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## A validated chiral LC method for enantiomeric separation of common intermediate of ritonavir and lopinavir using cellulose based chiral stationary phase

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### ABSTRACT

A rapid isocratic chiral LC method has been developed for the separation of (2R,3R,5R)-2-Amino-3-hydroxy-5-(tert-butyl oxy carbonyl) amino-1,6-diphenyl hemi succinic acid (R-BOC amine) from (2S,3S,5S)-2-Amino-3-hydroxy-5-(tert-butyl oxy carbonyl) amino-1,6-diphenyl hemi succinic acid (S-BOC amine). Good resolution with  $R_s > 3$  was obtained using cellulose based chiral stationary phase, chiralcel OD-H column (250 x 4.6 mm, 5 $\mu$ m particle size) and n-hexane, ethanol, trifluoroacetic acid and triethyl amine (950:50:1:1,v/v) as the mobile phase at ambient temperature. Flow rate was kept at 1.0 mL min<sup>-1</sup> and elution was monitored by UV detection at 210 nm. This method allowed for the detection and quantification of R-BOC amine of levels at 0.15 and 0.5  $\mu$ g mL<sup>-1</sup> respectively. The method was validated by following ICH guidelines. © 2009 Trade Science Inc. - INDIA

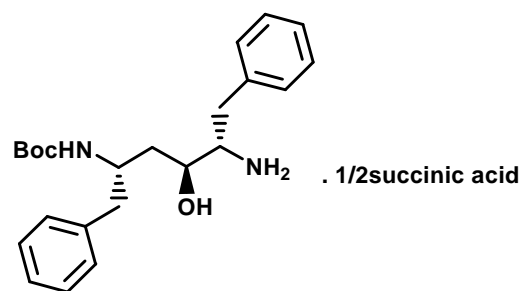
### KEYWORDS

Liquid chromatography;  
Enantiomer;  
Method validation;  
Chiral Stationary Phase;  
Resolution.

### INTRODUCTION

2-Amino-3-hydroxy-5-(tert-butyl oxy carbonyl) amino-1,6-diphenyl hemi succinic acid (BOC amine) (Figure 1) is a common intermediate for both Ritonavir (Figure 2) and Lopinavir (Figure 3). Lopinavir and ritonavir are antiretroviral drugs acting as inhibitors of human immunodeficiency virus (HIV) protease, a class of drugs that has markedly improved morbidity and mortality of HIV infected patients<sup>[1]</sup>. Within the highly active antiretroviral therapy (HAART) they are used in a fixed combination (33 mg ritonavir + 133 mg lopinavir) called Kaletra® with ritonavir added to boost lopinavir by inhibition of cytochrome P450 (CYP)

CYP3A isozymes and active transport by P-glycoprotein<sup>[2,3]</sup>. Even though HAART has markedly improved the clinical outcome of HIV-infected patients, virologi-

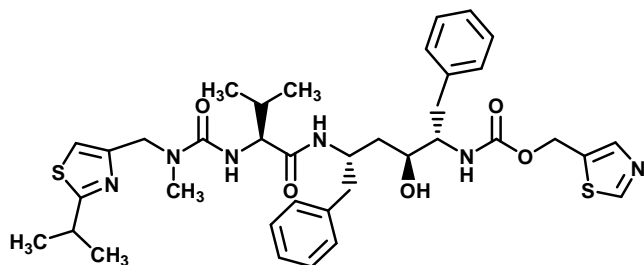


(2S, 3S, 5S)-2-Amino-3-hydroxy-5-t-butyl oxy-carbonylamino-1, 6-diphenylhexane hemi succinic acid

Figure 1 : Chemical structures of BOC-amine

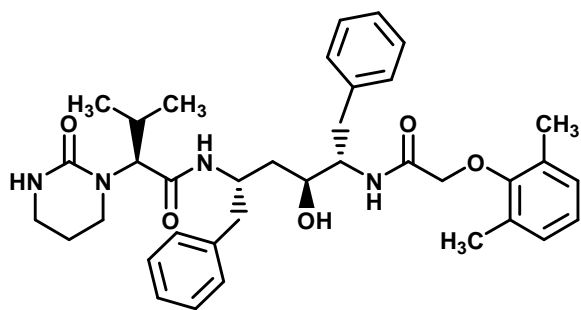
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cal treatment failure often occurs already within the first year of therapy<sup>[4,5]</sup>.



