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Cyclodextrin as solubility enhancer for Levosimendan Lyophilized injection

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ABSTRACT

The objective of this experiment is to formulate the lyophilized injection of water insoluble Levosimendan by using Cyclodextrin as solubility enhancer for Parenteral administration with better stability. Hydroxypropyl betacyclodextrin asSolubilizer, Trisodium citrate as buffer and Hydrochloric acid for pH adjustment were used with water for injection into 20 ml tubular vials. The filled vials were loaded into Lyophilizer and lyophilized them as per cycle. Different concentrations of Solubilizer and different pH concentrations of 5.0, 5.5, 6.0, 6.5, 7.0 and 7.5 were adjusted with 0.1 N Hydrochloric acid solutionswere usedto formulate thelyophilized formulation. The formulation F (3) which has been optimized, theresults of the cycle was observed to be optimised. Levosimendan was developed as lyophilized formulation for betterstability. The obtained results suggested that a stable formulation for drug Levosimendan was developed which wasComparable to reference listed product.

Keywords: Parenteral, Lyophilization, Levosimendan

INTRODUCTION

Levosimendan, an (2)-enantiomer of a racemic mixture of simendan, is a calcium-sensitizing agent that wasdeveloped as a safe positive inotropic drug for the treat-ment of heart failure (Fig. 1). Calcium sensitizers increasemyocardial contractility by generating more force for a given amount of cytoplasmic free calcium. This allows a greater positive inotropic effect without an increase inintracellular calcium [1]. The danger of arrhythmias due to calcium overload can be avoided, which is a very importantadvantage.Therapeutic concentrations of levosimendan in plasmaare achieved with intravenous infusion at doses of 0.05–0.2 mg/kg/min [2]. The clearance of levosimendan is 300–360 ml/min and it seems to bemainly eliminated by metabolism [3]. Continuous, controlled, and non-invasive delivery of levosimendan via a transdermal route might provide convenient administration with long duration of activity and less risk of excessive drug concentrations.For transdermal permeation, the drug molecule should have a small molecular weight, adequate solubility in the vehicle and appropriate lipophilicity [4-6].

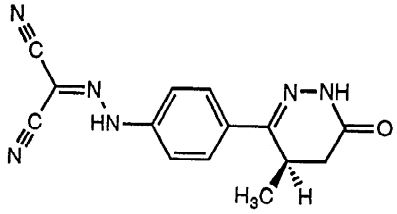

Fig. 1. Structural formula of levosimendan

The molecular weight of levosimendan is 280.29 and the partition coefficient (log *D* octanol / buffer) 1.1 at neutral pH[7]. Since levosimendan is a weak acid with a p*K* of 6.26, it is mainly negatively charged at physiological pH. When levosimendan was tested for transdermal delivery, we found that the solubility of levosimendan in 50 mM phosphatebuffer was 0.34 mg/ml at pH 7.4 and that the flux of levosimendan across the human skin in vitro is ca. 0.01 mg/ h. The stratum corneum, the major rate-limiting barrier for drug permeation in human skin, carries a net negative charge at neutral pH [8] and,therefore, may show electric repulsion of negatively charged levosimendan. In continuous treatment, the skin area employed for drug administration should be relatively small.

