run time was 15 min, The final fraction was concentrated to remove acetonitrile at 25°C using lab rotavapor and Freeze dried to remove trace level of water and acetonitrile.



C) Regular process D) FMT process

Figure4: 19FNMR spectra of purified peptide after regular concentration process C and after fraction minimizing technique D.

Elimination of Chromatographic Modifiers: Chromatographic modifiers, such as TFA, formic acid, and ammonium hydroxide, are widely used in many laboratories (including ours) to improve peak shape and enhance resolution during chromatographic separations. Although typically used in low concentrations, these additives are collected with the target compound during fractionation and concentrated during the initial stages of evaporation. The presence of these additives can, occasionally, lead to the decomposition of the final product; however, more commonly, they react with the product to form salts.

Using FNMR monitoring showed (Figure4) the effectiveness of fraction minimizing technique for removing TFA from fractions eluted from Prepative HPLC it is clearly representing fluorine absence after fraction minimizing technique when compare to regular concentration process. The advantage of TFA removal from fractions before evaporation has become a routine application for RP-HPLC purified compounds. Also analytical scientists chance to use

any type of additives to purify synthetic compounds to get better separation and recoveries followed by fraction minimizing technique.

RESULTS AND DISCUSSION:

The aim of our experiments was to obtain highly purified peptides relatively quickly. RP-HPLC followed by fraction minimizing technique is an easy and inexpensive means of purification and concentration of peptides. It enables rapid and efficient purification of peptides with minimal consumption of organic solvents and eliminates the need for sophisticated equipment.

At the outset of this study, a procedure to reduce the potential for salt formation, residual solvent and sample exposure to elevated temperatures for extended times during evaporation was envisaged. It was critical that this fraction minimizing technique did not decrease the quality of the final product or the amount recovered from our automated purification platform. It was also important that the cycle time from sample submission to availability for further testing did not increase. A final requirement was that minimal capital expenditure would be required to achieve this goal.

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