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Development of a Reversed-Phase Chiral HPLC Method for Concurrent Chiral, Achiral, Assay, and Identification Analysis of Boc-L-Valine

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Abstract

Boc-protected amino acids, such as Boc-L-valine, are widely used as key starting materials in the synthesis of pharmaceutical drug substances, necessitating efficient and reliable analytical control strategies. In this work, a streamlined reversed-phase chiral HPLC method was developed for the comprehensive characterization of Boc-L-valine without the need for derivatization. The method enables simultaneous determination of chiral and achiral impurities, assay, and compound identification within a single analytical run. This integrated approach simplifies the analytical workflow, increases throughput, and significantly reduces overall method validation efforts. Moreover, the method provides a robust and versatile platform readily applicable to routine quality control and method development for other Boc-protected or Fmoc-protected amino acids.

Keywords

Boc-L-Valine, Reversed-Phase Chiral HPLC, Polysaccharide Chiral Stationary Phase, Achiral Amino-Acid Impurities, Derivatization GC Benchmark

1. Introduction

Boc-protected amino acids serve as essential building blocks in the synthesis of pharmaceutical drug substances, therapeutic peptides, and fine chemicals [1]-[4]. L-Valine, featuring an isopropyl chiral center, is incorporated into several pharmaceuticals, including INGREZZA® (valbenazine; Neurocrine Biosciences, Inc.) [5], valacyclovir, valganciclovir, valsartan, and elvitegravir [4]. For instance, INGREZZA® is approved for reducing the severity of tardive dyskinesia and Huntington's chorea. Its drug substance is formulated as a single pure diastereomer

containing an L-valine moiety, which confers enhanced potency and safety through well-defined stereochemistry [5]. Use of a single, well-defined L-valine diastereomer maximizes the activity of the desired chemical while minimizing exposure to off-target stereoisomers that may reduce target engagement or introduce unexpected toxicity. This approach ultimately enhances both the potency and safety profiles of the resulting drug substance.

Numerous strategies for the enantiomeric separation of amino acids have been documented, such as o-phthalaldehyde (OPA) and thiol derivatization followed by reversed-phase high-performance liquid chromatography (HPLC) [1], in-situ derivatization with heptafluorobutyl chloroformate and gas chromatography with flame ionization detection (GC-FID) [6], and derivatization with heptafluorobutyl chloroformate/methylamine prior to gas chromatography-mass spectrometry (GC-MS) [7]. Chiral GC approaches have included trimethylsilyl (TMS) derivatization of Boc-L-valine, alongside explorations of alkylation, arylation, silylation, and acylation [8].

Chiral separations of Boc-protected amino acids have been achieved using various HPLC columns, including Chiralpak AD-H [1], Ultron Ovomucoid (OVM) [2], and Chiralpak IA, IC, and QNAX [3]. Among commercially available chiral stationary phases, polysaccharide-based CSPs have demonstrated the highest selectivity, with over 90% of enantiomeric excess (ee) estimations utilizing polysaccharide-based CSPs [5]. The most important interactions for polysaccharide-based CSPs are H-bridge, π-π, and van der Waals forces [9] [10]. However, no reports describe a single-run method enabling simultaneous chiral, achiral, and assay analysis of Boc-L-valine via reversed-phase gradient chiral HPLC.

This study introduces a simple and robust reversed-phase chiral HPLC method for the simultaneous chiral, achiral, assay, and identification analysis of Boc-L-valine without derivatization, addressing the need for an efficient, integrated approach.

Experimental

The chemicals and reagents are listed below:

Boc-L-Valine: >99.0%, Optical rotation: $[\alpha]^{20D} = -6.2^{\circ} \pm 0.5^{\circ}$, Millipore Sigma Catalog# 15528-25 g.

Boc-D-Valine: >98%, Optical rotation: $+6.5^{\circ} \pm 0.4^{\circ}$, ThermoFisher Scientific Catalog# AAL0919306.

Boc-L-alanine: >99.0%, Optical rotation: $[\alpha]^{20D} = -25^{\circ} \pm 1^{\circ}$, Millipore Sigma Catalog# 15380-25 g.

