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Amorphous solid dispersion of ledipasvir and sofosbuvir for enhancement of oral bioavailability

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ABSTRACT: The major goal of this research work was to augment bioavailability of the fixed-dose combination drugs, ledipasvir (LDV) and sofosbuvir (SBV) by developing orodispersible tablets and films and to estimate it by in vivo pharmacokinetic studies in rats. The pre-optimized amorphous solid dispersions of the LDV-SBV combination with HPMC E15 were made into orodispersible films (ODFs) and the pre-optimized inclusion complexes with dimethyl-β-cyclodextrin were made into orodispersible tablets (ODTs). The ODFs and ODTs were studied for the in vitro physical characterization studies and dissolution. Best of both the products were evaluated for in vivo pharmacokinetic studies in comparison with the marketed formulation of film coated tablets, Ledifos. The dissolution rate constants for the LDV from the optimized ODFs, ODTs and the marketed tablets were found to be 0.269, 0.13 and 0.073 min.-1 respectively. The dissolution rate constants for the SBV from the optimized ODFs, ODTs and the marketed tablets were found to be 0.302, 0.168 and 0.094 min.-1 respectively. Area under the curve (AUC) values for the LDV from the films, tablets and the marketed tablets were found to be 4231.4, 4050.3 and 3662.5 h*ng/mL respectively. AUC values for the SBV from the films, tablets and the marketed tablets were found to be 2173.2, 2084.6 and 1452.4 h*ng/mL respectively. Conclusion: The obtained results indicated that the optimized ODFs and the ODTs exhibited improved in vitro dissolution and in vivo bioavailability for the fixed-dose combination of LDV and SBV over the reference product.
KEYWORDS: Bioavailability; ledipasvir; sofosbuvir; orodispersible tablets; orodispersible films

1. INTRODUCTION

The fixed-dose combination drugs for the treatment of hepatitis – C infection, ledipasvir (LDV) and sofosbuvir (SBV)[1,2] are poorly soluble and belong to biopharmaceutic classification system (BCS) class II drugs. These drugs suffer dissolution limited bioavailability because of their poor aqueous solubility.[3] LDV takes around 5 hours to get the maximum plasma concentration (C_{max}) upon oral administration of the combination dosage form, HARVONI [3]. This could be attributed to the practically insoluble nature of LDV even though its permeability is good. Whereas SBV reaches C_{max} approximately in 1 hour after administration. But its elimination half-life is very low that is around 0.5 hours. Poor solubility of the SBV which causes slow absorption and this together with rapid elimination minimizes the C_{max} value. Even before absorbing sufficient amount of the drug, it gets eliminated quickly and hence the C_{max} value is lower resulting in the requirement of high doses. These issues with the LDV and SBV necessitates the desirability of improved solubility and dissolution rate thereby their bioavailability can be improved and the doses can be reduced possibly [4].
Oral bioavailability of these kinds of poor water-soluble drugs is enhanced majorly by improving the solubility and dissolution rate. Solubility is improved by numerous techniques like developing amorphous solid dispersions (ASDs), inclusion complexes (ICs), co-crystallization, and size reduction by nano-technological approaches etc., [5,6] But, ASDs and ICs remain best among those because of their stupendous advantages over techniques besides their ease of scale up [7,8]. The ASDs or the ICs of the poorly water-soluble drugs can be further be made into solid dosage forms with rapid disintegration in the oral cavity upon administration which are termed as orodispersible products. These have combined advantages of solid dosage forms and suspensions [9-11].

