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Development and Validation of Stability Indicating Method for the Determination of Levosimendan and its Related Impurities by RP-UPLC

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ABSTRACT

A simple, sensitive and stability indicating method was developed and validated for the determination of related substances in Levosimendan drug substance. An efficient chromatographic separation was achieved using Acquity; UPLC, BEH; C-18; 100 X 2.1mm; 1.7 μ m column with the mobile phase consisting of 0.05% Trifluoro acetic acid in water and 0.05% Trifluoro acetic acid in acetonitrile in a gradient elution mode within a short run time of 10 min at a flow rate of 0.3 mL min⁻¹. The eluents were monitored by a photodiode array detector at 220 nm an injection volume of 0.5 μ L. The drug substance was subjected to various stress conditions to investigate the stability indicating ability of the method and found that it undergoes significant degradation during acid, base and oxidative stress conditions. This method is proven to be capable to separate all known, degradation impurities from Levosimendan. The developed method was validated as per the current ICH quality guidelines with respect to specificity, precision, accuracy, linearity, robustness and solution suitability. The developed liquid chromatographic UPLC method was found to be specific, precise, sensitive and accurate for the determination of Levosimendan related substances in bulk and finished formulations.

Graphical Abstract



Keywords: Levosimendan, UPLC, impurities, stability indicating method, validation.

INTRODUCTION

Levosimendan, whose chemical name is (R)-N-(4-(4-methyl-6-oxo-1,4,5,6-tetrahydropyridazin-3yl)phenyl)carbonohydrazonoyl dicyanide has molecular formula and the weight of C₁₄H₁₂N₆O and 280.11 g mol⁻¹, respectively. Levosimendan it is a calcium sensitizer aimed for the treatment of congestive heart failure [1, 2]. Levosimendan is the pharmacologically active enantiomer of the racemate simendan. It acts by binding, in a calcium binding protein that regulates contraction of skeletal and cardiac muscles [3]. Levosimendan is an inotrope agent with a unique mode of action. It is a calcium sensitizer also called as inodilator, which increase cardiac contractility by enhancing the sensitivity of the myocardium to calcium [4, 5]. As a result, levosimendan produces positive inotropic effects that are independent of beta-receptors or cyclic AMP. It also has a vasodilator effect, by opening ATP-sensitive potassium channels in vascular smooth muscles, which results in smooth muscle relaxation [6]. The combination of inotropic and vasodilator actions results in an increased force of contraction with decreased preload and afterload in the myocardium. So the levosimendan can act as Inotropy, vasodilation and cardio protection [7]. Commercially levosimendan is available as SIMDAX IV. SIMDAX proven that it was more effective and well tolerated in large scale clinical trials of heart failure hospitalised patients. Total worldwide Over 1.500,000 patients were treated with SIMDAX.

Levosimendan having hemodynamic benefits, symptomatic benefits and sustained effects. Based on the drug's potential to fill a requirement of medical need, the FDA has granted Fast Track status for Levosimendan [8, 9]. Levosimendan activates stunned myocardium in patients after percutaneous transluminal coronary angioplasty (PTCA) or thrombolysis [10, 11]. The drug is still not official in any pharmacopoeia [12]. It is known that drugs can undergo physicochemical degradation during manufacturing and storage due to processes like oxidation, reduction, hydrolysis, racemization etc.[13, 14] Therefore, understanding drug degradation under stressed conditions is critical in pharmaceutical development because drug stability and degradation products have a significant impact on formulation development, analytical method development, package development, and storage conditions[15, 16]. Because of the chances of the active pharmaceutical ingredient may experience degradation, its products may result in activity loss or occurrence of adverse effects. Complete knowledge of API's stability profile is one of the key factors to prevent those risks during manufacturing and storage [17-20]. Thus the study of degradation of drugs should be carried out in stress conditions as recommended by the ICH.

