Section A -Research paper

QUANTIFICATION OF SOFOSBUVIR AND LEDIPASVIR IN BULK AND DOSAGE FORM BY HPTLC AND RP-HPLC METHODS IN PRESENCE OF ITS DEGRADATION PRODUCTS

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Abstract

The combine dosage form of Sofosbuvir (SFB) and Ledipasvir (LDV) used in the treatment of Hepatitis C virus genotype 1 infection. A simple,accurate and subsequent stability indicating HPLC and HPTLC methods for simultaneous estimation of SFB and LDV in their combined formulation was developed and validated as per the guidelines given by International Conference on Harmonization

In HPTLC, separations was achieved on aluminium plate precoated with silica gel 60 F_{254} using toluene:methanol:ethyl acetate: acetic acid (6 : 2 : 2 : 0.3, V/V/V/V) as mobile phase. The compact bands of SFB and LDV at 0.53 ± 0.01 and 0.37 ± 0.02 respectively were scanned at 254 nm. In RP-HPLC separation was achieved on Agilent 1260,binary pump , with C18 (250 cm × 4.6 mm) 5 μm column. The mobile phase composition of Phosphate buffer pH 2.5 : Acetonitrile: Methanol (60:30:10 (V/V/V) used for development with flow rate of 1.5 ml/min maintained at an room temperature. The retention time obtained for LDV and SFB were at 3.144

min and 5.732 min respectively. In HPTLC Linear regression analysis revealed linearity in the range of 1000 to 6000ng/spot for SFB and 100 to 600 ng/spot for LDV respectively. The standard solution in diluent was prepared and scanned in the UV range. Quantification was achieved with ultraviolet detection at 254 nm based on the overlay UV spectrum . For both the methods, dosage form was exposed to acid, alkali oxidative, dry heat and photolytic stress. The degradation products, shows well resolved peaks with significantly different retention time value in HPLC and with significantly different retention factor in HPTLC.The methods distinctly separated the drugs and degradation products even in actual samples. Hence the proposed methods are, precise, accurate for routine quantification of SFB and LDV in tablet formulation.

Keywords: High pressure liquid chromatography**,** High-performance thin-layer chromatography, Ledipasvir, Sofosbuvir, Method validation, Stability-indicating method

Introduction

Sofosbuvir chemically known as (S)-Isopropyl 2-((S)- (((2R,3R,4R,5R)-5-(2,4-dioxo-3,4-dihydropyrimidin1(2H)-yl)-4-fluoro-3-hydroxy-4-methyltetrahydrofuran2-yl)methoxy)-(phenoxy) phosphorylamino) propanoate. It has a molecular formula of $C_{22}H_{29}FN_3O_9P$ and a molecular weight of 529.45. Chemically Ledipasvir is known as Methyl [(2S)-1-{(6S)- 6-[5-(9,9-difluoro-7-{2-[(1R,3S,4S)-2-{(2S)-2-[(methoxycarbonyl)amino]-3-methylbutanoyl}2 azabicyclo[2.2.1]hept-3-yl]-1H-benzimidazol-6-yl}-9Hfluoren-2-yl)-1H-imidazol-2-yl]-azaspiro [2.4]hept-5- yl}-3-methyl-1-oxobutan-2-yl]carbamate. It has a molecular formula of $C_{49}H_{54}F_2N_8O_6$ and a molecular weight of 889.00 [1]. The chemical structure of SFB and LDV are shown in (**Figure 1)**.

Few methods for determining SFB and LDV by ultraviolet (UV) [2-4], high performance thin layer chromatography (HPTLC)[5] and high-performance liquid chromatography (HPLC) [6-12] have been reported in the literature. Therefore, the development of quick, sensitive, and stability-indicating HPTLC reversed-phase HPLC procedures is required for the simultaneous quantification of SFB and LDV in bulk and pharmaceutical dose form.