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The extensive literature survey on LDV and SBV indicated that there was only one research work was published regarding the development of fast disintegrating tablets of LDV only [12]. This indicates that there is a large scope for performing research on this newly approved fixed dose combination drugs towards improving their bioavailability. Basing on the literature review about the physicochemical and pharmacokinetic properties of the LDV and SBV, enhancing their solubility first and followed by developing immediate release dosage forms could be a better approach towards overcoming their poor bioavailability. ASDs can be developed using hydrophilic carriers like cellulose derivatives, povidone, polyethylene glycols (PEG) etc [13]. Among these, cellulose derivatives like HPMC can be used as a film former also. Hence in this work it was aimed that the pre-optimized ASDs developed with low molecular weight HPMC E15 were further made into orodispersible films (ODFs). Similarly, the pre-optimized ICs developed with dimethyl-β-CD were further developed into orodispersible tablets (ODTs) [14]. These two types of the products were studied for their *in vitro* characterization parameters. Then finally these were studies for *in vivo* pharmacokinetic/bioavailability studies on rats in comparison with one of the currently available marketed products.

2. RESULTS

2.1. Characterization studies on the ODFs

The ODFs for the combination of LDV and SBV were prepared using HPMC E15 as the polymer. Before preparing the films, the amount of the polymer was optimized by preparing solid dispersions with the polymers like methyl cellulose (MC) and HPMC. These polymers are efficient in order to impart hydrophilicity to and thereby increase solubility of the poorly soluble drugs. Hence, these studies are helpful in order to identify the correct amount of the polymer so as to obtain the maximum dissolution rates from the ODFs. HPMC E 15 at 1:3 ratio of the drug to polymer was found effective against other polymers and in other concentrations. Hence, ODFs were prepared using the HPMC E15 in order to maintain this ratio. Three different formulations were prepared using three different superdisintegrants in order to find the better disintegrants with this drug-polymer composition. The three ODFs were subjected to different physical characterization tests and the results were shown in the Table 1.

Table 1: Results* of physical characterization studies on the ODFs

Formulation	Thick-ness (μm)	Tensile strength (N/mm²)	% Elongation	Folding endurance	Disintegration time (sec)	Drug content (%)	
						LDV	SBV
ODF1 (CP)	51 ± 2	7.2 ± 0.5	22.1 ± 1.3	354 ± 17	68 ± 5	100.3 ± 1.5	99.1 ± 0.8
ODF2 (SSG)	53 ± 4	7.5 ±0.3	20.9 ± 1.7	316 ± 22	39 ± 3	99.6 ± 2.1	101.2 ± 1.3
ODF3 (CCS)	52 ± 2	7.6 ±0.6	21.5 ± 0.9	332 ± 14	53 ± 6	98.4 ± 1.2	98.9 ± 0.6

* Expressed as average ± standard deviation for n = 3

Thickness of the films depends on the viscosity of the pre-casting mixture which was kept constant for all the three films by taking same volume of the solvent. The thickness of all the three formulations was found to be around 50 μm. The tensile strength and the % elongation values were found to be in the range of 7.2 – 7.6 N/mm² and 20.9 – 22.1 % respectively. These values were considerably good enough to prevent any breakdown due to the external forces and can be considered flexible to overcome the external stress. This could be attributed to the sufficient amount of the PEG 400 added as the plasticizer. This flexibility and strength were further supported by the results of folding endurance which were found to be in the range of 316 – 354. All these physical characterization parameters together specified that the amount of the film former and the plasticizer were in best suitable combination so as to produce stable and flexible films. The results of drug content values further indicated that both the drugs were uniformly dispersed in the pre-casting mixtures and also in the obtained films after drying [24].

The results of the disintegration test inferred that all the three films were disintegrated rapidly. But among the three films, the ODF2 made with SSG exhibited much rapid disintegration among the three followed by CCS (ODF3) and then CP (ODF1). This highest disintegration rate and least time of 39 sec. in case

of the ODF2 could be attributed to the greatest swelling capacity of the SSG that it can swell up to 300 times to its initial volume [25]. The results obtained were in reasonable agreement with those testified by Zhao N *et al* [26].