Levosimendan (SIMDAX IV) formulation which is available in combination of poviodine, citric acid and ethanol combination in sterile form. Drug is a combination of citric acid and API it is necessary and important to study of acid and hydrolytic degradation and characterization of degradation products. Earlier some of analytical journals have been reported on enantiomer separations by chiral methods using HPLC and capillary electrophoresis [21-25]. Some other journals have published stability indicating assay method by HPLC for Levosimendan injectable dosage form [26], and estimated stability of prefilled syringes of Levosimendan along with the possibility of leaching of the compounds from the syringe during storage [27]. But they have not identified or characterized any impurities. The estimation of the drug from plasma matrixes using high performance liquid chromatography (HPLC) and Liquid Chromatography-Mass Spectroscopy (LCMS) have also been published in several articles [26-30]. The objective of the current work is to develop a stability indicating UPLC method with short run time. The objective is also to develop a stability indicating method by means of performing forced degradation studies on the bulk as well as finished formulations in order to study the impact of excipient on the developed method. The developed method need to be validated for the parameters as per the current ICH quality guidelines and USP general chapter for Analytical method validations. Hence the proposed validated method can

be used in quality control laboratories for routine as well as stability analysis of bulk and finished formulations.



Figure 1. UV Spectrum of Levosimendan.



Figure 2. Structure of Levosimendan and its related impurities.

MATERIALS AND METHODS

Chemical reagents and sample: Levosimendan was a gift sample from a reputed manufacturing unit in Hyderabad, India. Simdax IV injection was purchased from the local pharmacy. Acetonitrile of HPLC grade was purchased from Rankem chemicals (Mumbai, India). Formic acid, sodium hydroxide, hydrochloric acid and hydrogen peroxide were purchased from Merck chemicals (Darmstadt, Germany). HPLC grade water was obtained from milli-Q water purification system (Millipore, Milford, USA).

Equipment's: A prominence series Waters Acquity UPLC system equipped with a binary solvent manager pump, an auto sampler, and PDA detector used for method development, validation and stress degradation studies. The signal output was monitored and processed using Empower-3 software

on a Dell computer. Chromatographic separation was achieved on Acquity UPLC BEH C18 100*2.1 mm, with particle size of 1.7 μ m was used. Thermal degradation study was carried out in a hot air oven (Vision lab Equipment's), Ultrasonic bath sonicator was purchased from Thermo Analytics and photolytic degradation was carried out on photo stability chamber purchased from Thermo lab scientific instruments.

Chromatographic conditions: The objective of the present study is to develop a rapid stability indicating UPLC method for the estimation of impurities of Levosimendan with proper peak shape and resolution. Chromatographic separation was performed on Waters UPLC with Acquity UPLC BEH C18 100*2.1 mm, 1.7 μ m column. Mobile phase A was 0.05% Trifluoro acetic acid in water and mobile phase B was 0.05% Trifluoro acetic acid in acetonitrile. Diluent was prepared by mixing water and acetonitrile in the ratio of 50:50 (v/v). Injection volume was 0.5 μ L, Flow rate was 0.3 mL min⁻¹, analysis was carried out at a wavelength of 220 nm with data acquisition time of 10 min.

Preparation of buffer A: Dissolved accurately 0.5 ml of Trifluoro acetic acid in 1000 mL of milli-q water and mixed well.

Preparation of buffer B: Dissolved accurately 0.5 mL of Trifluoro acetic acid in 1000 mL of Acetonitrile and mixed well.

Preparation of standard solution: A working standard stock solution of Levosimendan was prepared by dissolving standard equivalent to 50 mg of Levosimendan into 100 mL volumetric flask, to this added 60 mL of diluent and sonicated for 5 min and then diluted to the volume with diluent to have a solution concentration of 500 ppm.

Preparation of sample solution: Transfer 20 mL of Levosimendan injection into 100 mL volumetric flask and added 60 mL of diluent and sonicated in ultrasonic bath for 20 min with intermediate shaking and diluted to the volume with diluent. Filter the solution through 0.45 μ m nylon membrane filter by discarding 4 mL of filtrate and injected the same solution (0.5 mg mL⁻¹).

Preparation of placebo solution: Povidone, Citric acid, Ethanol anhydrous sample solutions were used as excipient in the Levosimendan injection. So, in order to establish the specificity, Levosimendan and its excipients were prepared at the test concentrations individually (0.5 mg/ml) in diluent and injected in the proposed method to verify the spectral interference if any at the retention times of Levosimendan and its known impurities.

Method validation:

Specificity: Specificity is the ability of the method to measure the analyte response in presence of its potential impurities. Specificity of the developed method was carried out in the presence of blank, placebo and its known impurities i.e., imp-1, imp-2 and imp-3 for the accurate measurement of amount of impurities present in the sample. As a part of specificity, stress studies were carried out for Levosimendan drug substance, drug product and placebo under stress conditions like oxidation, acid, base, photolytic and thermal (120°C). These stress samples were analyzed using the proposed method at a test concentration of 500 ppm to separate all the three Levosimendan impurities along with its degradation impurities at a quantification level of 0.15 %. In these stress conditions the peak purity test was verified for the Levosimendan peak and its other known impurities by using photo diode array detector.

