Optimization of Vitamin B12- carbonyl-putrescine-FF-Boc synthesis

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Abstract

Objective: The present studies showed that Vitamin B12 has good potential to enable the oral delivery of protein drugs. Recently, we synthesized a Vitamin B12- carbonyl-putrescine-FF-Boc (VB12-FF-Boc) that was expected to utilize the absorption pathway of VB12 to achieve oral delivery of protein drugs. However, during the activation of the carboxyl, organic bases might trigger racemization at this site. Herein, we explore the effects of three factors on racemization: Organic Base, Addition Sequence, and Coupling Agent. The synthesis method of VB12-FF-Boc was optimized to increase the yield of VB12-FF-Boc and reduce the production of racemate.

Keywords

Vitamin B12, Racemate, Organic Bases, Addition Sequenc, Coupling Agent.

1. Introduction

The vitamin B12 uptake system had great potential for enhancing the absorption mechanisms of oral proteins, peptides and immunogens[1]. Vitamin B12 was taken up as a complex with the intrinsic factor protein (IF) in the ileum. After binding to the vitamin B12-IF complex to the vitamin B12-intrinsic factor receptor, the receptor and ligand were endocytosed. The receptor is recycled to the membrane while the vitamin B12-IF complex is routed to the lysosome. Here IF is broken down and vitamin B12 is eventually released into the portal circulation, where it is bound and transported by the transcobalamin II protein[2, 3]. In previous works, after vb12 modification, the degree of drug internalization of nanoparticles in the cell model was significantly higher than that of unmodified nanoparticles, and the amount of insulin transported was also increased[4-6].

Vitamin B12 was a highly functional molecule with a variety of methods for exposing its site of reaction[7]. Sites available for modification on VB12 include the peripheral amides, hydroxyl groups, the cobalt (III) ion and the phosphate moiety[1]. The 5'-OH site could react with an acid anhydride or CDI / CDT to further transformed into either a carboxylic acid or azide. It had been reported that the modification of the 5'-OH group at the ribose site of VB12 does not affect the binding ability of VB12 to the intrinsic factor (IF)[8, 9].

Self-assembly of diphenylalanine (FF) – based peptides had been the focus of considerable research in the supramolecular chemistry and biomaterials over the past few years[10]. Owing to the structural diversity, facile functionalization and excellent biocompatibility, the diphenylalanine-based assemblies are extremely attractive as building blocks for various applications such as nanofabrication and tissue engineering[11, 12]. The self-assembling nature of FF-based peptide building blocks providing broad prospects for the potential applications in biological fields, such as bioimaging biosensors and drug delivery[13]. Previously studies have shown that the Boc-Phe-Phe-OH building block could assembled into a homogeneous population of either spherical or tubular nanostructures[14].

In our previous work, the ribose 5'-OH of VB12 was activated with carbonylimidazole CDT to form an active carbamate which was further reacted with putrescine to produce VB12-carbonyl-putrescine. In this work, we activated the α-carboxyl group of BOC-FF with the coupling agent HATU under basic conditions to form an active amide intermediate, which reacting with VB12-carbonyl putrescine to obtain VB12-FF-BOC. Furthermore, we optimized the synthesis of VB12-FF-Boc by exploring

the effects of Organic Bases, Coupling Agents and Addition Sequences on the racemization of products.

2. Materials and Methods

2.1 Materials

Boc-Phe-Phe-OH was purchased from Bachem; N,N-Diisopropylethylamine (DIEA), Hexafluorophosphate Benzotriazole Tetramethyl Uronium (HBTU), Hexafluorophosphate Azabenzotriazole Tetramethyl Uronium (HATU), and N,N-Dimethylformamide (DMF) were purchased from Aladdin; All other chemicals and solvents were analytical or chromatographic grade. Vitamin B12- carbonyl- putrescine was produced by our laboratory.