2.2. Characterization studies on the ODTs

The poor aqueous solubility of both the LDV and SBV cannot make them suitable for developing fast dissolving tablets. Hence, both these drugs were converted into inclusion complexes using cyclodextrins before developing into ODTs. Different derivatives of the β-cyclodextrins at different ratios were used to prepare the inclusion complexes for the combination of the LDV and SBV. Considering the relatively high dose of 490 mg of the drugs mixture, the complex at 1:0.2 molar ratio with dimethyl-β-CD was chosen as the optimum which also exhibited sufficient improvement in solubilities of bot the drugs. Hence, 716 mg of the inclusion complex of the LDV and SBV containing one dose equivalent of both the drugs was taken per one tablet and developed the formulation. The final weight of the ODTs was adjusted not to cross the weight of the marketed tablet. Three different formulations were of the ODTs were developed by direct compression at three different concentrations of PVP K15 as the binder. In the direct compression technique, compressibility of the powder mixture largely depends on the cohesive forces among the particles. Hence, three different concentrations of the binder were taken to identify the better concentration. The results of the physical characterization studies of the three ODTs were shown in the Table 2.

Table 2: Results* of physical characterization studies on the ODTs

Formulation	Tensile strength (N/mm^2)	Packing fraction (P_f)	Friability (%)	Disintegration time (sec)	Drug content (%)	
					LDV	SBV
ODT1	0.79 ± 0.13	0.87 ± 0.03	0.24 ± 0.02	79.6 ± 5.1	98.7 ± 1.8	99.6 ± 2.1
ODT2	0.84 ± 0.06	0.91 ± 0.01	0.17 ± 0.05	91.8 ± 3.7	99.3 ± 0.7	98.5 ± 1.9
ODT3	0.86 ± 0.09	0.90 ± 0.02	0.14 ± 0.04	122.4 ± 8.3	98.1 ± 1.5	99.4 ± 1.3

* Expressed as average ± standard deviation for n = 3

The results of tensile strength were obtained in the range of 0.79 – 0.86 N/mm^2. These results designated that the tablets were sufficiently strong enough [27]. The tensile strength was found to be increased upon increase in the concentration of binder. Further the friability values which were found to be well below the maximum limit of 1% also showed that the tablets had enough strength to overcome the external stresses.[28] Though the ODTs were prepared by direct compression technique, the resulted relatively superior physical strength could be attributed to the micromeritic properties of the inclusion complex form of the drugs mixture. The inclusion complexes obtained from solvent evaporation technique results in the aggregate form of the solids which have good compressibility.[29] Thus direct compression technique even with minimum amount of direct compressible diluent resulted in ODTs with good physical strength. The packing fraction values were found to be in the range of 0.87 – 0.9 which indicated that there was a sufficient porosity for penetration of water and aid disintegration yet having enough strength [30].

The results of the disintegration test indicated that upon rising the PVP K15 concentration, the disintegration time was observed to be increased. This could be attributed to the improved cohesive forces and hence increased binding nature that could require more time for disintegration of the tablet. The results were in good agreement with those reported by Srikar G *et al.*[31]. The ODT1 with 5% w/w PVP K15 showed the least disintegration time of 79.6 sec.

2.3. *In vitro* Dissolution studies

The dissolution profiles of the ODFs were shown in the Fig 1 (a) for LDV and in the Fig 1 (b) for SBV. The results indicated that all the three formulations showed rapid and complete dissolution of both the drugs in around 20 min. The highly hydrophilic nature of the selected film former HPMC E15 at sufficient quantities and the superdisintegrant concentration could be responsible for this rapid dissolution [32]. Further, among the three formulations, ODF2 containing SSG as the superdisintegrant exhibited fastest dissolution than the other two formulations. This could be because of the rapid and very high degree of swelling of SSG [25]. All the three formulations exhibited first-order dissolution kinetics.

Dissolution studies on the ODTs were performed and the dissolution profiles were shown in the Fig 2 (a) for LDV and in the Fig 2 (b) for SBV. The results indicated that all the three formulations showed complete

dissolution of LDV in 60 min. and SBV in 45 min. Among the three formulations, ODT1 with 5% w/w of the binder exhibited greater dissolution rate than the remaining formulations. This might be because of the increased binding nature and hence higher disintegration time at higher concentrations of the binder that could decrease the dissolution rate [31].

