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# Chiral Method Development Using Polysaccharide-Based Chiral Stationary Phase (CSP) and Investigation of Chiral Column Degradation and Regeneration

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## Abstract

Two polysaccharide-based chiral stationary phase columns were evaluated to improve the previous partial chiral peak separation to a baseline-resolved separation of the INGREZZA® drug substance and its diastereomers. Moreover, the tailing factor (Tf) variation was studied to investigate chiral column degradation and regeneration and to optimize chiral column performance and efficiency.

## Keywords

Chiral Reversed-Phase HPLC Separation, Backflush, Chiral Column Regeneration, INGREZZA® (Valbenazine)

## 1. Introduction

Among 56% of all marketed drugs, 88% are administered as a racemate. In recent years, there has been an increasing trend in enantiomerically pure substances in medicinal chemistry to reduce the toxicity or side effects associated with the inactive enantiomer. Controlling enantiomeric purity and isolating the enantiomers from chiral drugs remain a crucial subject for analytical, clinical, and regulatory purposes, thus improving the drug safety profile [1] [2]. Nine out of ten best-selling drugs in 2018 and 2019 were chiral [3].

INGREZZA® (Valbenazine, Neurocrine Biosciences, Inc.) is one example of a pure diastereomer because the drug provides patients with additional potency and safety advantages. A normal-phase chiral method for Valbenazine, enantiomer,

and isomers has been developed [4], however, a reversed-phase chiral method is preferred due to less organic solvent usage and easier implementation. For the INGREZZA® drug substance (NBI-98854), a reversed-phase chiral method was initially developed using a ChiralCel OD-RH 4.6 × 150 mm, 5 µm column. Although diastereomer A had a resolution (Rs) of 2.6 from the main peak, it didn't have baseline separation, therefore spike recovery was highly dependent on the integration mode used, which can range from 69% to 139% as shown in **Figure 1**.

Among marketed chiral stationary phases, polysaccharide-based CSPs were the most efficient, and more than 90% of the chiral analytical evaluations have been performed using polysaccharide-based CSPs. [5] [6]. The most important interactions for polysaccharide-based CSPs are H-bridge, π-π, and van der Waals forces [7].

To achieve the baseline separation and to improve spike recovery, a simple reversed-phase chiral method was developed using Lux Cellulose-2, 4.6 × 250 mm, 3 µm column. The resolution improved from 2.6 to approximately 4.2 with the spike recovery consistently within 85% - 115% at the 0.5% wt level. The undesired diastereomer A sensitivity also improved from 0.3% wt to 0.2% wt in the presence of the API using this method. Both reversed-phase HPLC chiral methods have been validated per ICH Q2 guidelines.

The improved reversed-phase HPLC chiral method using the Lux Cellulose-2 column exhibited an issue with the USP tailing factor of the drug substance peak, which increased over time. This study aims to develop a refined reversed-phase chiral method and investigate the onset of chiral column degradation, methods for regenerating the column, and strategies to address the peak tailing issue. While backflush the chiral column with 100% isopropanol (IPA), methanol, or ethanol for 2 - 3 hours is a common practice and recommended by column manufacturers, we found that intensive backflush for 12 hours with the reversed-phase chiral method mobile phase, followed by 40 hours of re-equilibration, significantly improved the peak shapes, surpassing the initial peak shape, and extending the column lifetime.

This study evaluated three polysaccharide-based CSP chiral columns listed in **Table 1**.

**Table 1.** Three polysaccharide-based CSP chiral columns.

| Column | Size | Chiral Selector [8] |
|---|---|---|
| Lux Cellulose-2 (blue in **Figure 2**) | 4.6 × 250 mm, 3 µm | Cellulose tris (3-chloro-4-methyl phenyl carbamate) |
| Lux Cellulose-4 (orange in **Figure 2**) | 4.6 × 250 mm, 3 µm | Cellulose tris (4-chloro-3-methyl phenyl carbamate |
| ChiralCel OD-RH | 4.6 × 150 mm, 5 µm | Cellulose tris (3,5-dimethyl phenyl carbamate |

## 2. Experimental

### 2.1. Chemicals and Reagents

NBI-98854 Ditosylate Reference Standard: 99.3% wt, Neurocrine Biosciences lot 201907269999.

