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A Validated Chiral HPLC Method for the Enantiomeric Separation Of Levosimendan in Bulk Drug Substances

B.V. Rami Reddy[1,2], K. Subhash Chander Reddy[2], N.V.V.S.S. Raman[2], B. Sai Kumar[2], C. Rambabu[3*].

1. Department of Chemistry, Acharya Nagarjuna University, Dr.M.R.Apparow Campus, Nuzvid-521201, Krishna District, Andhra Pradesh, India.
2. Hetero Drugs Limited, Balanagar, Hyderabad-500018, Andhra Pradesh, India
3. Department of Chemistry, Acharya Nagarjuna University, Nagarjuna Nagar, Guntur-522210, Andhra Pradesh, India.

ABSTRACT

The present paper describes the development and validation of novel chiral HPLC method for the enantiomeric separation of S-Levosimendan from R-Levosimendan and quantitative determination of S-Levosimendan enantiomer in Levosimendan bulk drugs. The enantiomers of levosimendan were baseline resolved on normal phase chromatographic separation on Amylose tris (3,5-dimethylphenylcarbamate) immobilized on 5µm silica-gel-Based Chiral Stationary Phase, Chiral pak IA column (250mm ×4.6mm i.d.; particle size,5µ) at a temperature of 30°C using a mobile phase consisting of MTBE:Ethanol:TFA (650 : 350 : 1.0, v/v/v) at a flow rate of 1.0mLmin^{-1} with an injection volume of 10µL. Quantitation was achieved with UV detection at 383nm based on peak area with linear calibration curves. The elution times of S-Levosimendan and R-Levosimendan were 6.8 min and 11.0 minutes respectively. In this proposed chiral HPLC method, the resolution between S-Levosimendan and R-Levosimendan was found to be greater than 8.0. The developed method was validated with respect to linearity, accuracy, precision, solution stability, ruggedness, robustness, limit of detection and limit of quantification. The recovery obtained for S-isomer was in between 99.1 % and 107.9%. The detection limit obtained for S-isomer was 0.025µg.mL^{-1} and the quantification limit was 0.075µg.mL^{-1} respectively. Linearity was performed for the S-isomer from LOQ to 150%. The correlation coefficient obtained for S-isomer was more than 0.999. The solution stability of Levosimendan bulk drug was determined and the compound was found to be stable up to 48 hrs. As the method has less run time (20 min), it can be useful in quality control laboratories for routine analysis.

Keywords: R-Levosimendan, S-Levosimendan, High performance liquid chromatography and Method validation.

*Corresponding Author Email: crambabu694@gmail.com

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INTRODUCTION

The physiological environment within a living organism is mostly chiral. Therefore, chiral discrimination has been an issue in the development and use of pharmaceutical drugs. Enantiomers of racemic drugs often differ in pharmacokinetic behaviour or pharmacological action[1]. In recent years research has been intensified to understand the aspects of the molecular mechanism for stereoselective biological activities of the chiral molecules. The development of analytical methods for the assessment of enantiomeric purity is challenging due to the fact that enantiomers possess virtually identical properties[2]. Chiral separation by high-performance liquid chromatography (HPLC) using a chiral stationary phase (CSP) is one of the most efficient methods for separating enantiomers, not only on an analytical scale, but also on a preparative scale, and in the past two decades, many CSPs have been developed. Polysaccharides such as cellulose, amylose, and chitin are the most abundant optically active polymers on the earth and can be readily modified to carbamates and esters through the reaction with isocyanates and acid chlorides, respectively. The CSPs based on polysaccharide derivatives are some of the most popular ones and can separate a wide range of chiral compounds[3,4].Levosimendan (Figure1) chemically ({4-[(4'R)-4-Methyl-6-oxo-1,4,5,6-tetrahydropyridazin-3-yl]phenyl}hydrazono) propanedinitrile, is a calcium sensitiser used in the management of acutely decompensated congestive heart failure[5]. It is marketed under the brand name of Simdax (Orion Corporation). The Molecular formula of Levosimendan is $C_{14}H_{12}N_6O$ and the molecular weight is 280.2847.

