# Streamlining Peptide Purification Workflows through Analytical to Preparative with Improved Scale-up Strategy

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## 1. Introduction

Peptide therapeutics offer significant potential in the pharmaceutical industry, combining the advantages of small molecules and biologics. Nonetheless, the development of peptide therapeutics encounters significant challenges, especially in the purification process. The biological activity of peptides depends on their amino acid composition and structure. Therefore, precise isolation using reversed phase high-performance liquid chromatography (HPLC) is necessary for pharmaceutical applications.

Current purification workflows frequently become bottlenecks due to time and resource constraints. As shown in Fig. 1, the traditional one-step scale-up workflow, while effective for small molecules, often fails to provide optimal gradient profile for peptides, resulting in purification failures and operational inefficiencies.

To address these issues, this study introduces an improved scale-up strategy that incorporates rapid focused gradient validation into the purification process. This approach aims to enhance workflow efficiency, increase purification success rates, and facilitate the advancement of peptide therapeutics.

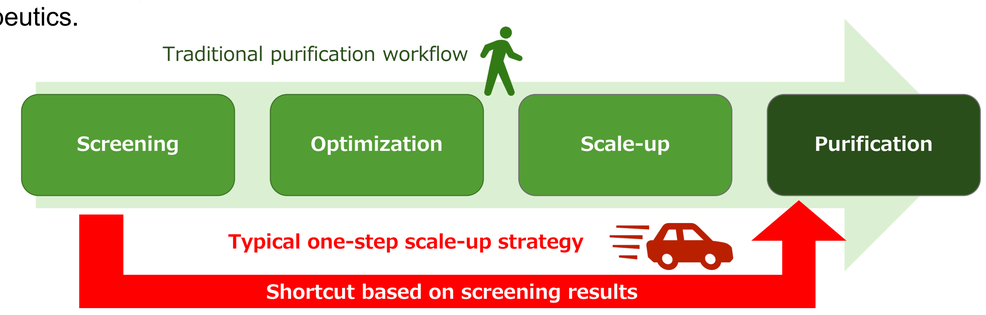


Fig. 1 Traditional purification workflow and typical One-Step Scale-Up strategy.

## 2. Methods

### 2.1. One-Step Scale-up Algorithm

The one-step scale-up algorithm was designed to automatically generate optimal focused gradient profile for purification. The initial concentration of solvent B (Int.B.conc.) in the preparative LC-MS was determined based on the retention time in the analytical LC-MS. The gradient profile in preparative LC-MS, involving the gradient duration and the rate of change in solvent B concentration, was standardized to ensure consistency across all runs. Notably, the target peaks were expected to elute at approximately the same time, around 8 minutes.

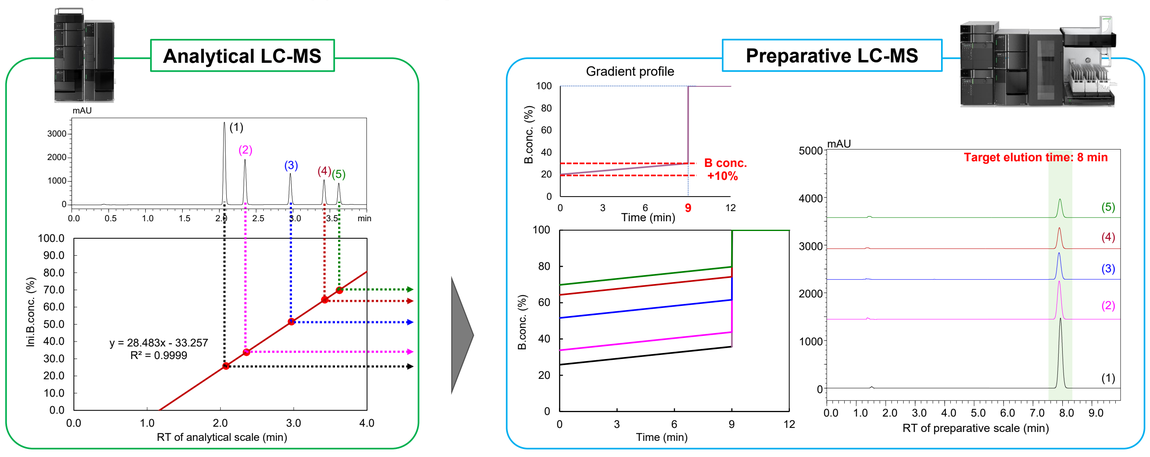


Fig. 2 Overview of the one-step scale-up algorithm from screening to purification