Precision: Precision of the analytical method is the closeness agreement for a series of measurement from multiple samplings as mentioned in ICH Q2 (R1). As per the quality guidelines, system precision, method precision and intermediate precision were analyzed on the homogeneous sample

and the % RSD of individual impurity for method precision and intermediate precision was calculated and reported.

LOD and LOQ: The LOD and LOQ values for Levosimendan and its impurities were determined at signal-to-noise ratio of 3:1 and 10:1 respectively by injecting a series of dilute solutions with known concentrations.

Precision at LOQ levels was also determined by injecting six individual preparations of mixtures of all impurities and Levosimendan at their LOQ level. The % RSD of the areas of each impurity and Levosimendan were calculated for precision studies.

Linearity: Linearity of the detector response was established for all the known impurities and Levosimendan with concentration ranging from LOQ to 150 % of the specification level (0.15 %) with respect to test concentration. The samples were analyzed as per the described test method. A linearity graph was plotted between the responses of impurity (Y-axis) against actual concentration in ppm (X-axis) and determined the correlation co-efficient and Y-intercept at 100 % response.

Accuracy: Accuracy of the analytical method is the closeness of agreement between the true value and experimental value. Accuracy of the three impurities was performed at 5 different levels ranging from LOQ to 150 % of the specification level of the impurity with respect to test concentration level. The % recovery was calculated by comparing the impurity level at each level of spiked sample with as such sample.

Robustness: The robustness of the method was evaluated to establish the capability of the method by changing the experimental conditions and studying its impact on the system suitability. Robustness was performed by changing the method parameters like mobile phase flow rate and column temperature.

Solution stability: Solution stability was carried out by storing the standard solution of Levosimendan 500 ppm spiked with 0.15% of each impurity at room temperature up to 48 h. This solution was injected at an interval of 0, 24, and 48 h. The impurity content and system suitability results were checked at each time interval.

RESULTS AND DISCUSSION

Method development and Optimization: As there was no stability indicating UPLC method reported for the determination of related substances in Levosimendan bulk and finished product. The intention of the method was to separate all the potential impurity peaks originate during the forced degradation study and stability studies with proper peak shape and resolution. Forced degradation sample was taken as reference, for the optimization of the UPLC method. Trials were taken by varying the pH value of the mobile phase buffer from 5.0 to 2.0. Finally Trifluoro acetic acid buffer was used based on the LC-MS compatibility. Forced degradation samples were injected and found that all the three acknowledged impurities cited above figure 2 were spectrally pure with longer run time and vast peak shapes in isocratic mode. In order to shorten the run time, gradient separation mode was optimized with adequate separation. Optimal separation was attain on Acquity UPLC BEH C18 column with dimensions 100*2.1 mm, 1.7 µm. Gradient elution was executed using the combination of 0.05% Trifluoro acetic acid in milli-q water buffer (pH~ 2.7) and 0.05% Trifluoro acetic acid in acetonitrile as organic modifier at a flow rate of 0.3 mL min⁻¹. UPLC detection was carried at a wavelength of 220 nm. Gradient programme was cited in table 1. Mobile phase A is 0.0.5% Trifluoro acetic acid in Milli-q water, Mobile phase B is 0.05% Trifluoro acetic acid in Acetonitrile.

Time (minutes)	Flow rate (mL min ⁻¹)	% of mobile phase-A	% of mobile phase-B
0.0	0.3	95	05
0.3	0.3	95	05
6.0	0.3	05	95
8.0	0.3	05	95
8.5	0.3	95	05
10.0	0.3	95	05

Table 1. Gradient Program for UPLC method

System suitability: System suitability solution was prepared by spiking all the impurities in the sample at the specification level (0.15%) and injected to evaluate the system suitability of the method and found that Levosimendan and its 3 known impurities were separated with good resolution, with typical retention times of 2.45, 3.44, 3.67 and 4.15 respectively. The system suitability results were given in table 2. The developed UPLC method was found to be specific for Levosimendan and its known impurities. Figure 3 shows separation of all the three known impurities from Levosimendan in the proposed method.