Cyclodextrins (CDs) are cyclic oligosaccharides composedof at least six D-(+)-glucopyranose units linked bya-(1–4) bonds (Fig. 1a) [9]. Natural CDs occur in the formof white crystalline powder, and they form stable hydrates.CDs have quite rigid structures (stabilized by hydrogenbonds between C2 and C3 hydroxyl groups) lacking freerotation in a-(1–4) bonds; thus they form torus-like molecules(truncated cone) (Fig. 1b) [9]. CD molecules have ahydrophilic outer surface (all hydroxyl groups in the ringare located in the exterior of torus) and a hydrophobic interior(there are skeletal carbons with hydrogen atoms andoxygen bridges inside the cavity). The non-bonding electronpairs of the oxygen bridges are directed toward theinside cavity, thereby generating high electron density [10, 11].While CDs were first isolated by Villiers in 1891, themain characteristics, preparation, and isolation techniquewere described by Schardinger [12, 13]. In the beginning, onlyvery small amounts of CDs were produced. It was only in the late 1970s when biotechnological development enabledproduction of purified CDs with high yield that the‘‘career’’ of CDs started.There are three main natural CDs: a-, b-, and c-CDcomposed of six, seven, and eight glucose units, respectively.They differ in ring size and physicochemical properties(Table 1). It is possible to achieve higher homologues,but because of their properties – large cavity dimension,high aqueous solubility, and weak complex formation –they cannot be of practical use. The CDs contain 18 (a-CD), 21 (b-CD), or 24 (c-CD) hydroxyl groups that canbe chemically modified. To improve some physicochemicalproperties of natural CDs, many types of derivatives havebeen developed: hydrophilic (methylated, hydroxyalkylated,and branched), hydrophobic (ethylated), ionic (sulphatedand phosphated) [10-13]. Derivatisation of parent crystalCDs usually leads to achieving amorphous mixtures of isomers;thus, their aqueous solubility is much higher [14].The most important attribute of CDs is the ability tocreate inclusion complexes with a large number of moleculesor their portions; however, not all molecules (drugs)can form stable complexes. There are some limitations, likevery high aqueous-soluble substances, that generally cannotbe included. Recently Martins et al. [15] reported thathigh soluble drug substances are able to create with CDs rather an association compound in which drug interactswith the hydrophilic outer surface of CD (hydroxyls atposition 3).The size – geometric factor of the molecule is mostimportant because it decides whether the molecule is ableto form ‘‘stable’’ inclusion with a-, b-, or c- CD. If the moleculehad adequate properties, it interacts with CD insidecavity without forming covalent bonds; this interaction is‘‘guest/host’’ type. CD inclusion complex is mainly formedvia the substitution of included water by the appropriate‘‘guest’’ molecule. Release of the enthalpy-rich water moleculesfrom the cavity decreases the energy of the system. Adecrease in the energy of the system is due to reduce thecontact surface area between the solvent and solute as wellas solvent (highly polar water) and imperfectly solvated(hydrophobic) CD cavity. Some other factors, such ashydrogen bonding, changes in surface tension, van derWaals’ interactions, and ring strain release, also can havesome influence on the complex formation. The complexationis usually a concentration-dependent process and themolar ratio (1:1, 1:2, 2:1, 2:2) can depend on the ‘‘guest/host’’ proportion. It is possible that in solution the moleculeinteracts with the outer surface of CD and CD complexesagglomerate (self-association) [16–18].

The association/dissociation equilibrium in aqueoussolution is one of the most characteristic features of inclusion.Drug release from the CD complex is mainly causedby dissociation due to dilution in fluids. In the case of topicalapplications, such as ocular, nasal, rectal, or dermal,with minimal or impossible dilution mechanism, the potentialmechanism of drug release from CD complex is preferentialdrug uptake by tissue [19]. As reported by Stella et al.[19] if the drug substances possesses physicochemical propertiesthat allow it to penetrate into or through biologicalmembranes (skin, mucosa, or cornea), then the tissue actsas a sink causing dissociation of the complex. Only a freefraction of drug that is in equilibrium with the complexedfraction may be available for absorption, thus CDs are able to increase bioavailability rather by deliver the drug substanceto absorption site and by minimization of drughydrophobicity than permeation by itself. The penetrationinto or permeation through the biological membranes ofinclusion complex and CDs are questionable because oftheir large mass (>1000 Da) and hydrophilicity [11, 19–24].However, there are few works that demonstrate someabsorption of CDs by pulmonary, dermal (occluded) ortransmucosal route, probably through paracellular pathway[25, 26]. There are also suggestions that certain CDs(especially methylated) are able to extract membrane components[11, 26]. Safety overview of selected cyclodextrins in Table-1