2.2 Synthesis of Vitamin B12- FF-Boc

HBTU, DIEA and Boc-FF were added to DMF and mixed evenly in a penicillin flask ultrasonic water bath 5 mins (70 W, 30 ° C) according to a molar ratio of 1.36:1.36:1. Then, under ultrasonic conditions one-fold DIEA molar equivalent of was Vitamin B12- carbonyl- putrescine added and the bath was sonicated for 2 h (70 W, 30℃). After the reaction, the mixture was precipitated with ethyl acetate (50ml), the precipitation was centrifugated at 7000g for 5 mins, the precipitates were lyophilized to obtain a solid product. The product was analyzed by reverse phase high performance liquid chromatography (RP-HPLC).

Based on the above methods, explore the effects of the following three factors on product synthesis, Organic Base, Addition Sequence, and Coupling Agent. According to the order in Table 1, the synthesis method of the latter changes with the conditions of the former screening.

Table 2- 1. The influencing factors of VB12-FF synthesis

Sequence	Factors	Group
1	Organic Base	With DIEA
		Without DIEA
2	Addition Sequence	Add HBTU first and then add VB12- carbonyl- putrescine
		Add VB12- carbonyl- putrescine first and then add
3	Coupling Agent	HBTU
		HATU

2.3 HPLC analytical method

For purification and preparation of products, separation was performed on an Waters SYMMETRY-C18 column (250mm*20mm,10um) column with mobile phase consisting of acetonitrile and water with gradient elute at the flow rate of 15ml/min. The UV wavelength used for detection was set at 361 nm, the injection volume was 4mL.

For analysis of purified products, separation was performed on a ZORBAX EDIPSE XDB-C18 column(250mm*4.5mm,5um)column with mobile phase consisting of acetonitrile and water with gradient elute at the flow rate of 1ml/min. The UV wavelength used for detection was set at 361 nm, the injection volume was 20μL.

2.4 Peak area ratio (%) and Racemization ratio (%)

Analyze the HPLC map, the Peak area ratio and Racemization ratio of VB12-FF-Boc were calculated by the peak area. Peak area ratio (%) and Racemization ratio (%) was calculated as follows:

$$\text{Peak area ratio}(\%) = \frac{S1}{St} \times 100 \quad (1)$$

$$\text{Racemization ratio}(\%) = \frac{S2}{S1+S2} \times 100 \quad (2)$$

Where S1 and S2 are the peak area of VB12-FF-Boc and Racemate, St represents the total peak area.

2.5 ESI-MS analysis of VB12-FF-Boc

The purified product VB12-FF-Boc was dissolved in 200 μl of acetonitrile, and passed through a 0.22 μm filter membrane,ensure sample clarification without particles. The VB12-FF-Boc was identified by electrospray ionization mass spectrometry (ESI-MS).

3. Results

3.1 Synthesis method trigger the generation of racemate

The carboxyl group of Boc-FF was dehydrated and condensed with the amine group of VB12-carbonyl putrescine to form an amide bond, thereby obtaining VB12-FF (Fig. 1 A). The results obtained by HPLC showed that during the synthesis of VB12-FF-Boc, the reaction produced two products, product A and product B (Fig. 1 B). Mass spectrometry was performed by collecting the eluates of the main absorption peaks of the two products, and the mass spectrometry results of the two products were the same, indicating that they are isomers (Fig. 1 C and D). The synthesis of vB12-FF was a mild, highly selective reaction. Only the amine group of VB12 carbonyl putrescine was coupled to the carboxyl group, while Boc-FF was prone to racemization upon coupling. Thus, the isomer in the synthesis product might be the racemate of VB12-FF-Boc.