The International Conference on Harmonization (ICH) (Q1A) guidelines require that analytical test procedures for stability samples be fully validated and that the assays be stability-indicating[13-14]. Simple, accurate, precise, selective, repeatable, and stability-indicating methods

have been developed. These methods can be used for routine analysis of drugs in bulk and dosage forms for both qualitative and quantitative research.

The methods were validated in accordance with ICH standards[15]. HPLC and HPTLC methods were used to determine the degradation products of SFB and LDV under various stress conditions.

Materials and Methods

2.1 Reagents and chemicals

Working standards of SFB and LDV were received as a gift sample from Mylan Laboratories,Nashik. Analytical grade solvents and reagents were purchased from Merck specialties Pvt. Ltd., Mumbai, India. And S.D.Fine-chemical Ltd. India

2.2 Instrumentation

HPTLC

The chromatography was carried out on 20 cm x 10 cm aluminium-backed HPTLC plates coated with 200-m layers of silica gel 60 RP-18 F254S. (Merck, Darmstadt, Germany, supplied by Merck India ,Mumbai, India). Prior to chromatography, the plates were developed in methanol and activated for 5 minutes at 105^0C. SFB and LDV were spotted in the form of 6 mm wide bands using a Camag microlitre syringe and the Camag Linomat V. (Switzerland). A constant application rate of 150 nl/sec was used, with a space of 15 mm between two bands. The slit dimension was kept at 6 mm x 0.45 mm micro, and the scanning speed was set to 20 mm/s. Using the linear ascending technique, the chromatogram was created in a twin trough glass chamber saturated with mobile phase. Camag TLC scanner III was used for densitometric scanning.

HPLC

Agilent technologies 1260 LC system with binary gradient pump , LC-10 AT VP solvent delivery system, Qualisial C18(250 cm × 4.6 mm) 5 μm column ,UV chamber (Camag),SPD M-10AVP photo diode array detector.

2.3 Optimized Chromatographic Conditions

After experimenting with various mobile phases, a good resolution and symmetric peak were obtained with toluene-methanol-ethyl acetate-acetic acid (6 : 2 : 2 : 0.3, v/v/v/v) for HPTLC and Phosphate buffer pH 2.5, acetonitrile, Methanol 60:30:10 (v/v/v) for HPLC. The overlain spectra of SFB and LDV taken on UV and selected 254 nm wavelength for quantification **(Figure 2)**.

HPTLC

The samples were spotted as 6 mm wide bands with a Camag microlitre syringe on a pre-coated silica gel aluminium plate 60F-254 with a thickness of 0.2 mm using a Linomat 5 sample applicator. A constant application rate of 150 nl/sec was used, with a space of 15.4 mm between two bands. The slit size was kept at 6 mm x 0.45 mm micro. As a mobile phase, a solution of toluene-methanol-ethyl acetate-acetic acid (6 : 2 : 2 : 0.3, v/v/v/v) was used. At room temperature, the optimal chamber saturation time for mobile phase was 30 minutes. The amount of mobile phase used per run was 10.3 ml. Plates formed using the ascending technique. The chromatogram run was approximately 80 mm long. At 254 nm, the chamber was kept at a constant temperature of 25±0.5ºC and relative humidity of 50–60%. **Figure 3** depicts an HPTLC chromatogram with well-resolved peaks.

HPLC

Agilent 1260, C18 column (250 cm × 4.6 mm) 5 μm was used as the stationary phase to produce the HPLC separation. Phosphate buffer pH 2.5, acetonitrile, and methanol 60:30:10 (V/V/V) were employed as the mobile phase, and UV detection at 254 nm was utilised to quantify the results. The flow rate of 1.5 ml/min at ambient temperature. **Figure 4** depicts an HPLC chromatogram with well-resolved peaks.