**10-Hydroxy-2-methyl-5-(1-methylethyl)-1-[2-(1-methylethyl)-4-thiazolyl]-3,6-dioxo-8,11-bis(phenylmethyl)-2,4,7,12-tetraazatridecan-13-oic acid, 5-thiazolylmethyl ester, [5S-(5R\*,8R\*,10R\*,11R\*)]**

**Figure 2 : Chemical structures of ritonavir**



**(2S, 3S, 5S)-2-(2, 6-Dimethyl phenoxy acetyl)amino-3-hydroxy-5-[2S-(1-tetrahydro-pyrimid-2-onyl)-3-methyl butanoyl] amino-1, 6-diphenylhexane**

**Figure 3 : Chemical structures of lopinavir**

Separation of enantiomers has become very important in analytical chemistry, particularly in the pharmaceutical and biological fields, reflecting the fact that different enantioselective properties can impact on pharmacokinetics and result in a variety of different pharmacological or toxicological effects<sup>[6-9]</sup>. As a consequence, regulatory authorities insist on stringent investigations when evaluating the safety and the effectiveness of chiral drugs. This places a significant importance on the application of enantiomeric separations to all stages of drug development and the commercialization process. The development of new methods for efficient chiral separations are mainly based on high performance liquid chromatography (LC), capillary electrophoresis (CE) or gas chromatography (GC). A large number of commonly employed LC methods are based on the use of chiral stationary phases (CSPs).

As per the requirement of various regulatory authorities, chiral method development for this vital intermediate, BOC amine is very essential. To the best of our knowledge a chiral LC method has not yet been reported any literature for the separation of BOC amine enantiomers. In this study, a simple, cost-effective and efficient chiral LC method was developed and validated as per the ICH guidelines<sup>[10,11]</sup>.

## EXPERIMENTAL

### Chemicals and reagents

HPLC grade n-hexane and ethanol were purchased from Merck (India) and trifluoroacetic acid (TFA), Triethylamine (TEA) were procured from Fluka (India). BOC amine enantiomers were obtained from the process development laboratory of Dr.Reddy's Laboratories, IPDO, Hyderabad, India.

### Instrumentation and chromatographic conditions

The resolution of the enantiomers was performed on Waters HPLC system, Alliance 2690 separation module consisting quaternary HPLC pump, equipped with an auto sampler and 2487 Dual channel absorbance detector (Waters Corporation, Milford, USA). The column used for the analytical separation was the cellulose tris (3,5-dimethylphenylcarbamate) coated on 5 $\mu$ m silica-gel particles known as Chiralcel OD-H (250 x 4.6 mm, 5  $\mu$ m) procured from Diacel Chemical Industries (Japan).

The mobile phase consisted of n-hexane, ethanol, Trifluoroacetic acid and Triethylamine (95:50:1:1, v/v). The flow rate was 1.0 mL min<sup>-1</sup>, the sample injection volume was 10  $\mu$ L.

The appropriate wavelength for the detection of enantiomers was determined by wavelength scanning over the range of 200-400nm using Waters 2996 photodiode array detector and the chromatograms were monitored by UV detection at a wavelength of 210nm.

### Preparation of solutions

A stock solutions of both the enantiomers of BOC amine were prepared at 1000  $\mu$ g mL<sup>-1</sup> by dissolving appropriate amount in the mobile phase as diluent.

## RESULTS AND DISCUSSION

### Optimization of the chromatographic conditions

In order to get optimum resolution and selectivity for the two enantiomers of BOC amine various experiments were conducted by using various CSPs containing cellulose and amylose derivatives. Enantiomeric separation is known to be achieved by the formation transient diastereomeric complexes, mostly based on hydrogen bonding, dipole-dipole and  $\pi$ - $\pi$  interactions<sup>[12]</sup>.

Various proportions of n-hexane / 2-propanol and n-hexane / ethanol were used as organic modifiers in our initial efforts to achieve normal-phase separation. But, it was found that addition of the both Trifluoroacetic acid and Triethyl amine led to the gaussian peak. Attempts to separate enantiomers on an amylose carbamate derivatized CSP column (Chiralpak AD-H) and cellulose ester derivatized columns (Chiralcel OJ and Chiralcel OB) were not successful. Further trials were made on Cellulose carbamate derivatized column (Chiralcel OD-H) using n-hexane, ethanol, trifluoroacetic acid and triethyl amine in the ratio of 950:50:1:1 (v/v) as mobile phase with flow rate of 1