Levosimendan is a calcium sensitiser-it increases the sensitivity of the heart to calcium, thus increasing cardiac contractility without a rise in intracellular calcium. Levosimendan exerts its positive inotropic effect by increasing calcium sensitivity of myocytes by binding to cardiac troponin in a calcium-dependent manner. It also has a vasodilatory effect, by opening adenosine triphosphate (ATP)-sensitive potassium channels in vascular smooth muscle to cause smooth muscle relaxation. The combined inotropic and vasodilatory actions result in an increased force of contraction, and a and a reduction in both preload and afterload, without adversely affecting diastolic function, without adversely affecting diastolic function. Moreover, by opening also the mitochondrial (ATP)-sensitive potassium channels in cardiomyocytes, the drug exerts a cardioprotective effect.

Levosimendan has one chiral centre and is used as a single enantiomer (R). Enantiomers of the racemic drugs often differ in pharmacokinetic behaviour or pharmacological action. The development of analytical methods for the quantitative analysis of chiral compounds are extremely challenging due to the fact that enantiomers posses virtually identical properties. Even though corresponding S-isomer is controlled in starting stages of Levosimendan synthesis, it is quite important to monitor the level of other isomer in finished product. Literature reveals that there are only few analytical methods for estimation of Levosimendan in plasma and dosage form by LC-MS/MS[6-12]. But none has reported the chiral separation of enantiomers of Levosimendan.

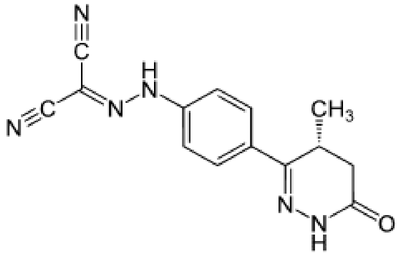

Figure1. Chemical structure of Levosimendan

MATERIALS AND METHODS

Chemicals and reagents

HPLC grade Methyl-tert-butyl ether (MTBE), Ethanol and Trifluoro acetic acid were purchased from Merck. All solutions were filtered through 0.22µm filters procured from Pall Pharmalab Filtration Pvt. Ltd(Mumbai, India). R-Levosimendan bulk drug and S-Levosimendan isomer were provided by Hetero Drugs Ltd, Hyderabad, India.

Instrumentation

An Agilent 1100 series HPLC with a variable wavelength detector was used for the development and validation of the proposed method. The output signal was monitored and processed using Chemstation software (Agilent Technologies, Waldbronn, Germany). Data acquisition and processing were conducted using the LC solutions software.

Preparation of Stock, Standard and Test Solutions

The stock solution of S-Levosimendan was prepared by dissolving 3.0mg of S-isomer in 100mL of mobile phase (6%). From this transferred 0.5mL into 20mL volumetric flask and diluted to the volume with diluent (0.15% solution). A series of dilutions prepared using 6% solution of S-Levosimendan for Limit of quantification, Limit of detection, Precision, Linearity and Accuracy. The sample solution was prepared by weighing about 10 mg of the R-Levosimendan bulk drug substance into a 20mL volumetric flask and dissolved and diluted to 20 mL with mobile phase.

Preparation of System suitability 10mg of Levosimendan racemic sample was dissolved in 20 mL of mobile phase. As a part of system suitability, two criteria were defined (i) resolution between R-Levosimendan and S-Levosimendan (ii) Tailing factor R-Levosimendan. The System suitability results are tabulated in Table 1.