2.4 Preparation of Solutions

HPTLC

Preparation of LDV standard stock solution

10 mg of LDV was weighed and transferred in 10 ml volumetric flask. The volume was made upto the mark with methanol which was used as solvent. The final concentration of the solution was 1000 µmg/ml.

Preparation of LDV working standard

1 ml of the above solution was placed in a 100 ml volumetric flask and diluted with 100 ml of methanol to obtain a final concentration of 10 mg/ml. 30 µl of this was used, with a concentration of 300 ng/spot.

Preparation of SFB Standard stock solution

10 mg of SFB was weighed and transferred in 10 ml volumetric flask. The volume was made upto the mark with methanol which was used as solvent. The final concentration of the solution was 1000 µmg/ml.

Preparation of SFB Working Standard

1 ml of the above solution was diluted in 10 ml of methanol to obtain a final concentration of 300 mg/ml. A volume of 10 µl was used, with a concentration of 3000 ng/spot..

HPLC

Preparation of LDV stock solution

5 mg of Ledipasvir was placed in a 10 ml volumetric flask, and 5 ml of Phosphate buffer pH 2.5: Acetonitrile: Methanol 60:30:10 (V/V/V) was added, sonicated to dissolve, and diluted to the desired concentration with mobile phase. Transfer 1 ml of the above solution to a 10 ml volumetric flask and dilute with the diluents. Dilute 1 ml of the above solution to 10 ml with the diluents.

Preparation of SFB stock solution

22 mg of Sofosbuvir working standard was accurately weighed and transferred to a 10 ml volumetric flask. Phosphate buffer pH 2.5:Acetonitrile: Methanol 60:30:10 (V/V/V) dissolved in 7 ml of diluents, sonicated, and diluted to volume with Phosphate buffer pH 2.5:Acetonitrile: Methanol 60:30:10 (V/V/V). Transfer 1 ml of the above solution to a 10 ml volumetric flask and dilute with the diluents. Dilute 1 ml of the above solution to 10 ml with the diluents.

Preparation of LDV and SFB working standard solution

Standard stock solutions were made from the above stock solutions, with a concentration of 22 mg/ml SFB and 5 mg/ml LDVs.

2.5 Analysis of Marketed Formulation

HPTLC

Twenty HARVONI tablets (containing 90 mg LDV and 400 mg SFB) were weighed and powdered. The average weight of the tablet was calculated. A small amount of methanol was added to a 100 ml standard volumetric flask containing 5 mg of LDV and 22 mg of SFB powder. Using methanol as a solvent, the volume was increased to the required level. The final solution was filtered through a 0.45m filter (Millifilter,MA).

The solution was appropriately diluted to achieve concentrations of 5 mg/ml LDV and 22 mg/ml SFB.Accurate aliquots from the stock solution were applied three times in the form of bands on the TLC plates for the HPTLC-densitometric method, which contains 500 ng/spot LDV and 2200

ng/spot SFB. In order to have the linearity range within Beer-Lambert's law limits, the above additional step is taken as the UV absorption of LDV is very high when compared to SFB.

HPLC

Twenty HARVONI tablets (containing 90 mg LDV and 400 mg SFB) were weighed. The average weight of each tablet was calculated and powdered. A small amount of methanol was added to a 100 ml standard volumetric flask containing 5 mg of LDV and 22 mg of SFB powder. To obtain the concentration (400 mg/ml), the solution was sonicated for 15 minutes and the final volume was made with the same solvent. The mixture was then filtered using a 0.45 mm nylon membrane filter. The above solution was diluted with mobile phase to obtain the final dilution of SFB and LDV (22 mg/ml and 5 mg/ml, respectively).

Method validation

The method was validated for its linearity range, accuracy, precision, sensitivity, and specificity. Method validation was carried out in accordance with ICH guidelines.

3.1 Linearity

The ability of an analytical method to produce test results that are directly proportional to the concentration of analyte in sample within a given range is referred to as linearity.