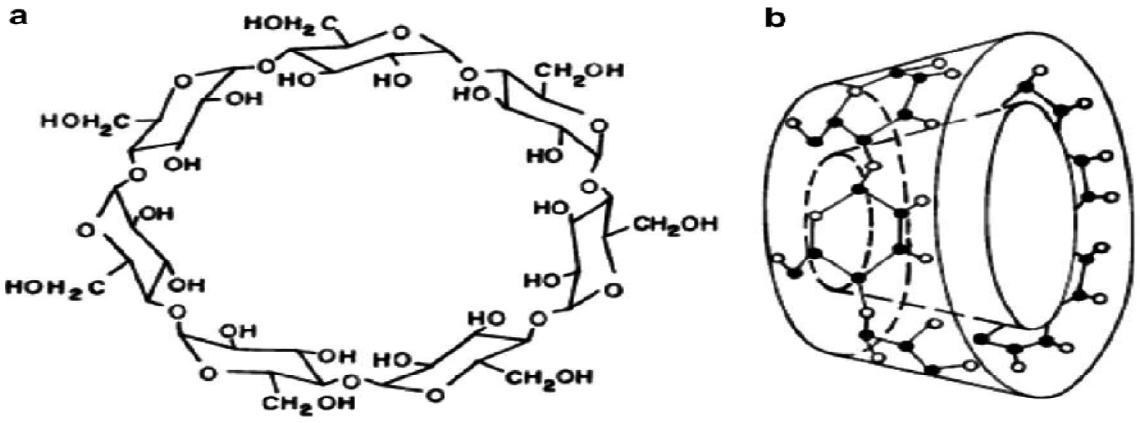

Fig. 1. Structure (a) and torus-like shape (b) of b-CD molecule [1]

Table 1 : Safety overview of selected cyclodextrins

Cyclodextrin	The pharmacokinetics in rats [a]			Acute toxicity, LD50 rat (g/kg) [b]		Maximum dosage in marketed products (mg/day)	
	t1/2 after iv injection (min)	Fraction excreted unchanged in urine	Oral absorption	IV	Oral	IV	Oral
α-Cyclodextrin (αCD)	25	90 %	2-3 %	0.5-0.8	>10	1.3	
β-Cyclodextrin (βCD)	20	90 %	1-2 %	1	19	Not for parenteral usage	170
2-Hydroxypropyl-β-cyclodextrin (HPβCD; Kleptose® HPB)	20	90 %	≤3%	10	>2	16,000	8000
Sulfobutylether β-cyclodextrin sodium salt (SBEβCD; Captisol®)				>15	>10	6000–14,000	-
Randomly methylated β-cyclodextrin (RMβCD)	18	95 %	0.5–12%	1.5-2.1	>8	Not for parenteral usage	-
6-O-Maltosyl-β-cyclodextrin (G2βCD)	23				>5	No product	No product
γ-Cyclodextrin (γCD)	20	90 %	b0.02%	4	>8	No product	No product
2-Hydroxypropyl-γ-cyclodextrin (HPγCD)					>2		

a - From [27-32].b - From [33-35].

The objective of this study is to formulate the lyophilized injection of water insoluble Levosimendan by using Hydroxy propyl beta Cyclodextrin as solubility enhancer for Parenteral administration with better stability.

MATERIALS AND METHODS

Materials: Levosimendan is an active ingredient, Hydroxypropyl betacyclodextrin as Solubilizer, Trisodium citrate as buffer and Hydrochloric acid for pH adjustment and water for injection as a vehicle for solubility were used for formulation. Activeingredient was procured from Gufic Biosciences Ltd. and all other ingredients used were AR grade.