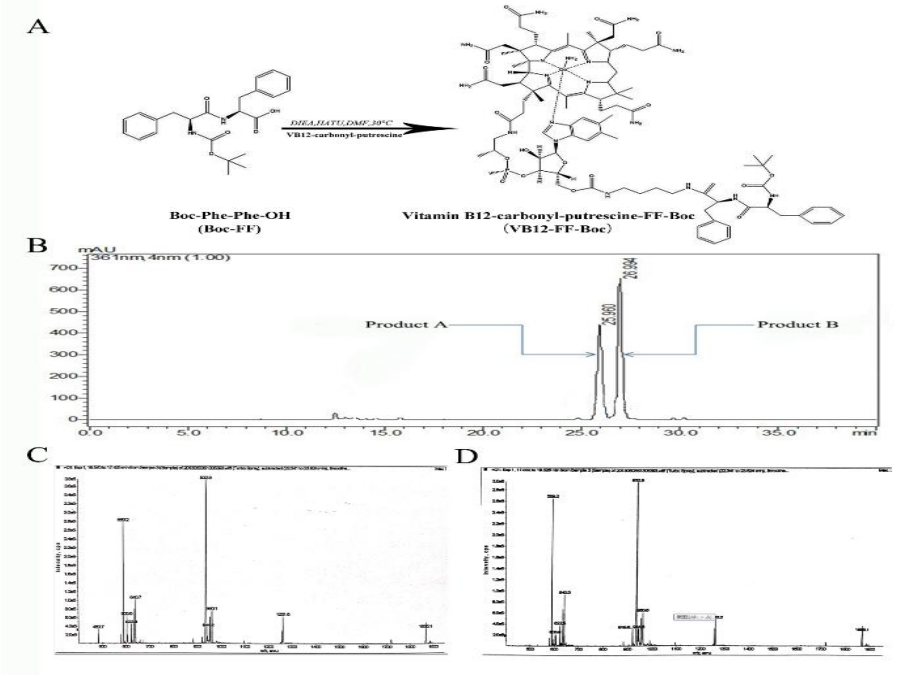

Fig. 1 (A) Synthesis of VB12-carbonyl-putrescine-FF-Boc (schematic). (B) HPLC analysis of the synthesis product of VB12-FF-Boc. (C,D) Mass spectrum of Product A and B.

3.2 Effect of the Organic Bases DIEA on Racemization of Products

In the synthesis of an amide, activation of a carboxyl group was necessary, but in the process, it might trigger racemization at the alpha carbon atom[13]. The two main pathways for racemization were enolization and the formation of oxazolone, while the presence of an organic base facilitates the formation of racemates[15, 16]. Here, we investigated the effect of organic base DIEA on the synthesis of Vb12-FF-Boc and further verified whether the resulting isomer was produced by racemization. The synthesis of vb12-ff-boc with the organic base DIEA still resulted in two products (Fig. 2 A). VB12-FF-BOC was synthesized without the organic base diatomic, and only a small amount of VB12-FF-BOC was synthesized. (Fig. 2 B). After the synthesis without organic base, the Peak area ratio and Racemization ratio were significantly reduced (Fig. 2 C). These results indicate that the organic base DIEA was a necessary condition for the synthesis of VB12-FF-BOC, and it was indicated that the product B is a racemate of VB12-FF-BOC.

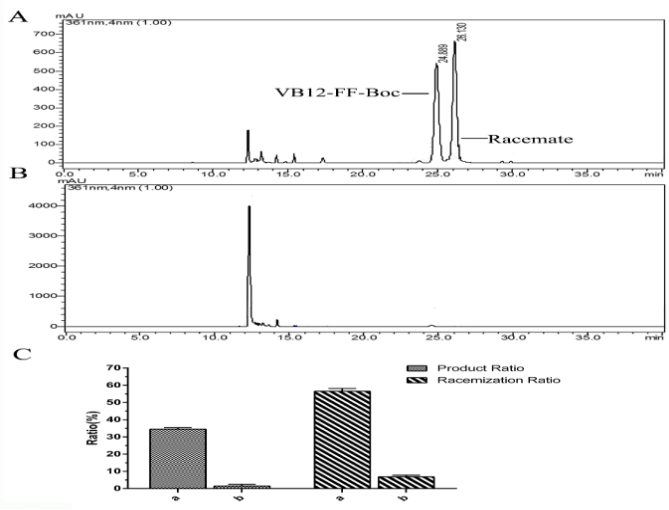

Fig. 2 (A) HPLC analysis the product of VB12-FF-Boc was synthesized with Organic Bases DIEA. (B) HPLC analysis the product of VB12-FF-Boc was synthesized without Organic Bases DIEA. (C) Effect of Organic Bases on Racemization of Products, "a" was the synthesized with Organic Bases DIEA, "b" was the synthesized without Organic Bases DIEA