2.4. Comparative dissolution studies

Based on the physical characterization studies results of the ODFs, the ODF2 formulation was chosen as the optimum formulation because of its rapid disintegration and dissolution while having sufficient physical strength. Similarly, basing on the disintegration and dissolution in case of the ODTs, the ODT1 formulation was chosen as the optimum tablet formulation. Even the 5% w/w of the binder exhibited almost similar physical strength as that of the other formulations with higher binder concentrations and hence the ODT1 was chosen as the optimum formulation. These optimized in-house formulations and the commercially available conventional tablets (Ledifos, film coated tablets containing LDV-90mg and SBV-400mg from Hetero Drugs Ltd.) were simultaneously studied for the dissolution and the results were compared. The comparative dissolution profiles were shown in the Fig 1 (a) for LDV and in the Fig 1 (b) for SBV; the dissolution kinetics were shown in the Table 3. These resulted indicated that, both the in-house formulations ODFs and ODTs exhibited significantly rapid dissolution than the marketed formulation in case of both the drugs. This could be due to two reasons; one is the inclusion of high soluble form of the drugs (solid dispersions with HPMC in case of the ODFs and inclusion complexes with dimethyl-β-cyclodextrin in case of the ODTs) and the second reason is inclusion of superdisintegrants. Dissolution is a direct function of solubility and hence the high solubility of the drugs in the ODFs and ODTs could be attributed to their rapid dissolution.[32,33] Further superdisintegrants make the formulations disintegrate readily as and when they are placed in the mouth and account for the rapid dissolution of the contained drugs. Between the ODFs and ODTs, the films exhibited more dissolution rates and less T90% values than those the tablets. This might be due to very higher surface area and lesser thickness of the films than those of the tablets [34] and these results were in reasonable agreement with the results shown by Brniak W *et al.* [35]

Table 3: Dissolution kinetics for both ledipasvir and sofosbuvir from the optimized formulations and the reference product

Formulation	Regression values		Dissolution rate constant (k, min^{-1})	T90%* (min.)	T50%* (min.)
	Zero-order	First-order			
			For Ledipasvir		
Opt. ODFs	0.834	0.971	0.31	7.52	2.26
Opt. ODTs	0.665	0.966	0.13	17.73	5.34
Ref. Tab	0.759	0.993	0.07	31.65	9.52
			For Sofosbuvir		
Opt. ODFs	0.820	0.946	0.37	6.23	1.87
Opt. ODTs	0.727	0.983	0.17	13.68	4.17
Ref. Tab	0.689	0.987	0.09	24.51	7.38

* the T90% and T50% values of ODFs and ODTs were compared with those of the Reference tablet using t-test and found to be statistically significant at $p < 0.05$

These *in vitro* dissolution test results indicated that the developed ODFs and ODTs were significantly better than the selected reference product. Further, the large size of the reference tablet makes it difficult for swallowing especially in case of dysphagia patients. Whereas the optimized ODFs and ODTs can be disintegrated with a little amount of the saliva present in the oral cavity and hence they can be swallowed as like a suspension without any difficulty. These characteristics make the optimized ODFs and ODTs better in terms of ease of administration and *in vitro* dissolution than the reference product (Figure 3.)

2.5. *In vivo* bioavailability studies

The calibration curve (linearity test) was constructed so as to quantify the drugs concentrations in the taken plasma samples using HPLC. The chromatograms observed in case of blank plasma, standard mixture of the drugs in the plasma and the test plasma samples taken from the rats after administration of the formulations were shown in the Fig 4. The retention times for LDV and SBV were found to be 8.2 and 4.37 min. respectively. The calibration curves for both the drugs were shown in the Fig 5. Under the taken

chromatographic conditions, both the drugs exhibited linearity with regression values of more than 0.999 in the range of 10 – 2000 ng/mL. These results indicated that these calibration curves can be used to calculate plasma drug concentrations accurately within this range for both the drugs from the test samples.