Diastereomer A Reference Standard (D-Valine version of NBI-98854): 98.7% wt, Neurocrine Biosciences lot NBI#2-017.

Diastereomer B Reference Standard (diastereomer of NBI-98854): 95.3% wt Neurocrine Biosciences lot NBI#2319-36.

Diastereomer C Reference Standard (diastereomer of NBI-98854): 96.9% wt Neurocrine Biosciences lot NBI#2319-7.

Acetonitrile, HPLC grade, Merck No. 1.00030.

Ammonium acetate: ≥98% Sigma-Aldrich A7262-500G.

Deionized Water: Milli-Q.

### 2.2. Solution Preparation

*Stock Solution of Diastereomer A, B, and C*: Accurately weigh 9 mg (±0.5 mg) each of Diastereomer A, B, and C Ditosylate into a 10 mL volumetric flask. Dilute to volume with diluent and mix until completely dissolved. Nominal concentration: 0.5 mg/mL free base impurity mix.

*Working Standard Solution of NBI-98854 Ditosylate (0.5 mg/mL of free base)*: Accurately weigh 23 mg (±1 mg) of NBI-98854 Ditosylate into a 25 mL volumetric flask. Add approximately 15 mL diluent to the flask and mix well by swirling. Sonicate briefly if necessary to dissolve solids. Dilute to volume with diluent and mix well.

*Resolution Solution of NBI-98854 with* 0.5% *Diastereomer A, B, and C*: With a volumetric pipette, accurately add 0.25 mL of Stock Solution of *Diastereomer A*, *B*, and *C* into a 50 mL volumetric flask. Dilute it to volume with the Working Standard Solution of NBI-98854 and mix thoroughly.

*Sample Preparation*: same as working standard solution.

### 2.3. Chromatographic Condition

Column: Cellulose_2, 250 × 4.6 mm, 3 µm, Part No: 00G-4456-E.

Mobile Phase: 20 mM Ammonium Acetate: Acetonitrile: 44:56.

UV detector: 280 nm.

Injection volume: 5 µL.

Column Temp: 25˚C.

ALS Temp: Ambient.

Flow rate: 1.0 ml/min., Isocratic.

Diluent: Water: Acetonitrile 50:50.

Run time: 18 min.

### 2.4. Equipment and Software

The HPLC system was an Agilent 1260 series HPLC unit.

The Cellulose 2 chiral column was purchased from the Phenomenex part number 00G-4456-E0. The acquisition software was OpenLab licensed from Agilent.

### 2.5. Chromatographic Parameters

**Table 2.** Chromatographic parameters for the Separation of NBI-98854 and diastereomer A.

| Chromatographic Parameters of the chiral method | | | |
|---|---|---|---|
| Compound | $\kappa'$ (capacity factor) | $\alpha$ (separation factor) | Rs (Resolution) |
| NBI-98854 | 4.4 | 1.2 | 4.2 |
| Diastereomer A | 5.5 | | |

## 3. Results and Discussion

The initial chiral method employing the ChiralCel OD-RH column can't achieve baseline separation of NBI-98854 and its diastereomer A, resulting in inconsistent spike recoveries at 0.5% wt. Due to the insufficient separation of diastereomer A from the NBI-98854 peak, Figure 1 illustrates that diastereomer A can be underestimated (as shown in panels A and C) or overestimated (as shown in panels B and D) depending on the integration mode applied.