Manufacturing Procedure:

Solubility study for Levosimendan in Bulk solution: Different compositions of Hydroxypropyl betacyclodextrin, Trisodium citrate and Hydrochloric acid has been used for the experiment which was placed in table-1. 60 ml water for injection was collected in glass beaker below 30°C. Weighed quantity of Hydroxypropyl betacyclodextrin was added and dissolved by stirring until a clear solution was formed. Then trisodium citrate was weighed and transferred to the above solution and dissolved by stirring for minimum duration of 10 min below 30°C. Levosimendan was weighed and transferred to the above solution and dissolved by stirring until clear solution was formed. The pH of the solution was checked and adjusted the pH with 0.1 N Hydrochloric acid slowly below 30°C. The solution was diluted and made upto 70 ml, by WFI below 30°C. pH was checked.

Table 2: Solubility study for Levosimendan in Bulk solution

Batch No .	Levosimendan` (2.5% Overages)		Hydroxypropyl betacyclodextrin		Trisodium citrate		0.1 N Hydrochloric acid		Water for injection	
	Mg/Vial	Qty./70 ml	Mg/Vial	Qty./70 ml	Mg/Vial	Qty./70 ml	Mg/Vial	Qty./70 ml	ml/Vial	Qty./70 ml
S1	12.81	128.1 mg	100	1.0 gm	50	500 mg	q.s. to pH 6.0	q.s. to pH 6.0	q.s. to 7	Qty./70 ml
S2	12.81	128.1 mg	150	1.5 gm	50	500 mg	q.s. to pH 6.0	q.s. to pH 6.0	q.s. to 7	Qty./70 ml
S3	12.81	128.1 mg	200	2.0 gm	50	500 mg	q.s. to pH 6.0	q.s. to pH 6.0	q.s. to 7	Qty./70 ml
S4	12.81	128.1 mg	250	2.5 gm	50	500 mg	q.s. to pH 6.0	q.s. to pH 6.0	q.s. to 7	Qty./70 ml
S5	12.81	128.1 mg	300	3.0 gm	50	500 mg	q.s. to pH 6.0	q.s. to pH 6.0	q.s. to 7	Qty./70 ml
S6	12.81	128.1 mg	350	3.5 gm	50	500 mg	q.s. to pH 6.0	q.s. to pH 6.0	q.s. to 7	Qty./70 ml
S7	12.81	128.1 mg	400	4.0 gm	50	500 mg	q.s. to pH 6.0	q.s. to pH 6.0	q.s. to 7	Qty./70 ml
S8	12.81	128.1 mg	450	4.5 gm	50	500 mg	q.s. to pH 6.0	q.s. to pH 6.0	q.s. to 7	Qty./70 ml
S9	12.81	128.1mg	500	5.0 gm	50	500 mg	q.s. to pH 6.0	q.s. to pH 6.0	q.s. to 7	Qty./70 ml
S10	12.81	128.1 mg	500	5.0 gm	-	-	q.s to pH 5.5 to 7.5	q.s to pH 5.5 to 7.5	q.s. to 7	Qty./70 ml
S11	12.81	128.1 mg	-	-	50	500 mg	q.s to pH 5.5 to 7.5	q.s to pH 5.5 to 7.5	q.s. to 7	Qty./70 ml

Freeze drying procedure:

For optimization of lyophilized cycle, dummy trials were taken as per batch F0. Until the moisture content of the lyophilized vial was within the acceptance limit (Max. 5.0%) and the formation of good cake was observed in the vials. Based on these parameters, freezing temperature, primary drying time, secondary drying time and vacuum during drying was decided.