Table 1: System suitability Results

S.No	Parameter	1.0 mLmin^{-1}
01	The resolution between S-isomer and R-Isomer	10.15
02	The tailing factor of S-Isomer from system suitability solution	1.12

Chromatographic Conditions

The chromatographic column used was chiral pak IA (250mm ×4.6mm i.d.; particle size, 5µ), make of Chiral Technologies, USA. Using solvents like Methyl tert-Butyl ether, Ethanol and trifluoro acetic acid(650:350:1.0, v/v/v) was optimized as mobile phase as well as diluent. The flow rate of the mobile phase was 1.0 mL.min^{-1} and the column temperature set at 30°C. The injection volume was 10µL. The concentration of the sample taken was 0.5mg.mL^{-1}. The wavelength of method was set at 383nm.

RESULTS AND DISCUSSION

Method Development and Optimization

The HPLC method carried out in this study aimed at developing chromatographic system capable of eluting and resolving Levosimendan Enantiomers (R-Levosimendan and S-Levosimendan) and also complied the general requirements of system suitability criteria. The main challenge was to get good resolution between Levosimendan enantiomers with acceptable peak shapes and trace level detection. With this objective several trials were carried out initially, on Chiral pak AD-H and Chiral pak IC, chiral cell OD-H columns with respect to different flow rates and different mobile phase compositions. The results showed that the resolution was not more than 1.0 and peak shapes was poor with these columns. Later Chiral pak IA 250x4.6mm, 5microns column was considered, which gave good resolution and Gaussian peak shapes with the optimized mobile phase. With respect to mobile phase, initially n-Hexane and 2-propanol with trifluoro acetic acid as additive was used which giveless resolution between both enantiomers. To improve separation MTBE and Ethanol was used as a mobile phase component and within 20 mins both enantiomers were resolved with very good peak shapes. Therefore the finally optimized mobile phase consists of 65% of MTBE and 35% of Ethanol and 0.1% trifluoroacetic acid as additive, with a flow rate of 1.0mL.min^{-1} and column oven temperature of 30°C. The run time was 20 minutes. The blank, system suitability chromatograms Levosimendan enantiomers were shown in Figures 2, 3and 4 respectively. The sample concentration of

0.5mg.mL^{-1} was considered based on the detection level. In the present study 0.15% specification kept for S-isomer.