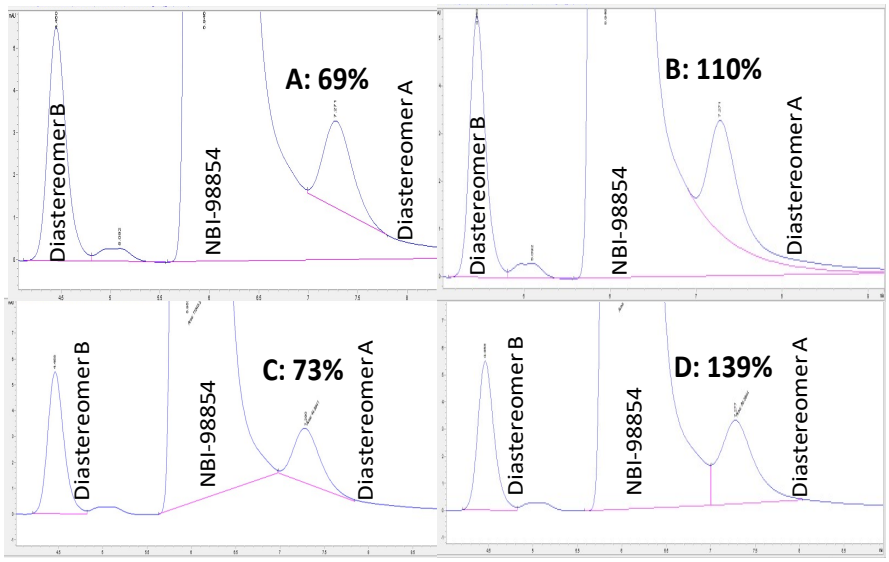


**Figure 1.** Due to partial baseline separation in the initial chiral method using the ChiralCel OD-RH column, the spike recovery of diastereomer A varied significantly, ranging from 69% to 139% depending on the integration modes.

Based on a previous reversed-phase chiral method development experience on Boc-L-valine and Boc-D-valine using polysaccharide-based CSPs (Cellulose-1, Cellulose-2, Cellulose-3, and Cellulose-4) columns, Cellulose-2 and Cellulose-4 columns have been selected to develop a reverse-phase chiral method for NBI-98854. The mobile phase was selected based on two pKa of NBI-98854.

NBI-98854 has four chiral centers. The diastereomers B and C yielded baseline separation from the NBI-98854 peak on both chiral and achiral methods. However, diastereomer A can only be separated from NBI-98854 by the chiral method since the only difference is that diastereomer A has a D-valine portion, while NBI-98854 has an L-valine portion. The structures of NBI-98854, its diastereomers, and two CSPs are shown in Figure 2.