The plasma drug concentration time profile for the three formulations administered into three different groups of the animals were shown in the Fig 6 (a) for LDV and in the Fig 6 (b) for SBV. Further, these results were subjected to non-compartmental analysis suing PK Solver software. The obtained results of different pharmacokinetic parameters for both the drugs from the three formulations were shown in the Table 4. The main bioavailability indicating parameters which are C_{max}, time for the C_{max} (t_{max}) and the area under curve ($AUC_{0-\infty}$) were compared for all the three formulations. It was observed that the C_{max} and $AUC_{0-\infty}$ found to be increased; and t_{max} was found to be decreased for the in-house orodispersible formulations when compared to the taken marketed tablet. This indicated that the drug absorption rate was increased in case of the developed orodispersible formulations which could be attributed to rapid disintegration followed by rapid dissolution of the drugs from the ODT and ODF formulations [35]. Further, C_{max} and $AUC_{0-\infty}$ were found to be increased for the in-house orodispersible formulations. In case of LDV, the AUC was found to be increased by 10.6% for the ODTs and 15.5% for the ODFs when compared to the reference product. In case of SBV, the AUC was found to be increased by 43.5% for the ODTs and 49.7% for the ODFs when compared to the reference product. This could be due to the increased solubility, dissolution rate and stability of the drugs in the GIT because of their solid dispersion of inclusion complex form that resulted in more amount of drug absorption.[37] But, the elimination kinetic parameters like elimination rate constant (k_e) and its half-life ($t_{1/2}$) were found to be almost same for all the three formulations. Changes in the formulation of the drugs into different products can influence only absorption parameters but not the elimination parameters because the drugs are same from all the three products and once absorbed will show same kinetic properties. These kinetic parameters altogether indicated that the ODF and ODT formulations are having more bioavailability than the reference product. Hence, the development of ODTs and ODFs for the poor soluble LDV and SBV was successful in terms of improving their dissolution limited bioavailability. Besides, ODFs exhibited much better bioavailability than that of the ODTs which could be attributed to rapid dissolution of the former [36].

Table 4: Results of non-compartmental analysis of the data from the *In vivo* bioavailability/pharmacokinetic studies of the optimized ODF and ODT formulations in comparison with the reference product

S. No.	Pharmacokinetic parameter	Ledipasvir			Sofosbuvir		
		Ref	ODF	ODT	Ref	ODF	ODT
1	C_{max} (ng/mL)	304.8	392.1	360.8	483.6	672.1	610.4
2	T_{max} (h)	5.0	2.0	4.0	2.0	0.5	1.0
3	$AUC_{0-\infty}$ (h*ng/mL)	3662.48	4231.4	4050.3	1452.4	2173.2	2084.6
4	$t_{1/2}$ (h)	5.12	5.03	5.37	0.81	0.85	0.88
5	k_e (1/h)	0.135	0.138	0.129	0.858	0.811	0.786
6	MRT (h)	9.69	8.64	9.56	2.3	2.4	2.54
7	V_d (L/kg)	5.04	4.29	4.78	8.92	6.3	6.78
8	Cl_T (L/kg/h)	0.68	0.59	0.62	7.64	5.11	5.33

3. DISCUSSION

The fixed-dose combination drugs LDV and SBV suffer with poor solubility and dissolution limited bioavailability besides the large size (around 1 g) of the commercially available tablet. Hence, in this work it was aimed to prepare orodispersible formulations for the pre-optimized solid dispersions and inclusion complex forms of the drugs mixture. Orodispersible formulations, ODFs and ODTs improve dissolution limited bioavailability of the drugs besides making the administration easy without need of water. Solid dispersions with the HPMC E15 were made into ODFs as the contained HPMC is a film former. Inclusion complexes with the dimethyl-β-CD were developed into ODTs. The optimized ODFs and ODTs were compared with the reference product for *in vitro* dissolution and *in vivo* bioavailability studies. The results indicated that both the ODFs and ODTs were significantly ($p < 0.05$; 95% confidence intervals) better over the taken reference product in terms of both dissolution rate and AUC. Further, ODFs exhibited greater dissolution and bioavailability that could be due to their higher surface area and lesser thickness.