Table 1 Screening conditions

| LC Conditions | | MS conditions | |
|---|---|---|---|
| Column | : Shim-pack Scepter C18-120 (50 mm x 3.0 mm I.D., 3 µm) *1 | Ionization | : ESI/APCI (DUIS), Positive mode SCAN (*m/z* 200-2000) |
| Mobile phase | : (A) 0.1% Formic acid in water (B) 0.1% Formic acid in water in acetonitrile | Nebulizing Gas Flow | : 2.0 L/min ($N_2$) |
| Flow rate (Mobile phase) | : 1.5 mL/min | Drying Gas Flow | : 5.0 L/min ($N_2$) |
| Gradient profile | : B Conc. 5%(0 min)→90%(3.0-4.0 min) | Heating Gas Flow | : 7.0 L/min ($N_2$) |
| Column temp. | : 25 ℃ | DL Temp. | : 200 ℃ |
| Detection (PDA) | : 220 nm (SPD-M40, standard cell) | Desolvation Temp. | : 450 ℃ |
| Sample | : DMSO solution | Interface Voltage | : 1.0 kV |
| Injection volume | : 2 µL | | |

*1 P/N: 2227-31015-01

Table 2 Purification conditions

| LC Conditions | | | |
|---|---|---|---|
| Column | : Shim-pack Scepter C18-120 (150 mm x 20 mm I.D., 5 µm) *2 | MS make-up solution | : Methanol |
| Mobile phase | : (A) 0.1% Formic acid in water (B) 0.1% Formic acid in water in acetonitrile | Flow rate (MS make-up) | : 1.5 mL/min |
| Flow rate (Mobile phase) | : 20 mL/min | **MS conditions** | |
| Gradient profile | : B Conc. x *3%(0 min)→(x+10)%(9 min) →100%(9.01-12.0 min) | Ionization | : ESI/APCI (DUIS), Positive mode SCAN (*m/z* 200-2000) |
| Column temp. | : Ambient temperature | Nebulizing Gas Flow | : 2.0 L/min ($N_2$) |
| Detection (PDA) | : 220 nm (SPD-M40, preparative cell) | Drying Gas Flow | : 5.0 L/min ($N_2$) |
| Sample | : DMSO solution | Heating Gas Flow | : 7.0 L/min ($N_2$) |
| Injection volume | : 200 µL | DL Temp. | : 200 ℃ |
| | | Desolvation Temp. | : 100 ℃ |
| | | Interface Voltage | : 1.0 kV |

*2 P/N: 227-31102-03 *3 Values calculated by the scale-up algorithm

### 2.2. Improved Scale-up Strategy with Rapid Focused Gradient Validations

The one-step scale-up method often results in purification failures for peptides, as many compounds elute later than the target elution time. This delay varies based on peptide properties, indicating a failure to design appropriate focused gradients and highlighting the need for improved strategies. To overcome these challenges, an enhanced scale-up strategy has been developed, integrating rapid focused gradient validations. This approach tailors gradient profile to the unique characteristics of each peptide. Rapid validation through analytical LC-MS optimizes the purification conditions, which increases success rates and reduces operational inefficiencies. Fig. 3 provides an overview of the improved scale-up strategy.

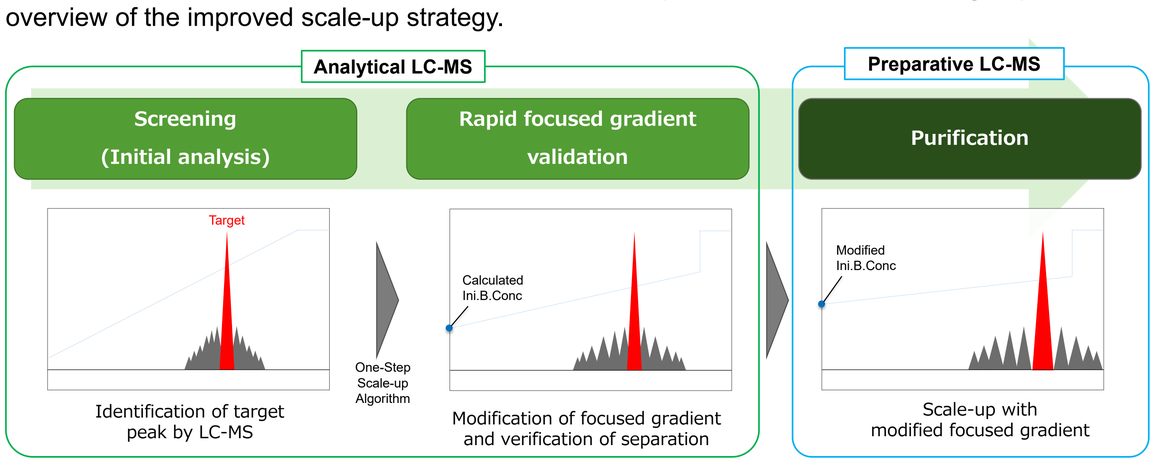


Fig. 3 Overview of the Improved Scale-up Strategy

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The focused gradient modification method for purification involves the following steps:

**1. System volume and hold-up time measurement**: Measure the system volume of the analytical LC-MS and the column hold-up time ($t_0$) to determine the gradient delay time ($t_D$).

**2. Gradient profile development and run**: Create a gradient profile using retention times from screening analyses, executed on the same system and column as the screening.

**3. Retention time identification**: Identify the retention time of the target peak ($t_e$) and calculate the organic solvent ratio at elution ($C_e$) using:

$$C_e = Ini.B.Conc + Gradient.Slope \times (t_e - t_D)$$

**4. Adjustment value of ini.B.Conc. Calculation**: Calculate the adjustment value of ini.B.Conc. ($C_A$) as:

$$C_A = C_e - (Ini.B.Conc + 10)$$

**5. Application to focused gradient in purification**: For scale-up, apply the modified Ini.B.Conc. as:

$$Modified\ Ini.B\ Conc. = Ini.B\ Conc. + C_A$$

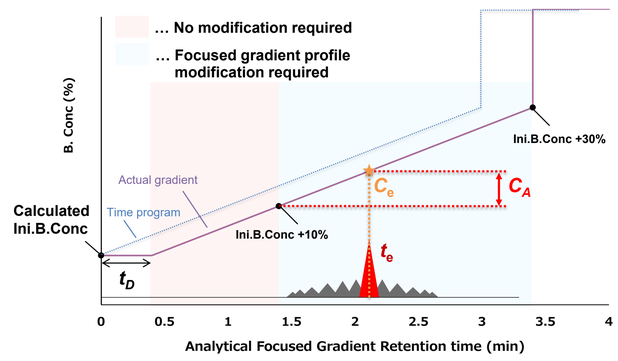


Fig. 4 Graphical explanation of the focused gradient modification method

## 3. Results and discussion

### 3.1. Achievements with the Improved Scale-up Strategy

The effectiveness of the scale-up—particularly the ability to establish appropriate focused gradients—was assessed through the retention times of target peaks in preparative LC-MS. A successful scale-up was confirmed when target compounds eluted within a target elution time of 8 minutes ±2 minutes, as designed by the One-Step Scale-up Algorithm.

The scale-up success rate was assessed across 130 unpurified peptide samples with diverse characteristics, including sequences composed solely of natural amino acids, those containing non-natural amino acids, as well as linear and cyclic structures. When the One-Step Scale-up Algorithm was applied, the success rate was only 10% (13 samples). In contrast, when the Improved Scale-up Strategy was implemented, the success rate dramatically increased to 91.5% (119 samples). This significant improvement demonstrates that just a few minutes of rapid focused gradient validations can significantly boost the success rate of scale-up processes, thereby improving the overall efficiency of purification operations.

### 3.2. Practical examples with peptide compounds

Chromatograms resulting from the application of the One-Step Scale-up and the Improved Scale-up Strategy for unpurified peptides are illustrated in Figs. 5 and 6. In cases where the One-Step Scale-up Strategy was used, most unpurified peptides caused target compounds to elute later than the desired elution time, resulting in co-elution with impurities during column washing at the gradient endpoint.

Conversely, when the Improved Scale-up Strategy was applied, target compounds eluted close to the desired elution time. The adjustment value of ini.B.Conc. ($C_A$), calculated through rapid focused gradient validations, varied among different peptides. This indicates that just adjusting the values from the One-Step Scale-up Algorithm would not be sufficient to create optimal gradient profile for each peptide. This emphasizes the utility of the Improved Scale-up Strategy in enhancing purification outcomes.