HPTLC

The solutions of SFB and LDV were spotted on a TLC plate to obtain final concentrations of LDV of 100-600 ng/spot and SFB of 1000-6000 ng/spot. Each concentration was applied to the TLC plate five times. After developing the plate with the previously described mobile phase, a calibration curve was constructed by plotting the peak area vs corresponding drug concentration.

HPLC

The working range solutions for LDV and SFB were set at 2–12 µg /ml and 5–60 µg /ml, respectively, from the standard stock solution. Three identical injections of the prepared solutions were made. Regression analysis, which was performed using the least square regression approach, was used to determine linearity.

3.2 Accuracy

Accuracy is measured as a percentage of recovery. For both methods, a known amount of LDV and SFB standard drug powder corresponding to 80, 100, and 120 percent of label claim was added, mixed, and analyzed by running chromatograms in optimized mobile phase.

3.3 Precision

Precision of the method was assessed by repeatability, intra-day and Inter-day. The precision measures the similarity of measurements obtained from multiple samplings of the same homogeneous sample under the specified conditions. The intra-day precision was determined by analysing standard drug solutions three times on the same day within the calibration range of individual drugs. Inter-day precision was determined by analyzing drug solutions over a week on three different days within the calibration range.

3.4 Ruggedness

Different analysts used aliquots from homogenous lots and operational and environmental circumstances to assess an analytical method's robustness. The assay was conducted utilizing the parameters, such as in various settings, by various analysts, and on various dates.

3.5 Robustness

Robustness was studied by comparing the results obtained for deliberate changes in chromatographic conditions.

HPTLC

Toluene-methanol-ethyl acetate-acetic acid (6.5.1.5:2.5:0.3 v/v/v), (5.5:2.5:2.5:0.2 v/v/v), and (7:3:3:0.3 v/v/v) mobile phase compositions were tried, and chromatograms were run. The amount of mobile phase was varied by ±5%, and the time from spotting to chromatography and chromatography to scanning was varied by ±10 minutes. The effect on the retention factor and peak parameter was investigated, as well as the effects on the results were examined.

HPLC

Flow rates ranging from 1.4 ml/min to 1.6 ml/min and 1.7 ml/min were tested. The mobile phase ratio was changed by 5%, resulting in 50%, 55%, 65% Phosphate buffer. The pH of the mobile phase was varied between 3, 3.5, and 4. The impact on retention time and peak parameter was investigated.

3.6 Limit of Detection (LOD) and Limit of quantitation (LOQ)

Concentrations in the calibration curve's lower linear range were used to determine the detection and quantification limits. The amount of drugs used versus the average response (peak area) was plotted, and the regression equation was determined. Response standard deviations (S.D.) were computed. The average of standard deviations was calculated from this data (A.S.D.). LOD was

calculated using the formula (3.3 xA.S.D.)/b, and LOQ was calculated using the formula (10 xA.S.D.)/b, where "b" corresponds to the slope obtained in the method's linearity study.

3.7 Specificity

Specificity of the method was determined by means of entire separation of standard drugs in the presence of other excipients normally present in the dosage forms.

HPTLC

The method's specificity was determined by analysing standard drug and sample. At various Rf values, the mobile phase resolved all of the drugs very efficiently. The LDV and SFB spot was confirmed by comparing the Rf and spectra of the spot to those of the standard. The peak purity spectra of LDV and SFB were evaluated by comparing the spectra of drug and sample at the spot's peak start (S), peak apex (M), and peak end (E).

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HPLC

A study was conducted to establish the interference of blank and placebo. Diluent and placebo were injected into the system under the above mentioned chromatographic conditions and the chromatograms of blank and placebo were recorded.

3.8 System suitability

The suitability of the system was evaluated in order to ensure the chromatographic system's quality performance. Six replicates of Ledipasvir and Sofosbuvir working standards samples were injected, and parameters such as capacity factor (K), injection repeatability tailing factor (T), theoretical plate number (N), and resolution (Rs) for the main peak and its degradation product were tested. The system suitability parameters were revealed to be within acceptable limits.