Formulation of Levosimendan injection by Lyophilization: Different compositions of Hydroxypropyl betacyclodextrin, Trisodium citrate and Hydrochloric acid has been used for the experiment which was placed in table-2. 1200 ml WFI of was collected in a S.S vessel below 30°C, weighed quantity of Hydroxypropyl betacyclodextrin was added and dissolved by stirring until a clear solution was formed. Then Trisodium citrate was weighed and transferred to the above solution and dissolved by stirring for minimum duration of 10 min below 30°C. Levosimendan was weighed and transferred to the above solution and dissolved by stirring until clear solution was formed. The pH of the solution was checked and adjusted the pH with 0.1 N Hydrochloric acid slowly below 30°C. The solution was diluted and made upto 1.4 Lit, by WFI below 30°C. pH was checked. The final solution was filtered by using 0.22µm membrane filter. The solution was filled into 20 ml tubular vials (20 mm neck) and half stopped the vials with 20 mm grey bromobutyl full slotted rubber stopper. The filled vials were loaded into lyophilizer and lyophilized them as per standard cycle. After completion of Lyophilization cycle, Stopering the vials without breakage of vacuum. Using hydraulic system. Now break the vacuum of the plant with help of the sterile nitrogen.Unload the vials for sealing and seal the vials.Temperature of room should be below 30^{o}C and humidity below 40%

Table 3: Formulation of Levosimendan injection by Lyophilization of Various Batches

Batch No.	Levosimendan` (2.5% Overages)		Hydroxypropyl betacyclodextrin		Trisodium citrate		0.1 N Hydrochloric acid		Water for injection	
	Mg/Vial	Qty./1.4 lit	Mg/Vial	Qty./1.4 lit	Mg/Vial	Qty./1.4 lit	Mg/Vial	Qty./1.4 lit	ml/Vial	Qty./1.4 lit
F0	-	-	500	100 gm	50	10 gm	q.s. to pH 6.0	q.s. to pH 6.0	q.s. to 7	q.s. to 1.4 lit
F1	12.81	2.56 gm	500	100 gm	50	10 gm	q.s. to pH 5.0	q.s. to pH 5.0	q.s. to 7	q.s. to 1.4 lit
F2	12.81	2.56 gm	500	100 gm	50	10 gm	q.s. to pH 5.5	q.s. to pH 5.5	q.s. to 7	q.s. to 1.4 lit
F3	12.81	2.56 gm	500	100 gm	50	10 gm	q.s. to pH 6.0	q.s. to pH 6.0	q.s. to 7	q.s. to 1.4 lit
F4	12.81	2.56 gm	500	100 gm	50	10 gm	q.s. to pH 6.5	q.s. to pH 6.5	q.s. to 7	q.s. to 1.4 lit
F5	12.81	2.56 gm	500	100 gm	50	10 gm	q.s. to pH 7.0	q.s. to pH 7.0	q.s. to 7	q.s. to 1.4 lit
F6	12.81	2.56 gm	500	100 gm	50	10 gm	q.s. to pH 7.5	q.s. to pH 7.5	q.s. to 7	q.s. to 1.4 lit

Analytical procedure for Levosimendan:

HPLC analysis was carried out with a column C8 (250 x 4.6mm) (5µm), flow rate was 1.0ml/min, detector was UV detector at 360 nm and injection volumewas 20µl, with the runtime of 10min.

Mobile phase: Buffer: Methanol (35: 65)

Buffer: Dissolve 1.8gm of sodium Dihydrogenphosphate & 2.0ml of Phosphoric acid in 900ml of water, Adjust pH 2.0 with 1M Sodium Hydroxide & diluted to 1000ml with water.

Standard solution : Dissolve 25 mg of Levosimendan WS in 1 ml of Dimethylsulphoxide. Then make it up to 25 ml volume with diluent [methanol : water : 80 : 20]. Take 1 ml from this solution and make it up to 10 ml with diluent. (100 mcg/ ml)

Test solution: Reconstitute the sample of 4 Lyophilized vials. Each vial reconstituted with 10 ml water for injection. Mix all the reconstituted solution in 500 ml volumetric flask. Add sufficient amount of water for injection. Sonicate for 5-7 minutes and dilute up to the mark to 500 ml with water for injection. (100 mcg/ ml)

Procedure: Separately inject the equal volumes (about 20µl) of the standard preparation (replicate) & the test preparation (duplicate) into the chromatograph, record the chromatograms, and measure the responses for the major peaks. Calculate the quantity of Levosimendan in percentage from the mean peak area of standard, sample peak and percentage potency of working standard used. The assay percentage should be within 90.0% to 110.0%.