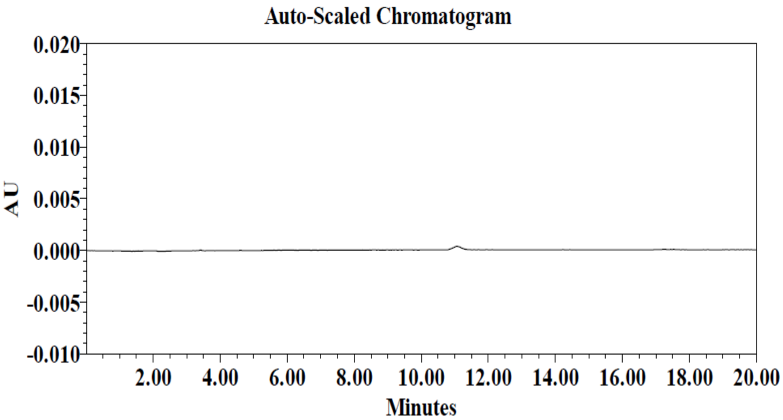

Figure 2: A typical HPLC chromatogram of Blank solution

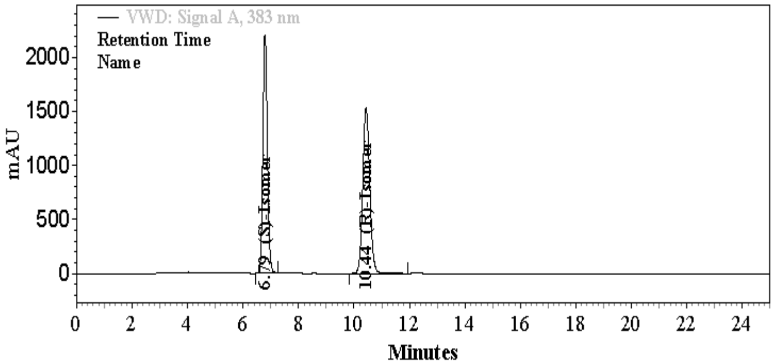

Figure 3: A typical HPLC chromatogram of System suitability solution

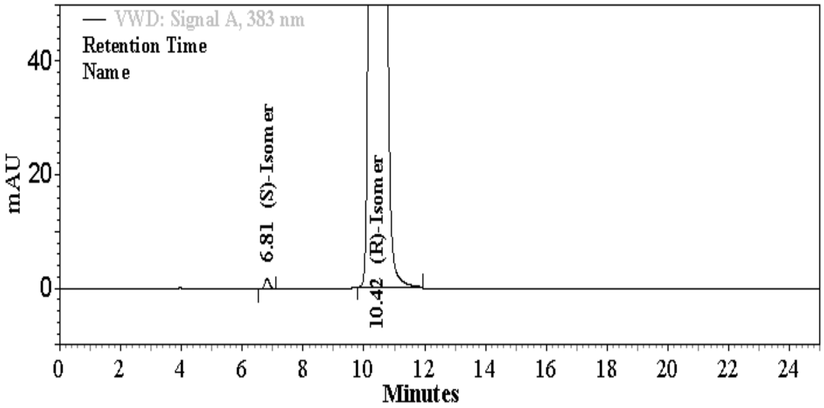

Figure 4:A Typical HPLC chromatogram of Levosimendan Test solution

Method Validation

The validation work was conducted according to the ICH (International Conference on Harmonization) guidelines. The validated method parameters were LOD, LOQ, Accuracy, Precision, Linearity, Range, Ruggedness and Robustness. Sensitivity was determined by establishing the Limit of detection (LOD) and Limit of quantitation (LOQ) for S-Levosimendan estimated at a signal-to-noise ratio of 3:1 and 10:1 respectively, by injecting a series of dilute solutions with known concentration. The detection limit (LOD) of the S-isomer was found to be about 0.025µg/mL and the quantification limit (LOQ) was about 0.075µg/mL. The precision study was also carried out at LOQ level by injecting six individual preparations of S-Levosimendan. Calculated the %RSD for the areas of S-isomer. The precision values at LOQ concentration for S-isomer was below 2%. The recovery at LOQ concentration level for S-isomer was 99.1%.

Precision

The precision of the chiral method was performed by injecting six individual preparation of R-Levosimendan (0.5mg.mL^{-1}) test sample. The %RSD for area% of S-isomer was calculated. Precision study was also determined by performing the same procedures at different day on different system. The cumulative %RSD values for precision and intermediate precision study were below 2%.

Linearity and Range

A linearity test solutions for chiral method were prepared by diluting the S-Levosimendanstock solution at six concentrations from LOQ to 150% of the 0.15% level of the S-Levosimendan (i.e. LOQ, 50%, 80%, 100%, 120% and 150%) was subjected to linear regression analysis with the least square method. The peak area versus concentration data was performed by least-squares linear regression analysis. Calibration equation obtained from regression analysis was used to calculate the corresponding predicted responses. The correlation coefficient for S-Levosimendan was more than 0.999. The linearity calibration curve for (S)-Isomer was shown in Figure 5.