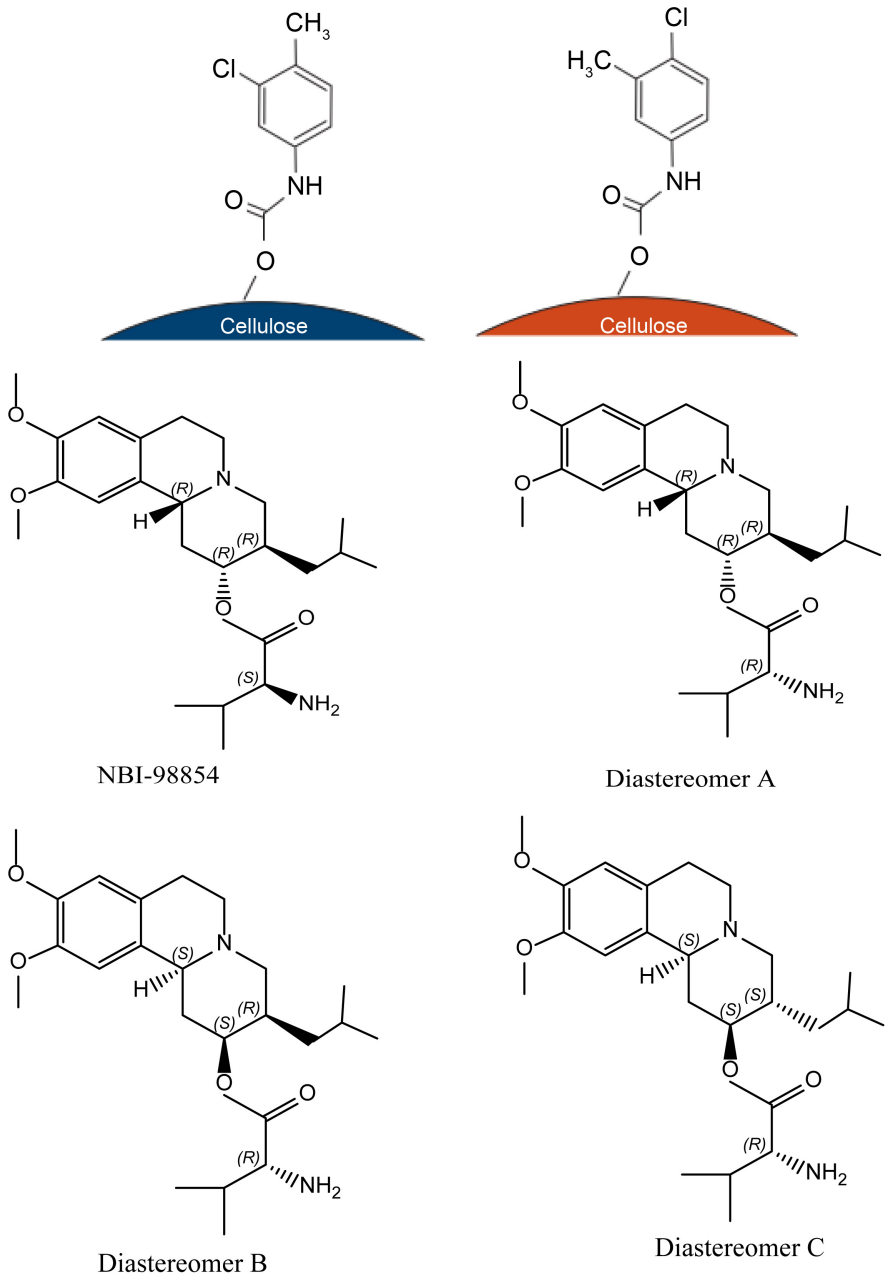


Figure 2. Chiral stationary phases Lux Cellulose 2 (blue) and 4 (orange) [8] and structures of NBI-98854 and diastereomers.

The structures of Chiral stationary phases Lux Cellulose 2 and 4 are similar.

Both gradient and isocratic conditions were tested, and Lux Cellulose-2 resulted in better separation between the NBI-98854 and diastereomer A, as shown in Figure 3. The mobile phase components started at 20 mM Ammonium Acetate: Acetonitrile 50:50 and then optimized to 44:56.