4. CONCLUSION

In conclusion, the results indicated that the set objective of the work of developing high bioavailability and easily administrable orodispersible formulations for the DLV and SBV combination was successfully achieved.

5. MATERIALS AND METHODS

5.1. Materials

LDV and SBV were acquired from Hetero Drugs Pvt. Ltd, Visakhapatnam; HPMC E15, dimethyl-β-CD and PEG 400 were purchased from Sigma Chemicals Co.; Sodium starch glycolate, isopropyl alcohol (IPA) and Tween 80 were procured from SD Fine Chemicals, Mumbai; All remaining chemicals of analytical grade were used.

5.2. Methods

5.2.1. Preparation of ODFs

The pre-optimized ASDs of LDV-SBV with HPMC E15 at drugs to carrier ratio of 1:3 was taken for development into ODFs (optimization of the ASDs was published elsewhere). The films were prepared using solvent casting method [15] 1470 mg of HPMC E15 was dissolved in the 10 mL of the solvent mixture containing isopropyl alcohol and water at 6:4 ratio containing 0.2% v/v of tween 80. This mixture was subjected to vertex mixing until homogenous liquid was formed and then 90 mg of LDV and 400 mg of SBV were added into it and the mixing was continued. 235 mg of crospovidone (CP) as the disintegrant and 150 mg of PEG 400 as the plasticizer were added to the above mixture and subjected to mixing till the formation of homogenous dispersion. Then this dispersion was carefully transferred into a plastic mold having an area of 25 cm^2 and allowed for solvent evaporation. After 48 hours, the obtained films were collected and stored properly for further studies. Two more formulations of the films with other superdisintegrants like sodium starch glycolate (SSG) (ODF2) and croscarmellose sodium (CCS) (ODF3) in place of crospovidone (ODF1) were also prepared in the same way. All the three films were characterized to identify the best superdisintegrant among them.

5.2.2. Preparation of ODTs

The inclusion complexes (ICs) of the LDV-SBV prepared with dimethyl-β-CD at a molar ratio of 1:0.2 were used to develop ODTs by direct compression technique (optimization of the ICs is in consideration for publication elsewhere). 716 mg of the inclusion complex which was equivalent to LDV-90 mg and SBV-400 mg was mixed with 76 mg of croscarmellose sodium as the disintegrant (at 8% w/w), 47.5 mg of povidone (PVP K15) as the binder (at 5% w/w), 72.5 mg of microcrystalline cellulose and 28.5 mg mannitol as the vehicles. This blend was mixed properly with a glass pestle in a mortar. Finally, 4.75 mg of magnesium stearate and 4.75 mg of aerosil were added finally and mixed. The quantities of all the ingredients mentioned above were per one tablet of final weight of 950 mg. A batch of 50 tablets was prepared by taking proportionate quantities of all the ingredients. After suitable mixing, the blend was subjected to compression in 12 mm die cavity of a multi-stage rotary tablet press. Two other formulations with different binder concentrations which were 7.5% (ODT2) and 10% w/w (ODT3) were also prepared in the same manner keeping the remaining formulation composition same. The tablets obtained were stored appropriately for characterization studies to study the influence of the binder concentration.

5.2.3. In vitro characterization studies of the ODFs

Physical characterization studies: Thickness, folding endurance, tensile strength and percentage elongation were studied as per the procedures reported by Bansal S *et al.* [16] and Nirmala D *et al.* [17]

Drug content: An ODF of 25 cm^2 as casted was taken and placed in a beaker containing 80 mL of the dissolution medium i.e., 10 mM potassium phosphate buffer containing 0.0075 mg/mL butylated hydroxytoluene and 1.5% tween 80 with pH 6.0. This was placed in a rotary shaker and periodically samples were withdrawn and subjected to spectrophotometry. This was continued until the maximum absorbance was observed and that was used to calculate the drug contents of LDV and SBV.