3.9 Forced degradation of LDV and SFB

A stock solution of Ledipasvir and Sofosbuvir was prepared. This solution was used for forced degradation to provide an indication of the stability-indicating property of the method.

Acid and base induced degradation

1 ml of 2 M methanolic HCl and 2 M methanolic NaOH were added to SFB and LDV solutions. For 8 hours, the solutions were kept at room temperature. These solutions were diluted with the mobile phase to achieve a final concentration of SFB and LDV of 10 mg/ml. The solutions (1

ml) were taken and neutralised in HPTLC before being diluted up to 10 ml with methanol. The resulting solutions were applied in triplicate (10 L each, i.e. 1000 ng/spot) to a TLC plate. The solutions were diluted with the mobile phase in HPLC to a final concentration of 10 mg/ml of SFB and LDV before being injected.

Hydrogen peroxide induced degradation

2 ml of 30% hydrogen peroxide was added to SFB and LDV solutions. These solutions were kept at room temperature and shielded from light for 8 hours. In HPTLC, the solution (1 ml) was diluted to 10 ml with methanol and the resulting solutions were applied in triplicate i.e. 1000 ng/spot) on TLC plates. After the time intervals specified, the solutions were diluted with the mobile phase to achieve a final concentration of 10 mg/ml of SFB and 10 mg/ml of LDV in HPLC. Following the previous treatments, the solutions were filtered through a 0.45-mm filtration disc before being injected into the column..

Dry heat degradation products

SFB 10 mg and LDV 10 mg were stored in oven at 55^0 C for 3 hours separately. They were transferred to a 10 ml volumetric flask containing methanol, and the volume was increased to the required level with methanol. In HPTLC, the solutions were diluted with the mobile phase to achieve a final concentration of 10 mg/ml of SFB and 10 mg/ml of LDV on TLC plates in triplicate i.e. 1000 ng/spot. By injecting the sample into the column, the chromatograms were generated.

Light heat degradation products

Separately, 10 mg of SFB and LDV were dissolved in 10 ml of methanol. The solutions were exposed to sunlight for 8 hours. 1 ml of the above solutions were taken and diluted with methanol to a volume of 10 ml. The treated powder solution was then prepared in HPTLC, and 1000 ng/spot was applied to a plate in triplicate. The solutions were diluted with the mobile phase in HPLC to achieve a final concentration of 10 mg/ml SFB and 10 mg/ml LDV. By injecting the sample into the column, the chromatograms were generated..

4.Results

HPTLC Method

The proposed HPTLC method allows for the quantification of LDV and SFB in bulk and dosage forms in a timely, accurate, and precise manner. The calibration plots' linear regression analysis data showed a good relationship over the concentration ranges of 100-600 ng/spot for LDV and

1000-6000 ng/spot for SFB (r2=0.998 for LDV and 0.999 for SFB). The proposed methods' dependability and analytical performance, including linearity, range, precision, accuracy, detection, and quantitation limits, were statistically validated. The results for precision (repeatability, intraday and interday)shown in **Table 1** . Good recoveries for both the drugs in the range of 98.03-99.07% for LDV and 98.16-100.57% for SFB respectively. The results are shown in **Table 2**. Signal-to-noise ratios of 3:1 and 10:1 were obtained for LOD and LOQ, respectively. By using the values of slopes and intercepts of the calibration curves for both the drugs the LOD and LOQ were calculated . The LOD and LOQ for SFB were found to be 0.298 and 0.852 respectively. The LOD and LOQ for LDV were found to be 0.238 and 0.997 respectively The standard deviation of peak areas was calculated for each parameter, and the % RSD was found to be less than 2. The low values of the % RSD indicated the robustness of the method. The ruggedness of the proposed method was evaluated by two different analysts under different condition. The results for SFB and LDV were found to be 99.88%, 99.85% and 100.19%, 100.69%, respectively. The peak purity of SFB and LDV was assessed by comparing their respective spectra at the peak start, apex, and peak end positions of the spot, that is, r(S, M) = 0.9991 and r(M, E) = 0.9993. A good correlation (r = 0.9989) was also obtained between the standard and sample spectra of SFB and LDV, respectively. Experimental results obtained during the assay shows that the amount of SFB and LDV in tablets was in good agreement with the labeled claim and suggest there is no interferences from other excepients present in dosage form. The drug content was found to be 99.69% for SFB and 100.67% for LDV respectively. Summary of validation parameter is shown in **Table 5.**