3.3 Effect of the Addition Sequence on Racemization of Products

The effect of racemization was investigated by changing the order of addition between HBTU and VB12. High performance liquid chromatography first analyzed the product obtained by the addition of HBTU, indicating that the absorption peak area of the racemate was greater than VB12-FF-Boc (Fig. 3 A). The product obtained by first adding Vb12 carbonyl putrescine had a smaller absorption peak of the racemate than Vb12-FF-Boc (Fig. 3 B). The Peak area ratio and Racemization ratio of the product obtained by the first addition of vb12-carbonyl putrescine were 14.07% and 71.53%, respectively(Fig. 3 C). These results shown that the addition of HBTU followed VB12-carbonyl-putrescine could increase the formation of the product and reduce the formation of the racemate.

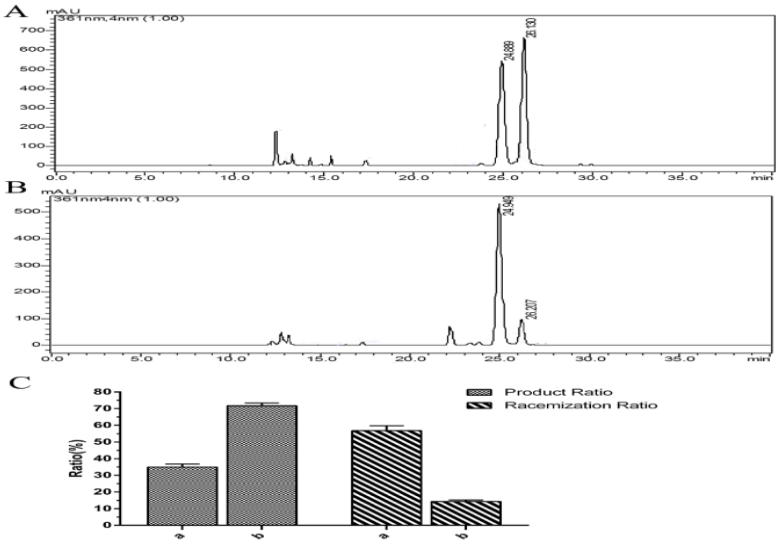

Fig. 3 (A) HPLC analysis of VB12-FF-Boc was obtained by adding HBTU first. (B) HPLC analysis of VB12-FF-Boc was obtained by VB12-carbonyl-putrescine first. (C) Effect of Sample Addition Sequence on Racemization of Products, "a" was the VB12-FF-Boc was obtained by first adding HBTU first, "b" was the VB12-FF-Boc was obtained by adding VB12-carbonyl-putrescine first.

3.4 Effect of the Coupling Agent Type on Racemization of Products

Investigate the effect of Coupling Agent on Racemization of Products by using HBTU/HATU to participate in the synthesis of VB12-FF-Boc. When HBTU and HATU were used as coupling agents, the HPLC analysis showed that the absorption peak area of VB12-FF-Boc was larger than that of the

racemate (Fig. 4 A and B). In the reaction involving HATU, the Racemization ratio and Peak area ratio reached 79.61% and 7.08% (Fig. 4 C). These results indicate that since the activity of HATU is higher than that of HBTU, the reaction can be completed more quickly, thereby increasing the yield of VB12-FF-Boc and reducing the formation of the racemate.