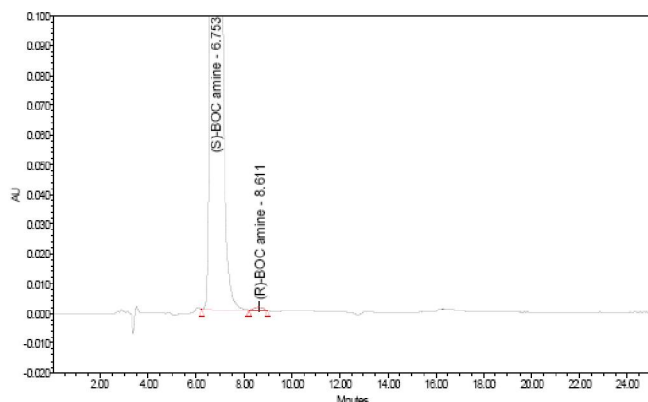


Figure 4 : Typical chromatogram of (S)-Boc amine spiked (R)-Boc amine

mL min<sup>-1</sup>. Hence, the chiral separation was optimized using isocratic conditions as these offers more rapid analysis attributable to the presence of column re-equilibration steps. The typical retention times of S-BOC amine and R-BOC amine were about 6.8 and 8.6 min (Figure 4), respectively

### Limit of detection and quantification

The lowest LOD and the LOQ were determined based on signal-to-noise ratios using analytical responses of 3 and 10 times to the background noise, respectively<sup>[13]</sup>. The LOD and the LOQ for R-BOC amine were calculated to be 0.15 and 0.5  $\mu\text{g mL}^{-1}$ , respectively.

### Linearity

Under the optimized working conditions, plotted standard calibration curve for R-BOC amine was linear over the concentration range of 0.5  $\mu\text{g mL}^{-1}$  (LOQ) to 2.0  $\mu\text{g mL}^{-1}$ . The results of the statistical analysis of the experimental data, such as slope, the intercept and the correlation efficient obtained by the least squares treatment of the results were 180476, 28 and 0.9992, respectively. The standard calibration curve was linear with the linear regression equation

$$y = 28 + 180476x$$

### Precision

The repeatability (intra-day) and the intermediate precision (inter-day) of the method was evaluated by the determination of peak area percentage RSD of R-BOC amine for six replicate injections of spiked sample at the levels of 0.5  $\mu\text{g mL}^{-1}$  (LOQ) and 1  $\mu\text{g mL}^{-1}$ . The intermediate precision (Ruggedness) of the method was evaluated by different analyst using different column and different instrument in the same laboratory. The results were summarized in the TABLE 1.

TABLE 1 : Results of Intermediate precision

Sr. No.	Parameter	Variation	%RSD for % Area of R-BOC amine	%RSD for RT of R-BOC amine	Resolution between enantiomers
1	Different system	a. Waters 2487 VWD	0.3	1.2	3.8
		b. Agilent 1100 series PDA	0.6	1.9	3.5
2	Different column	Column-1	0.7	1.2	3.8
		Column-2	0.7	0.8	3.5
3	Different analyst	Analyst-1	0.3	1.2	3.8
		Analyst-2	0.6	1.3	3.5

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### Accuracy

Accuracy of the method was demonstrated at the four different concentration levels in triplicate. The analysis was carried out at the concentration levels of 0.5  $\mu\text{g mL}^{-1}$  (LOQ), 1  $\mu\text{g mL}^{-1}$  and 1.5  $\mu\text{g mL}^{-1}$ . The percentage recoveries were in between 98 and 102.

### Robustness

The Robustness of the method was studied by varying number of method parameter. The experimental conditions were deliberately varied in order to determine the impact on resolution of enantiomers. The flow rate on resolution was studied by varying the flow rate by  $\pm 0.2 \text{ mL min}^{-1}$ . The temperature effect was studied by varying  $\pm 5^\circ\text{C}$ . In addition, the percentage of ethanol in the mobile phase was varied  $\pm 10\%$ . No significant change observed in the resolution of enantiomers, results were shown in the TABLE 2, illustrating the robustness of the developed method.

TABLE 2 : Results of Robustness study

Parameter	Variation	USP resolution (Rs)
Flow rate	0.8 $\text{mL min}^{-1}$	3.9
	1.2 $\text{mL min}^{-1}$	3.4
Temperature	22 $^\circ\text{C}$	3.6
	32 $^\circ\text{C}$	3.7
Organic ratio	45 mL Ethanol	3.9
	50 mL Ethanol	3.7
	55 mL Ethanol	3.4

### CONCLUSION

An isocratic enantioselective HPLC method that enables sensitive determination of R-BOC amine in S-BOC amine was developed. The method was found to be simple, sensitive, precise, accurate and robust. Hence, this method can be used in quality control laboratories for the routine analysis.

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