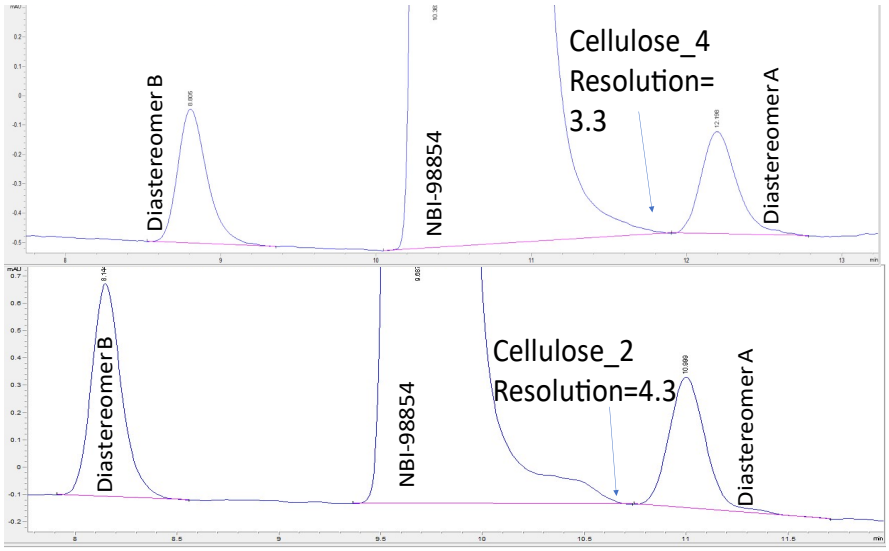


**Figure 3.** Method Development on two CPSs to enhance the resolution from 2.6 to 4.3 with a baseline separation.

For the improved method using the Cellulose-2 column, the tailing factor of the drug substance peak increased over about three months of usage. To understand when degradation occurs, how to regenerate the chiral column, and how long the regenerated column can restore and improve performance were investigated. Several column conditioning solutions, equilibration, and column regeneration conditions were evaluated.

As summarized in Table 3, procedure 1 began by conditioning the packing solvent in the column using (90:10) Methanol/Ethanol mixture for 1 hour at 0.5 mL/min in preparation for reversed-phase chromatography. The column was then equilibrated for 2 hours at 0.5 mL/min using the mobile phase. Once the system was fully equilibrated, the flow was increased as specified in the method, and the injection sequence (Table 4) started once the system pressure was stable. 81 injections were performed, with the Tf of NBI-98854 increasing from 1.9 to 2.9 after 28 hours of continuous injections.

The column direction was reversed and flushed for 12 hours at 0.5 mL/min using the mobile phase. Then the column was reverted to the correct flow direction and the column was re-equilibrated for 7 hours at 0.5 mL/min using the mobile phase. The flow rate was increased as specified in the method and the injection sequence was started once the pressure stabilized.

168 injections were made, with 56 hours between the first and last injection of the entire sequence. The Tf of NBI-98854 remained at 1.0 over this period of continuous injections as seen in Figure 4.

**Table 3.** Summary of column degradation and regeneration studies.

| Procedure | 1 | | 2 | 3 |
|---|---|---|---|---|
| Conditioning | MeOH/EtOH (9:1) for 1 hr at 0.5 mL/min | | IPA for 4 hrs at 0.25 mL/min | IPA for 4 hrs 0.25 mL/min in the reverse direction |
| Equilibration | Mobile phase for 2 hrs at 0.5 mL/min | | | |
| >20 hrs Continuous Injection | Tf initial | 1.9 | 2.0 | 2.5 |
| | Tf final | 2.9 | 0.7 | 3.5 |
| **Backflush** | Mobile phase for 12 hrs at 0.5 mL/min | | | NA |
| **Re-Equilibration** | Mobile phase for 7 hrs | | Mobile phase for 40 hrs | |
| >12 hrs Continuous Injection | Tf initial | 1.0 | 1.2 | |
| | Tf final | 1.0 | 1.2 | |
| Advantages | The peak shape is sharper and more symmetrical than the initial peak. Extend column lifetime to 2 years. | | | NA |
| Disadvantages | Required 12 hrs backflush & 40 hrs re-equilibration to achieve consistent retention time | | Required additional conditioning time than procedure 1 | Peak tailing was the worst of all 3 procedures |

**Table 4.** Injection sequence used for the chiral HPLC method.

| Sample | Number of Injections |
|---|---|
| Blank (Diluent) | 3 until a steady baseline is achieved |
| Sensitivity Solution | 1 |
| Resolution Solution | 1 |
| Blank | 1 |
| Working Standard Solution | 5 |
| Blank | 1 |
| Sample 1 through 6 | 1 each |
| Working Standard Solution | 1 |

The extensive 12-hour backflush with the mobile phase significantly enhanced column performance, as evidenced by a reduction in the peak tailing factor from 2.9 to 1.0. The improved peak shape (Tf = 1.0) was maintained over 50 hours of continuous injections. A retention time shift was observed in Procedure 1. As illustrated in **Figure 5**, the retention time required approximately 40 hours of re-equilibration following the backflush to return to the initial values. Once restored, the retention time remained stable, as demonstrated in Procedures 1 and 2.