HPLC method

Several mobile phases were tried using various proportions of different aqueous phases and organic modifiers. Ultimately, Phosphate buffer pH 2.5:Acetonitrile: Methanol 60:30:10 (V/V/V)selected as a mobile phase. The chromatogram obtained from the analysis of standard solution of LDV and SFB was SFB(Rt=5.732 min) and LDV (Rt=3.144 min), at a flow rate of 1.5ml/min. Quantification was achieved with ultraviolet detection at 254 nm. The detector response was found to be linear in the concentration ranges of 2-12 mg/ml and 5-60 mg/ml for LDV and SFB, respectively, and the correlation coefficients for both drugs were 0.9992 and 0.994 for SFB and LDV, respectively. Validation in accordance with ICH guidelines demonstrated the suitability of this HPLC method for quantitative determination of the

compounds. Precision was calculated for both drugs based on inter-day and intra-day variations. The percent relative standard deviations for estimating LDV and SFB under intra-day and inter-day variations were found to be less than 2. The results are shown in **Table 1**. The proposed method's accuracy was determined, indicating agreement between the true and found values. The results are shown in **Table 2**.The LOD and LOQ for both the drugs were calculated using the values of slopes and intercepts of the calibration curves. The LOD and LOQ for SFB were found to be 0.348 and 1.052 respectively. The LOD and LOQ for LDV was found to be 0.278 and 0.789 respectively . Good peak with resolution between two drugs is > 1.5, asymmetric factor <2 shows that the three drugs were better separated. The results were tabulated in **Table 3**. Robustness was investigated in all deliberately varied conditions, and the percent relative standard deviations were found to be less than 2%, indicating that the method is robust. The experimental values obtained in tablet for determining LDV and SFB were within the claimed limits. Two different analysts assessed the robustness of the proposed method. The SFB and LDV results were 99.68 percent, 99.95 percent, and 99.98 percent, 100.11 percent, respectively. Because chromatograms of blank and placebo showed no peaks at the retention times of the LDV and SFB peaks, the method is said to be specific. This shows that the diluents and placebo used in sample preparation had no effect on the simultaneous estimation of LDV and SFB. A summary of validation parameters are Shown in **Table 4.**

Stability-indicating property

A forced degradation study was used to determine the stability of LDV and SFB. The chromatograms of samples degraded with acid, base, hydrogen peroxide, dry heat, and light revealed well separated spots of pure LDV and SFB, as well as some additional peaks at different retention factors in HPTLC (shown in **Figure 5**) and retention times in HPLC (shown in **Figure 6**).

The number of degradation products with retention factor and percentage recovery were calculated and listed in **Table 5**.

The number of degradation products with their retention time of LDV and SFB and percentage recovery were calculated and listed in **Table 6**.