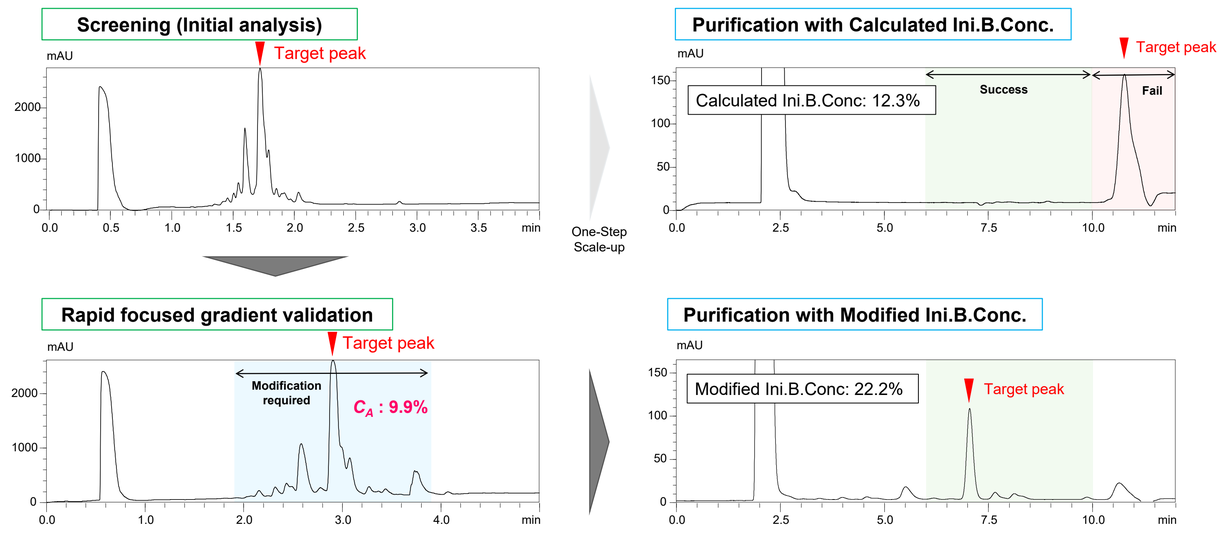


Fig. 5 Chromatograms of the Improved Scale-up Strategy applied to unpurified peptide A

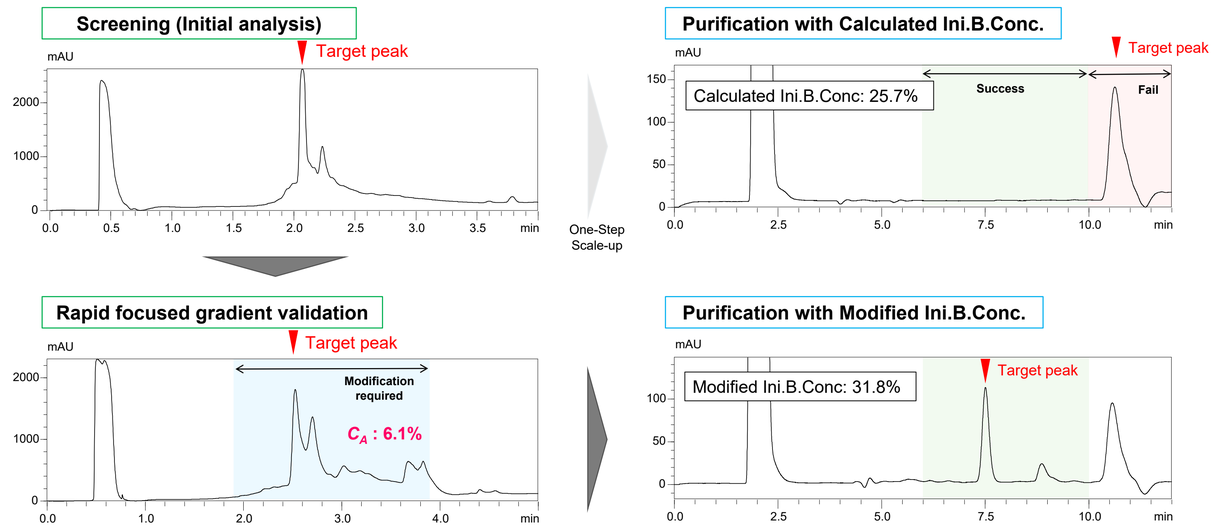


Fig. 6 Chromatograms of the Improved Scale-up Strategy applied to unpurified peptide B

## 4. Conclusion

The implementation of the Improved Scale-up Strategy significantly enhances the efficiency and success rates of peptide purification workflows. By integrating rapid focused gradient validations, this approach effectively tailors purification conditions to the specific characteristics of each peptide, overcoming the limitations of traditional methods. The substantial increase in success rates—from 10% to 91.5%—demonstrates the effectiveness of this strategy in achieving optimal purification outcomes. This advancement not only streamlines the purification process but also supports the broader development of peptide therapeutics in the pharmaceutical industry.