Boc-L-phenylalanine: >99%, Optical rotation: $[\alpha]^{20D} = -25^{\circ} \pm 2^{\circ}$, ThermoFisher ScientificCatalog#AC275640050.

Boc-L-tyrosine: >99%, Optical rotation: $+3.0^{\circ} \pm 0.4^{\circ}$, ThermoFisher Scientific-Catalog#AC275740250.

Acetonitrile: HPLC grade, Merck no. 1.00030.

Trifluoroacetic Acid: >99%, Sigma-Aldrich, Catalog #91707 — 10 × 1 mL.

Water: Milli Q or equivalent.

Chiral HPLC Conditions

High-performance liquid chromatography (HPLC) analyses were performed using an Agilent 1260 system or equivalent. Separations were performed on a Lux Cellulose-2 column (250 × 4.6 mm, 3 μm; Phenomenex, no. 00G-4456-E0) or an equivalent column, maintained at 25˚C. The mobile phase consisted of (A) 0.1% trifluoroacetic acid (TFA) in Milli-Q water and (B) acetonitrile. The flow rate was set at 0.8 mL/min, with an injection volume of 10 μL. Detection was achieved via UV absorbance at 224 nm (bandwidth: 4 nm). Samples were prepared in a diluent of water: acetonitrile (1:1, v/v) and maintained at 25˚C during analysis.

Gradient elution was employed as follows: 0 - 14 min, 80% - 30% A (linear); 14 - 14.5 min, 30% A (isocratic); 14.5 - 14.6 min, 30% - 80% A (linear); 14.6 - 16 min, 80% A (isocratic). Sample preparation involved weighing 100 mg of the sample into a 10 mL volumetric flask, followed by dilution to volume with the diluent and vigorous shaking to ensure complete dissolution.

2. Results and Discussion

Accurate determination of Boc-L-valine's chiral purity is essential. Initial methods employed chiral and achiral gas chromatography (GC) using Trimethylsilyldiazomethane (TMS) derivatization. In a benchmark workflow, chiral and achiral impurities were analyzed on two separate GC columns—chiral and achiral—because no single GC stationary phase provided sufficient resolution for both sets of impurities in a single injection, highlighting the efficiency advantage of the unified chiral HPLC method.

TMS was selected as the derivatization reagent due to its safety, non-explosive nature, cost-effectiveness, and relatively low toxicity [11]. The recoveries from the derivatization process, as outlined in the GC sample preparation, were thoroughly evaluated. The derivatization efficiency of Boc-L-valine and Boc-D-valine into their respective methyl esters (Boc-L-valine methyl ester and Boc-D-valine methyl ester) was assessed and compared to the corresponding standards. Excellent recoveries (100.6% - 109.5%) were achieved when using a 2-equivalent excess of TMS, with low relative standard deviations (RSD 0% - 2.6%).

The chiral GC column, CP-ChiralSil-L-Val, was used to separate Boc-L-Val-Ome and Boc-D-Val-Ome. To optimize this separation, different initial oven temperatures and ramp rates were assessed. Given the high boiling points (250˚C - 300˚C) of Boc-L-Val-OMe and Boc-D-Val-OMe, lower initial oven temperatures (35˚C or 40˚C) were unable to achieve baseline separation, even at higher ramp rates (15˚C or 18˚C/min). However, starting at a higher initial oven temperature (100˚C) with a lower ramp rate (5˚C/min) produced the best separation between Boc-L-Val-OMe and Boc-D-Val-OMe. At lower initial oven temperatures, the high-boiling Boc-L-Val-OMe and Boc-D-Val-OMe derivatives exhibited poor volatility and weak on-column focusing, leading to broad, partially overlapping peaks and preventing baseline resolution even when steeper temperature ramps were applied.

To streamline analysis, a reversed-phase chiral HPLC method was developed, enabling simultaneous chiral, achiral, and assay determinations, as well as compound identification, without derivatization. Both GC and HPLC methods, validated per ICH guidelines, detect undesired isomers at 0.1 - 0.2% wt, well below the 1% wt specification for Boc-D-valine and related Boc-L-amino acids.