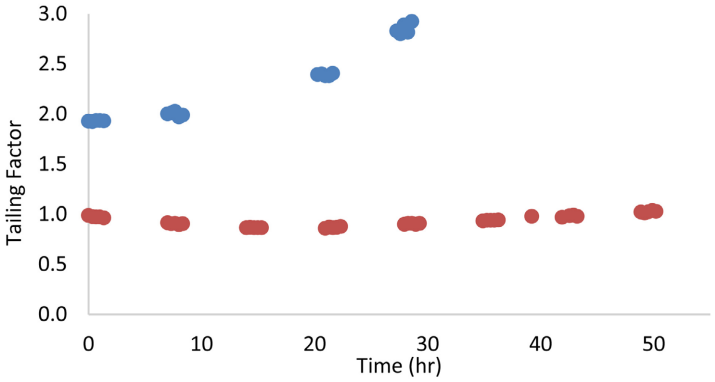


**Figure 4.** The tailing factor of the drug substance increased during 28 hours of continuous injections (blue). After performing a backflush, the tailing factor improved and stabilized at 1.0 over 56 hours of continuous injections (red).

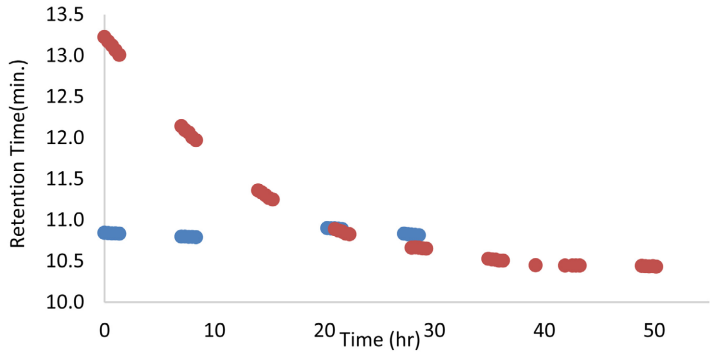


**Figure 5.** After the backflush, the retention time requires 40 hours of re-equilibration (red) to restore the initial retention time (blue) and remain consistent.

The column vendor (Phenomenex) suggested that insufficient conditioning might be present when flushing out the packing solvent when conditioning the column with MeOH/EtOH (9:1). The vendor recommended changing the conditioning to 100% isopropanol for at least 20 column volumes while keeping the pressure under 150 bar.

To further investigate column degradation and regeneration, isopropyl alcohol (IPA) was tested in Procedure 2 and Procedure 3 using new columns. In Procedure 2, the column was conditioned with IPA at 0.25 mL/min for 4 hours to maintain low pressure, then equilibrated as in Procedure 1. After 20 hours of continuous injections, the column showed front tailing with a tailing factor of 0.7. The column was reversed, flushed as in Procedure 1, and re-equilibrated for 40 hours. After repeating the injection sequence, the Tf improved to 1.2. Procedure 2 confirmed that backflushing enhances column performance and reproducibility, but the IPA flush caused a retention time shift of 0.5 minutes longer than the original.

In Procedure 3, to explore if the backflush can replace the column conditioning step and improve the column performance, a new column was backflushed with IPA at 0.25 mL/min for 4 hours, then equilibrated in the mobile phase at 0.5

mL/min for 2 hours, also in reverse. After flipping the column back to its original direction, the sequence was started once the pressure stabilized at the method-defined flow rate. The tailing factor increased from 2.5 to 3.5 after just over 20 hours of continuous injections, following a similar trend to Procedure 1. However, IPA caused higher back pressure than the MeOH/EtOH mixture, requiring a lower flow rate and longer flush time, providing no advantage over Procedure 1. Therefore, the rest of the experiment was not conducted.