Table 2. System suitability criteria

S.No	Name	Rt	Purity Angle	Purity Threshold	RRT	USP Resolution	USP Tailing	USP Plate count
1	Impurity-2	2.45	0.53	1.155	0.59	NA	1.21	77128.29
2	Impurity-1	3.44	0.62	1.094	0.83	23.35	1.18	83560.85
3	Impurity-3	3.66	0.64	1.233	0.88	4.53	1.18	85394.24
4	Levosimendan	4.15	0.17	0.329	1.00	9.62	1.2	109181.48



(Rt-Retention time, RRT-relative retention time)

Figure 3. Typical chromatogram of Levosimendan and its impurities.

Precision: Method precision was performed by analyzing the spiked sample with known impurities at the specification level (0.15%) and % impurity was calculated. The % relative standard deviation was found to be 0.35, 0.61, 0.27 and 1.07 for impurity-1, impurity-2, impurity-3 and Levosimendan respectively. Intermediate precision was performed on a different day, different UPLC system using different UPLC column of the same manufacturer. The relative standard deviation for these spiked samples was also within 2.0% limit. The results were mentioned in table 3. Hence the developed method is precise for its intended use.

LOD and LOQ: The determined limit of detection and limit of quantification results of Levosimendan and its related substances were in the range of 0.0001-0.0003 μ g mL⁻¹ (LOD) and 0.001-0.0004 μ g mL⁻¹ (LOQ) respectively indicating the higher sensitivity of the method. The %RSD

Method precision			Intermediate precision					
S No. % impurity		% impurity						
5. 1N0	Imp-1	Imp-2	Imp-3	Levosimendan	Imp-1	Imp-2	Imp-3	Levosimendan
1	7842	7620	8103	1613348	8309	7698	8056	1587223
2	7889	7590	8098	1610785	8370	7613	8017	1561218
3	7820	7552	8141	1637090	8370	7656	8113	1577668
4	7879	7564	8152	1604906	8297	7622	8150	1589532
5	7883	7677	8142	1649176	8277	7659	8044	1568130
6	7877	7632	8132	1630229	8170	7572	8138	1568577
Avg	7865.00	7605.83	8128.00	1624255.67	8298.83	7636.67	8086.33	1575391.33
SD	27.55	46.58	22.28	17298.96	73.91	43.80	54.69	11359.36
% RSD	0.35	0.61	0.27	1.07	0.89	0.57	0.68	0.72

Table 3. Method precision and intermediate precision data

values for precision at LOQ level of peak areas for six injections were below 3.41%. All the detailed values were reported in table 4. The LOQ mixture chromatogram was mentioned in figure 4.

	LOQ precision							
S.No	S.No % impurity							
	Imp-1	Imp-2	Imp-3	Levosimendan				
1	1756	2375	1836	3317				
2	1770	2332	1826	3305				
3	1763	2290	1829	3284				
4	1756	2360	1834	3315				
5	1806	2360	1802	3306				
6	1751	2309	1798	3265				
Avg	1767.00	2337.67	1820.83	3298.67				
SD	20.22	33.22	16.57	20.23				
% RSD	1 14	1 4 2	0.91	0.61				

 Table 4. LOD and LOQ establishment for Levosimendan and its related known impurities



Figure 4 (a). LOQ (b) LOD mixture typical chromatograms.

Linearity and range: Linearity of the developed method was evaluated for six different levels of Levosimendan and its three known impurities. The concentrations ranged from LOQ to 150 % of impurity specification limit (0.15% with respect to test concentration). The respective peak area was recorded and plotted against standard (impurity) concentration and the graph resulted in straight line.

The correlation coefficient values were found 0.9998, 0.9984, 0.9993 and 0.9995 respectively. The values are tabulated for Levosimendan and its known impurities. The compiled results were tabulated below in table 5.

S No.	Concentration	Area response					
5.110	(%)	Levosimendan	Impurity-1	Impurity-2	Impurity-3		
1	LOQ	3302.00	1763.00	2332	1830		
2	50	794703.00	4114.00	3824	4093		
3	75	1191218.67	6324.33	5744	6120		
4	100	1579484.67	8067.00	7614	8098		
5	125	1983777.33	10217.00	9301	10202		
6	150	2341903.67	12648.33	11254	11974		
Correl	ation coefficient	0.9998	0.9984	0.9993	0.9995		

Table 5. Linearity results for for Levosimendan and its related known impurities

Accuracy: Recovery studies were performed to judge the accuracy of the test method. The study was evaluated by spiking the known quantity of impurities at various levels on the placebo. From the amount of impurity found the % recovery was calculated. Recovery was performed at four different levels ranging from LOQ to 150 % of the specification level. The % recovery of each impurity was found to be within the acceptance criteria of 95% to 105%. So the method is accurate for the determination of Levosimendan and its impurities. The mean recovery values for the impurities were tabulated in table 6.