The initial step in developing the chiral HPLC method involved using a chiral HPLC screening system for direct analysis. The mobile phases consisted of 0.1% TFA in water (phase A) and acetonitrile (phase B), mixed at a 50:50 ratio. The system included four Phenomenex reversed-phase chiral columns, each featuring polysaccharide-based chiral stationary phases (CSPs) with dimensions of 4.6 × 150 mm. Both Cellulose 2 and Cellulose 4 columns demonstrate baseline separation, likely due to hydrogen bond interactions between the polysaccharide-based chiral stationary phases (CSPs) and Boc-L-valine and Boc-D-valine. In contrast, the Cellulose 3 columns show partial separation, while the Cellulose 1 columns exhibit no separation, as shown in **Figure 1**.

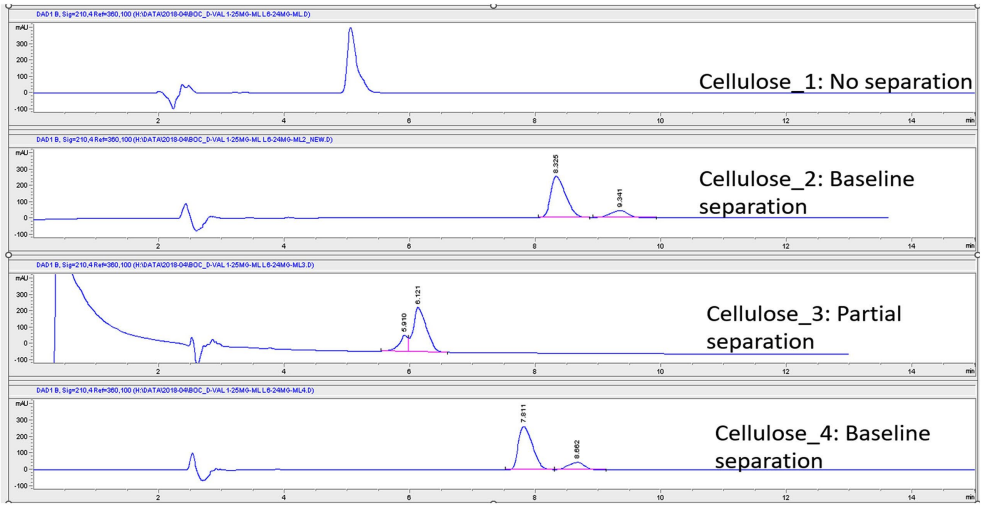

Figure 1. Overlaid HPLC chromatograms of a 5% Boc-D-valine solution in a 2 mg/mL Boc-L-valine sample were analyzed under isocratic conditions.

Based on the initial chiral screening results, Cellulose 2 and Cellulose 4 were selected for further development, including analysis of three additional achiral impurities: Boc-L-alanine, Boc-L-phenylalanine, and Boc-L-tyrosine. Boc-L-phenylalanine could not be eluted under isocratic conditions, requiring the development of a gradient method. However, when analyzed using Lux Cellulose 4 on two different column sizes (4.6 × 150 mm and 4.6 × 250 mm), Boc-D-valine and Boc-L-tyrosine co-eluted.

To resolve the coelution issue between Boc-D-valine and Boc-L-tyrosine, a 4.6 × 250 mm Lux Cellulose 2 column was selected based on the results obtained with the 4.6 × 150 mm Lux Cellulose 2 column. Using the 4.6 × 250 mm column, the resolution between Boc-L-valine, Boc-D-valine, and Boc-L-tyrosine was significantly improved compared to the shorter column. To further enhance the separa-

tion between Boc-L-valine and Boc-D-valine and to improve the peak shape of Boc-L-alanine from asymmetric to symmetric, additional optimizations, such as adjustments to the gradient profile, injection volume, and flow rate, were performed. These modifications increased the resolution of the critical pair (Boc-L-valine and Boc-D-valine) from 1.7 to 2.2, as illustrated in Figure 2. The three optimized conditions are summarized in Table 1.