Stability studies:
Accelerated stability study was conducted for the optimized batch under various temperature and humidityconditions. The water content, assay and pH were determined and compared with standard conditions.

RESULTS AND DISCUSSION

Results of Solubility study observation and pH were summarized in below Table no 4.

Table 4: Solubility Study of Levosimendan in HP- Beta Cyclodextrin

Batch No	Description of Solution	pH
S1	Yellow turbid solution	-
S2	Yellow turbid solution	-
S3	Yellow turbid solution	-
S4	Yellow turbid solution	-
S5	Yellow turbid solution	-
S6	Yellow turbid solution	-
S7	Yellow turbid solution	-
S8	Yellow turbid solution	-
S9	Yellow to orange Clear solution	5.97
S10	Yellow turbid solution	-
S11	Yellow turbid solution	-

Based on the above results for solubility & pH, S9 formulation has given clear solution and was selected for further studies.

Optimization of Freeze drying procedure
Freeze drying cycle was optimized as, freezing of the product under the lyophilizer at -50°C for four hours. Then Primary drying at -40°C for seven hours along with vacuum 100 to 150 mtor & then at -30°C for 6 hours along with vacuum 100 to 150 mtor, then -10°C for 18 hours along with vacuum 100 +to 150 mtor then +10°C for 10 hours along with vacuum 100 to 150 mtor. Secondary drying was carried out at +30°C for 2 hours along with vacuum 10 to 50 mtor.

Optimization Levosimendan injection by Lyophilization
The further batches (F1-F6) were planned to observe the effect of pH by adjusting with 0.1 N Hydrochloric acid based on solubility study of levosimendan (S9).

Table 5: Observation of Lyophilized Levosimendan Injection

Batch No	Description of cake	pH	Water content	Assay
F1	Yellow to Orange lyophilized cake	4.99	2.14%	101.48%
F2	Yellow to Orange lyophilized cake	5.46	2.37%	101.62%
F3	Yellow to Orange lyophilized cake	5.97	2.23%	102.87%
F4	Yellow to Orange lyophilized cake	6.41	2.59%	100.53%
F5	Yellow to Orange lyophilized cake	6.97	2.46%	99.89%
F6	Yellow to Orange lyophilized cake	7.43	2.38%	99.17%

Stability Studies:
The accelerated stability study was conducted for theoptimized batch F3 for 6 months at 30°C± 2°C/65% RH ± 5% RH. The stability study chart of batch F3 whichwas optimizedand results has depicted in Table-6

Table 6: Evaluation of Levosimendan lyophilize injection of optimised batches (Accelerated study)

Batch No	Description of cake		pH		Water content		Assay		Total Impurities	
	3 Months	6 Months	3 Months	6 Months	3 Months	6 Months	3 Months	6 Months	3 Months	6 Months
F3	Yellow to Orange lyophilized cake	Yellow to Orange lyophilized cake	5.99	6.01	2.95%	3.75%	102.11%	101.27%	0.92%	1.15%

From stability study data, it was found that, minimum impurities found in optimized batch (F3). Total impurities was found to be 1.15% and the limit is NMT 3.0%. The assay of the optimized batch was101.27%.

CONCLUSION

Parenteral formulation of Water insoluble Levosimendan available in market as in non aqueous alcohol based formulation and as Lyophilized aqueous formulation with limit of total number of impurities was 6.0%, therefore it was developed as lyophilized formulation for better solubility,Stability and lower amount of degradation products (Not more than 3%). The lyophilized cycle was optimized with direct four step freezing at-50°C and changing vacuum with post heat up to 30°C. Levosimendan lyophilized Injection 12.5mg was compatible with 20mL clear glass USP Type I vial, Greybromobutyl rubber closure. The formulation was stable for 6 months on accelerated stability studies. In conclusion a stable aqueous lyophilized Levosimendan injection with minimum level of impurities profile was developed.

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