Table 6. Accuracy results for Levosimendan and its related impurities

S No	Cono loval	% Mean recovery				
3.1NO	Conc level	Impurity-1	Impurity-2	Impurity-3		
1	LOQ	100.23%	100.23%	99.48%		
2	50%	99.48%	100.33%	99.88%		
3	100%	99.88%	100.03%	99.99%		
4	150%	100.35%	99.96%	99.41%		

Solution stability: There was no change in the area counts of the Levosimendan and its respective impurities, when both the standard and sample solutions were monitored periodically for a period of 0, 24 h and 48 h at room temperature (not more than 27° C) and at a temperature of $5 \pm 3^{\circ}$ C. It was observed that the standard solution and sample solution was stable for 48 hrs at $5 \pm 3^{\circ}$ C.

Specificity: The specificity of the method was evaluated by verifying the peak purity of the sample. The method was found to be specific as there was no interference from blank and placebo at the retention time of main peak. No degradant peaks were observed at the retention time of Levosimendan during the forced degradation and stability study indicates that the method is stability indicating and also peak purity index for all the impurities and Levosimendan were less than the purity threshold indicates that there was no spectral co-elution for any of the peaks in this method and also the resolution between the neighbouring peak was greater than 2.0. Typical chromatograms of (a) placebo (b) blank and (c) standard were mentioned in figure 5.

Forced degradation studies: Forced degradation studies were performed to establish the stability indicating power of the method. In this study Levosimendan raw material, finished product and placebo were subjected to acidic, basic, peroxide, thermal and photolytic stress studies on sample concentration of 0.5 mg mL⁻¹ in diluent. Sample equivalent to 50 mg of Levosimendan was placed into 100 mL volumetric flask added 60 mL of diluent and sonicated for 10 min with intermediate shaking and then added respective degradant (Acid, Alkali, Oxidant) and performed the stress study. Samples were neutralized after degradation and then diluted to the volume with diluent and injected to verify the stability indicating power of the analytical method. Stress conditions under which the study





Table 7. Force degradation conditions and results for Levosimendan

Parameters	RT	% Assay	RS Purity	% Degradation in RS
Acid Degradation Sample (5 mL 1N HCl/10h,60°C)	4.16	89.80	89.34	10.66
Base Degradation Sample (5 mL 1N NaOH/10h,60°C)	4.16	88.53	88.33	11.67
Peroxide Degradation Sample (5 mL 10% H ₂ O ₂ /48h, Rt)	4.16	81.30	81.39	18.61
Thermal Degradation Sample 120°C 48h	4.16	99.13	99.32	NA
UV Degradation Sample	4.16	99.11	99.189	NA



Figure. 6. Typical chromatograms of (a) Levosimendan-API (b) acid (c) base (d) peroxide (e) Thermal and (f) UV condition.

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was performed, the amount of Levosimendan remains, % impurities generated, drug purity and % assay results were tabulated in table 7. The chromatograms for forced degradation study were summarized figure 6.

APPLICATION

Till date there was no methods have been reported for the estimation of Levosimendan and its related substances by chromatographic techniques in bulk and finished dosage forms and also in pharmacopoeias. This method is shorter, sensitive, precise and mass compatible to detect low level of process related impurities and degradation products of Levosimendan.

CONCLUSION

The degradation behavior of Levosimendan was studied under various stress conditions as per International Conference on Harmonization (ICH) prescribed guidelines. A novel, reverse phase simple gradient ultra-performance liquid chromatographic method with a low run time of 10 minutes, LC-MS compatible UPLC method has been developed and validated for the determination of a stability-indicating assay of Levosimendan and its related substances in bulk drugs for the first time. The developed method has been found to be selective, precise, linear, accurate, robust, sensitive and rugged. It is applicable for detecting process related impurities and other possible degradation products which may be present at trace levels in bulk drugs. Thus, the method can be used for process development, stability analysis as well as quality control of Levosimendan in bulk drugs

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