Disintegration time: It was determined by taking 25 cm^2 ODF in a petri plate comprising 6 mL of phosphate buffer pH 6.8. [16]. The film was observed and the time when the film was completely dispersed was noted down as the disintegration time.

Dissolution study: It was conducted by taking the 900 mL of the USFDA suggested dissolution medium i.e. 10 mM potassium phosphate buffer containing 0.0075 mg/mL butylated hydroxytoluene and 1.5% tween 80 with pH 6.0 in a paddle apparatus. Six films from each formulation were taken and placed separately in the vessels containing the medium. Samples of dissolution medium were taken at every 5 min. The obtained samples were quantified after suitable dilutions for both the LDV and SBV using spectrophotometer. The data obtained was fitted to zero-order and first-order kinetic models to identify the order of dissolution kinetics and also to calculate T90% values [17].

5.2.4. In vitro characterization studies of the ODTs

Physical characterization studies: Friability and disintegration tests were performed referring to the method specified in the Indian pharmacopoeia. Tensile strength was performed according to the standard procedure reported by Pitt KG *et al.* [18] Packing fraction (P_f) was calculated according to the procedure reported by Malik K *et al.* [19] Porosity was calculated by subtracting the obtained P_f value from one.

Dissolution studies: The dissolution study for the ODTs was conducted similar to that of the ODFs as mentioned above.

5.2.5. In vivo bioavailability studies

Calibration curve in rat plasma: High performance liquid chromatography (HPLC) method with necessary modifications to the methods reported by Farid NF *et al.* [20] and Mastanamma SK *et al.*[21] was used to develop calibration curve for the simultaneous estimation of LDV and SBV. Mixture of acetonitrile and water at 55:45 ratio was taken as the mobile phase. Stock solution of the drugs in the mixture of mobile phase was prepared so as to contain 2 µg/mL of each drug. Different volumes of this solution were spiked with same volume of rat plasma so to get working concentrations in the range of 10 – 2000 ng/mL for constructing calibration curve (linearity test). For every concentration, 200 µL was taken and mixed with 100 µL of diethyl ether so as to separate the plasma proteins. The mixture was subjected to high-speed centrifugation at 8000 rpm at 4°C for about 5 min. the clear supernatant was collected and 25 µL was loaded into the HPLC system with the Luna Phenyl Hexyl 250 4.6 mm, 100mm column which was maintained at a temperature of 25°C. The mobile phase was flown at 1.0 mL/min rate for 10 min. Detection of LDV and SBV were performed using photodiode array (PDA) detector at a λ_{max} of 260 nm.

5.2.6. Animal study protocol

The study was performed in the animal house facility of Aditya College of Pharmacy and the approved institutional animal ethical committee (IAEC) protocol number for the study is SAIPSR/IAEC.Clear/11/20-21. 24 Male Wister rats of average weight of 250 g were used for the study and all the animals were allocated into 4 groups. Group 1 was treated as control; Group 2 was administered with the reference product (Ledifos); Group 3 was administered with the optimized ODF; and the Group 4 was administered with the optimized ODT. The dosage for the later three groups was 2.5 mg/kg of LDV and 11.11 mg/kg of SBV as per the USFDA. Dose equivalent products were added to 1.5 mL water and administered through oral gavage immediately. After administration, the plasma samples were collected at 0.5, 1, 2, 3, 4, 6, 9, 12, 18, and 24 h from tail vein [22]. The collected samples were extracted using liquid – liquid extraction method, injected into HPLC column and responses were obtained as discussed previously.

5.2.7. Pharmacokinetic estimation

The obtained plasma drug concentration time data was subjected to non-compartmental analysis using PK Solver software for the estimation of bioavailability parameters and their comparison among the different formulations [23].

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