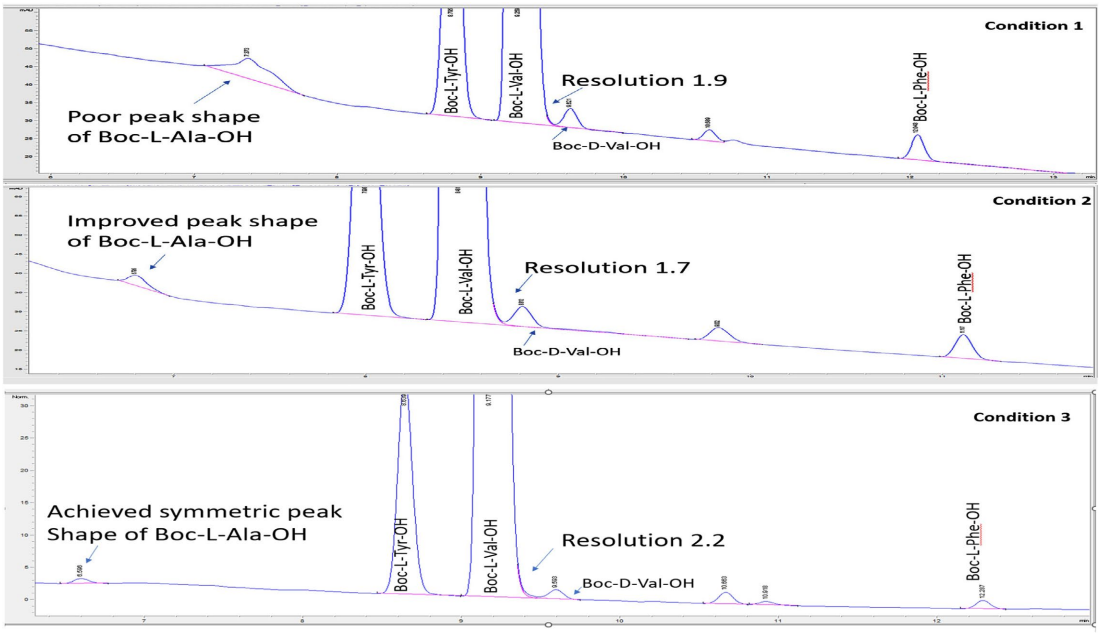

Figure 2. HPLC method development was conducted using 0.5% wt of chiral and achiral resolution solutions under three conditions on a Lux Cellulose 2 column (4.6 × 250 mm) to optimize the separation of Boc-protected amino acids.

Table 1. Three modified conditions were tested on a Lux Cellulose 2 column (4.6 × 250 mm), with Condition 3 yielding the optimized results.

Parameters	Condition 1			Condition 2			Condition 3		
Injection (μL)	20			20			10		
Flow rate (mL/min)	0.9			0.9			0.8		
Gradient	Time	A	B	Time	A	B	Time	A	B
	0	92	8	0	92	8	0	80	20
	4	70	30	2	70	30	14	30	70
	15	30	70	14	30	70	14.5	30	70
	15.5	30	70	14.5	30	70	14.6	80	20
	15.6	92	8	14.6	92	8	16	80	20
	18	92	8	16	92	8			

Note: Time (min), A:MP A% (0.1% TFA in water), B:MP B% (Acetonitrile).

The 4.6 × 250 mm Lux Cellulose 2 column was selected for its optimal perfor-

mance, achieving a resolution of 2.2 between Boc-L-valine and Boc-D-valine, while also improving the peak shape for Boc-L-alanine and successfully resolving the coelution of Boc-D-valine and Boc-L-tyrosine (**Figure 3**). Additionally, the method was capable of detecting 0.2% wt of the other three Boc-protected amino acids. During routine use, on-column sample loads were typically limited to ≤ 20 μg to maintain column performance and extend column life. Higher sample loads on the polysaccharide stationary phase led to gradual loss of chiral selectivity and occasional backpressure increases, consistent with stationary-phase fouling. In this method, however, even with an on-column load of 100 μg of Boc-L-Val and appropriate column-washing procedures, no backpressure or performance issues were reported by two Contract Manufacturing Organizations (CMOs) after extended use. The mechanism of column fouling was not further investigated. Two small late-eluting peaks (10.6 and 10.9 min) were observed only in analytical resolution samples containing Boc-L-Tyr (relative response factor 53). Their absence in chromatograms of the neat starting materials indicates that these minor components originated from impurities in Boc-L-Tyr rather than Boc-L-Val.