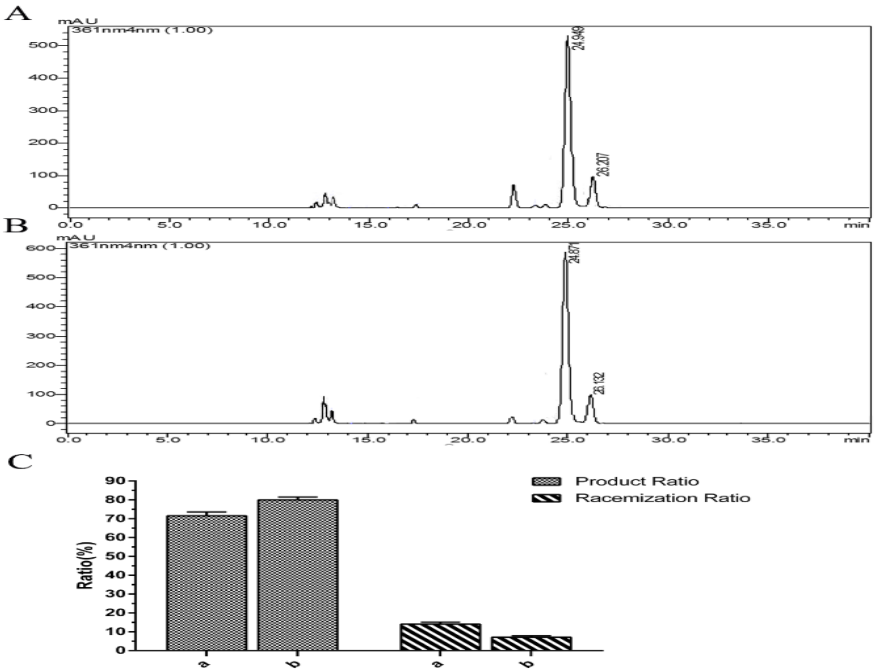

Fig 4. (A) HPLC analysis of purified VB12-FF-Boc. (C) EMI-MS analysis of VB12-FF-Boc (VB12-FF-Boc).

3.5 RT-HPLC and EMI-MS analysis of purified product

The crude product was purified by semi-preparative HPLC, and according to HPLC analysis, the purity of the collected sample was above 99% (Fig. 5 A). Mass spectrometry results showed that the corresponding molecular weight of VB12-FF-Boc was 1885[H+], consistent with the predicted value (Fig. 5 B). These data demonstrate the successful synthesis of VB12-FF-Boc.

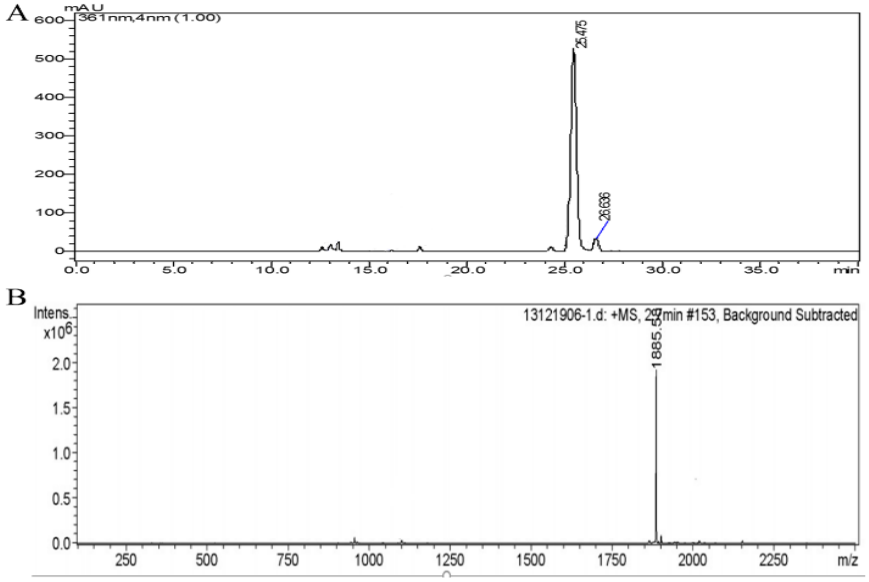

Fig 5. (A) HPLC analysis of purified VB12-FF-Boc. (C) EMI-MS analysis of VB12-FF-Boc (VB12-FF-Boc).

4. Discussion

In summary, we provide a method for Vitamin B12 modified Boc-FF, and we explored the effects of organic bases, sample addition sequences, and coupling agent types on racemization of the product. After adding DIEA, VB12 carbonyl putrescine and Boc-FF, HATU was added for coupling, the product yield was up to 80%, and the racemization rate was reduced to below 10%. The molecular weight of VB12-FF was consistent with the predicted value, confirming the successful synthesis of VB12-FF-Boc. The synthesis process of VB12-FF-Boc was optimized, the yield of VB12-FF-Boc was increased, and the yield of racemate was reduced. Its laid a good foundation for the development and utilization of VB12-FF-Boc.

Acknowledgements

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