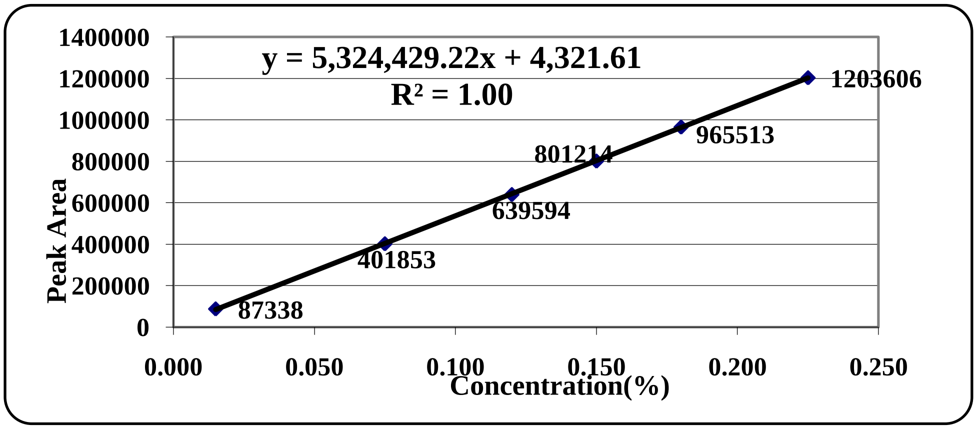

Figure: 5 Typical Linearity curve of (S)-Levosimendan

Accuracy

Accuracy of the method was determined by analyzing R-Levosimendan (*n*=3) spiked with (LOQ, 50% to 150%) 0.075%, 0.15% and 0.225% of the S-isomer. The recoveries obtained for S-isomer at all levels were in between 99.1% and 107.9% respectively. The percentage of recoveries of S-isomer was calculated and tabulated Table 2.

Table 2: Accuracy Results

Level of Accuracy	Preparations	Isomer added (µg/mL)	Isomer Recovered (µg/mL)	%Recovery(n=3)
LOQ	1	0.075	0.074	99.1
	2	0.075	0.081	108.0
	3	0.075	0.079	105.7
50%	1	0.075	0.077	103.07
	2	0.075	0.075	100.00
	3	0.075	0.076	101.33
100%	1	0.150	0.150	100.00
	2	0.150	0.149	99.33
	3	0.150	0.158	105.33
150%	1	0.225	0.230	102.22
	2	0.225	0.243	107.87
	3	0.225	0.233	103.56

Ruggedness

Ruggedness of the method was performed by doing precision study by injecting six individual preparations of ($0.5 mgmL^{-1}$) R-Levosimendan spiked with 0.15% of S-isomer (100% level) using different lot column, different analyst and different system from the same laboratory. The %RSD for area% of S-isomer was calculated. The %RSD obtained for S-isomer was below 2.0%.

Robustness

To determine the robustness of the developed method, experimental conditions were deliberately changed and the resolution (*Rs*) between R-Levosimendan and S-Levosimendan was evaluated. The flow rate of the mobile phase was $1.0 mLmin^{-1}$. To study the effect of flow rate on the developed method, 0.2 units of flow was changed (i.e. 0.8 and $1.2 mLmin^{-1}$). The effect of column temperature on the developed method was studied at 28°C and 32°C instead of 30°C. The effect of Mobile phase composition on resolution of isomers was studied by varying ± 5% of MTBE and Ethanol. System suitability injected in the above changed conditions to check the resolution and RRT of both enantiomers. No peak is merged with each other and the resolution between two isomers was more than 8.0. The Robustness system suitability data was tabulated Table-3.

Table 3: System suitability Results (Robustness)

S.No	Parameter	0.8 $mLmin^{-1}$	1.2 $mLmin^{-1}$	28°C	32°C	+5% MTBE	-5% MTBE
01	The resolution betweenS	10.28	9.05	9.67	9.49	10.64	10.39

	and R-Isomer						
02	The Tailing factor from system suitability solution	1.15	1.12	1.15	1.13	1.14	1.16

Solution Stability Solution study was performed for sample, system suitability solutions upto 48 hours at room temperature and the solution was found to be highly stable at room temperature during 48 hours study and no impurities observed during this study.

CONCLUSION

A simple, sensitive and novel Chiral HPLC method was developed and validated for the quantitative determination of S-isomer related to Levosimendan. Compared with the previously reported methodologies there is no specific chiral method was reported for Levosimendan Active pharmaceutical ingredient. This method utilizes a Chiral pak IA column which is readily available in most of the quality control testing laboratories in the pharmaceutical industry and relatively simple to use. This method is sensitive enough to detect S-isomer 0.025 $\mu g.mL^{-1}$ and can quantify upto 0.075$\mu g.mL^{-1}$. The run time of the method was only 25 minutes, so it can reduce solvent consumption as well as analysis time.

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