Among the three procedures evaluated, the optimal approach involved conditioning the packing solvent with a 90:10 MeOH/EtOH mixture at 0.5 mL/min for at least one hour. This was followed by column equilibration with the mobile phase at 0.5 mL/min for at least two hours. Initial runs typically produced a tailing factor of approximately 2.0, which remained within system suitability limits of 2.5 for at least 24 hours of continuous injections. If the Tf exceeded 2.5, the limit was backflushed with the mobile phase at 0.5 mL/min for 12 hours, then re-equilibrated with the mobile phase for at least 40 hours. This procedure effectively restored and improved column performance, achieving a Tf of approximately 1.0, as shown in Figure 6.

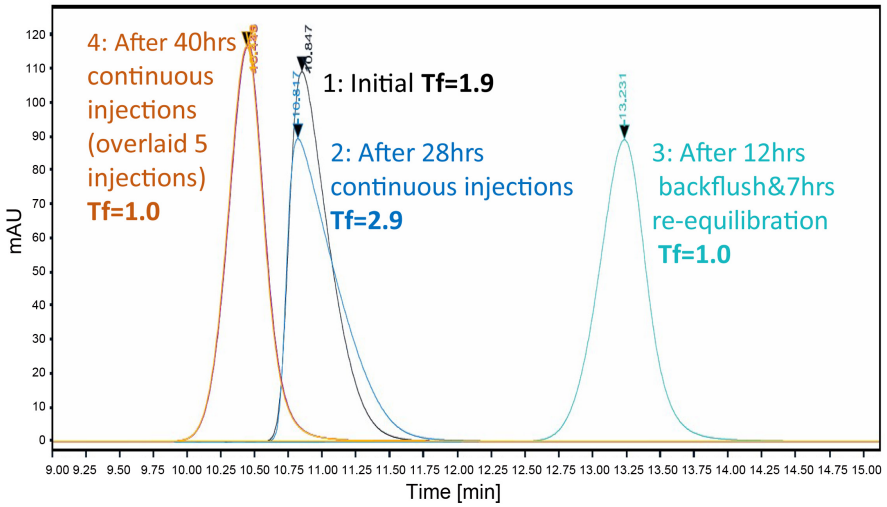


**Figure 6.** In Procedure 1, the tailing factor and peak shape improved compared to the initial state (1). Tf increased to 2.9 after extensive injection, (2), and then the improvement was observed following a 12-hour backflush and 7-hour re-equilibration (3), with Tf remaining stable after 40 hours of continuous injections (4).

The column vendor, Phenomenex, recommends regenerating Lux chiral columns by flushing them with 100% methanol or ethanol for 2 - 3 hours. Backflush is also suggested as an alternative cleaning procedure [8]. However, the literature survey did not identify any studies reporting the application of backflush techniques on Lux chiral columns using the specified mobile phase. This approach demonstrated improved column performance and efficiency beyond the initial operating conditions, while also contributing to an extended column lifespan.

In this study, extensive backflush for 12 hours using a mobile phase comprising

20 mM ammonium acetate in water and acetonitrile (44:56), followed by 40 hours of re-equilibration, significantly improved the performance of polysaccharide-based chiral stationary phase (CSP) columns. This process restored and stabilized retention times, yielding sharper and more symmetrical peaks with a tailing factor of 1.0. This improvement may be attributed to a reduction in hydrogen bonding and π–π interactions between the CSP and NBI-98854, along with its diastereomers. The retention time changes observed after backflush, as shown in Figure 5 and Figure 6, provide strong evidence of alterations in H-bonding or π–π interactions. The cyano group of acetonitrile may play a significant role in modifying π–π interactions following extensive backflush with a mobile phase containing 56% acetonitrile. Additionally, a higher concentration of sample injections may help reduce the re-equilibration time by facilitating faster stabilization of the interactions between the chiral stationary phase (CSP) and NBI-98854, compared to a lower sample concentration of 0.5 mg/mL.