The 4.6 × 250 mm Lux Cellulose 2 column also exhibited excellent chiral separation of the NBI-98854 drug substance, which contains a valine group, and was selected as the chiral method column for the chiral analysis of the NBI-98854 drug substance [5].

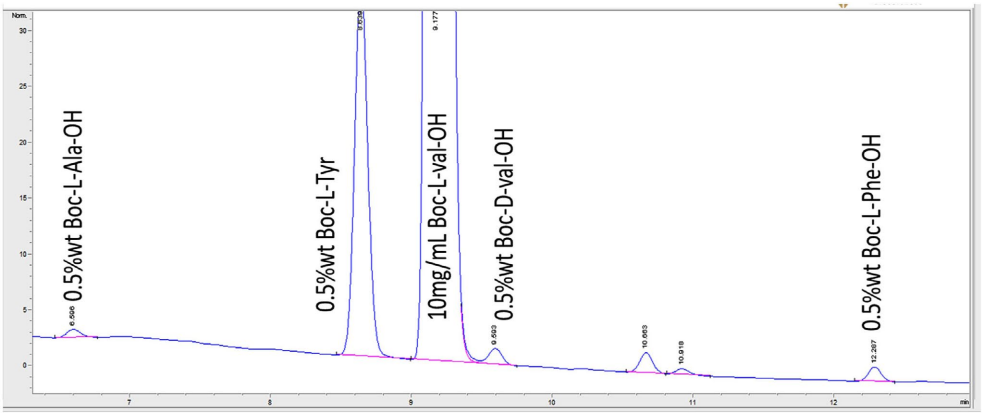

Figure 3. Chromatogram of the chiral and achiral resolution solution (0.5% of each chiral and achiral impurity) using a single reversed-phase gradient chiral HPLC method for Boc-L-valine.

3. Conclusions

To enhance the sensitivity of chiral and achiral impurity analysis for Boc-L-valine, alternative GC methods with derivatization have been developed for both chiral and achiral impurities. Additionally, a gradient-based reversed-phase chiral HPLC method has been developed for Boc-L-valine, which eliminates the need for sample derivatization while allowing the simultaneous detection of three achiral impurities in a single run. This method provides comprehensive results, including identification, assay, chiral purity, and achiral impurity data, all within one analysis. The quantitation limit for both the undesired isomer and the three Boc-pro-

tected amino acids is 0.2%. The method has been validated by two CMOs following ICH guidelines, ensuring reliable quantitation down to 0.2% for both the undesired isomer and the achiral impurities.

This approach can be extended to other Boc-protected and Fmoc-protected amino acids, enabling direct analysis with simultaneous chiral, achiral, and assay results in a single run, eliminating the need for derivatization. It also substantially reduces the overall qualification and validation effort. This broader applicability is supported by the shared carbamate functionality and hydrophobic side chains present in Boc- and Fmoc-protected amino acids, which engage in similar hydrogen-bonding, π–π, and dispersive interactions with the cellulose tris(3-chloro-4-methylphenylcarbamate) selector of the Lux Cellulose-2 stationary phase under reversed-phase conditions used in this work.

Authors' Contributions

Hua Zhao authored the original manuscript, developed the reversed-phase HPLC method for chiral, achiral, and assay analysis, and two GC methods, conducted all experiments, and analyzed the data. Jiangwei Li and Frank Wu contributed to the review and editing.

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Conflicts of Interest

H. Zhao, J. Li, and F. Wu are employees of Neurocrine Biosciences, Inc. and own stock or share options.

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