Notably, this study focused exclusively on the reversed-phase mode, leaving the normal-phase mode unexplored.

The resolution between diastereomer A and NBI-98854 remained stable at 4.2 - 4.3 over 28 hours of continuous injections, with a slight decrease of 9.5% to 3.8% observed after backflush. Despite this minor reduction in resolution, baseline separation was maintained, accompanied by sharper peak shapes (tailing factor, Tf = 1.0) as shown in Figure 7.

Furthermore, spike recoveries for all three diastereomers remained consistent at approximately 100% both before and after backflush.

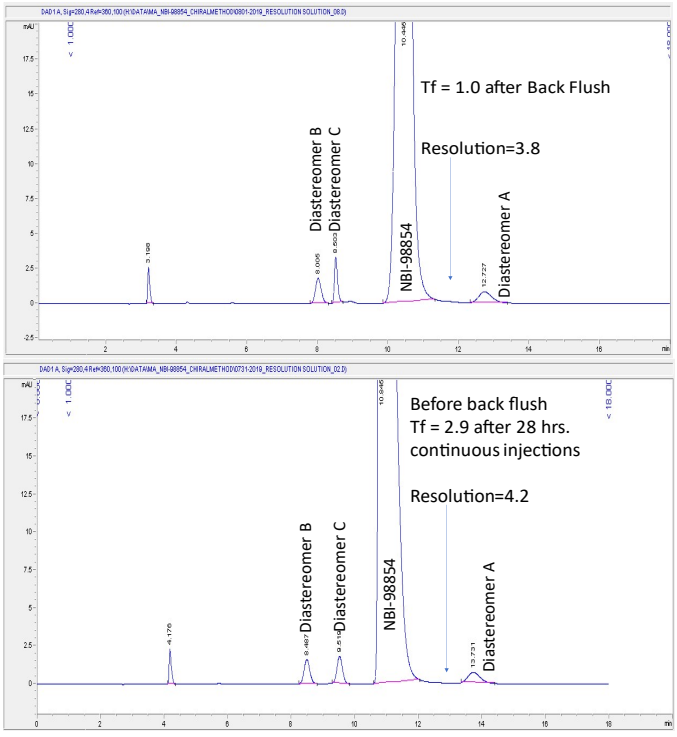


**Figure 7.** Resolution comparison before and after backflush.

## 4. Conclusions

A simple reverse-phase chiral method using polysaccharide-based CSPs was developed, achieving an improved resolution from 2.6 to approximately 4.2, with spike recovery within 85% - 115% at the 0.5% wt level for diastereomer A. The sensitivity in detecting the undesired diastereomer A improved from 0.3% to 0.2% wt in the presence of the drug substance NBI-98854. The improved chiral reversed-phase chiral method using Lux Cellulose-2 was transferred to two contract manufacturing organizations (CMOs) and successfully validated per ICH guidelines. After implementing the backflush and re-equilibration procedure, both CMOs observed the same improvement in peak shape. The CMOs extended the chiral column's lifespan from 3 - 6 months to 2 years for routine release and stability testing. This practical strategy to enhance chiral column performance and extend its lifespan is highly beneficial, although some underlying mechanisms are not fully understood.

The study demonstrated that the chiral column degrades after 28 hours of continuous injections. A 12-hour backflush with the mobile phase, followed by 40 hours of re-equilibration, effectively restores and enhances the performance of reversed-phase chiral columns with polysaccharide-based CSPs. This extensive backflush and re-equilibration procedure can be applied to both chiral and achiral columns, significantly improving column performance and extending column lifespan.

## Acknowledgements

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## Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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