Discussion

For the routine analysis of SFB and LDV in API and dosage forms, simple, rapid, accurate, and precise stability-indicating HPTLC and HPLC analytical methods have been developed and validated. The proposed methods' reliability was determined by validating the methods' linearity, precision, accuracy, detection limit, quantitation limits, robustness, and ruggedness. The repeatability and intermediate precision (intraday and interday) were expressed as a percentage of RSD. Because of the low percentage RSD value, the proposed method provides an acceptable intraday and interday variation. Percent recovery confirmed the accuracy. The percent RSD was observed to be less than 2. The method's accuracy was indicated by the low percent RSD values. Forced degradation testing was used to determine the stability of SFB and LDV. The chromatograms of samples degraded with acid, base, hydrogen peroxide, dry heat, and light revealed well separated spots of pure SFB and LDV, as well as a few additional peaks at various Rf and Rt values. These methods performed admirably in terms of sensitivity and speed. The methods have been demonstrated to be stability-indicating and can be used for routine analysis of production samples as well as to ensure the stability of drug substances. The method is being applied to pharmaceutical formulations one at a time; no chromatographic interferences from tablet excipients were observed. The suitability of HPTLC and HPLC methods for quantitative compound determination is proved by validation in accordance with the requirements of ICH guidelines.

Conclusion

Validated stability indicating HPTLC and RP-HPLC methods were developed and validated as per ICH guidelines and found to be simple and robust. The standard deviation and % RSD calculated for the proposed methods are low, indicating high degree of precision and the results of the recovery studies performed show the high degree of accuracy for the proposed methods. The HPTLC and RP-HPLC methods gives the details of quantification of LDV and SFB in presence of its degradation products hence; it can be employed as a stability indicating method. From the results of the experimental data it can be concluded that the developed stability indicating HPTLC and HPLC methods are simple accurate, precise and selective and can be adapted successfully for the estimation of LDV and SFB in tablet dosage form. The proposed methods can separate the drug from its degradation products, related substances, and excipients

found in dosage forms, and they can be used to analyse samples obtained during accelerated stability experiments. The proposed methods can be used to determine the shelf life of pharmaceutical formulations.

Abbreviations

NaOH: Sodium hydroxide; HCl: Hydrogen chloride; RP-HPLC: Reversed-phase high performance liquid chromatography; HPTLC: High-performance thin-layer chromatography; ICH: International Conference on Harmonization; LOD: Limit of detection; LOQ: Limit of quantitation; BIC: Bictegravir; ETB: Emtricitabine; TAF: Tenofovir Alafenamide; SD: Standard deviation;RSD: Relative standard deviation; RF: Retention factor;UV: Ultraviolet.

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8. References

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Table 1: Precision values of LDV and SFB

Method	**Drug**	**Concentration (µmg/ml)**	**Intra-day (% RSD,n=3)**	**Inter-day (% RSD,n=3)**	**Repeatability (% RSD,n=6))**
HPTLC	**SFB**	2200ng/spot	1.04	0.89	0.88
	LDV	500ng/spot	1.02	1.01	0.84
HPLC	**SFB**	22 µmg/ml	1.04	1.13	0.91
	LDV	5 µmg/ml	1.41	1.12	0.78

Table 2: Recovery studies of LDV and SFB

Drug	**HPTLC method**			**% RSD**		
	80%	**100%**	**120%**	**80%**	**100%**	**120%**
SFB	98.16	99.85	100.57	0.85	0.96	1.02
LDV	98.03	99.07	99.04	1.05	0.88	1.05
Drug	**HPLC method**			**% RSD**		
	80%	**100%**	**120%**	**80%**	**100%**	**120%**
SFB	99.12	99.74	99.56	0.65	0.75	1.14
LDV	99.45	100.02	100.45	0.75	0.55	0.45

***Average of Six reading**

Table 3: System suitability parameter

System Suitability Parameters	Observations	
	LDV	**SFB**
Retention time (T_R)	3.14	5.73
Capacity factor ($K^{'}$)	0.75	0.76
Theoretical plate (N)	6234	7767
Tailing factor (T)	0.97	1.07
Resolution	4.53	6.78

Table 4: Validation parameter

Parameter	HPTLC Method		HPLC Method	
	SFB	**LDV**	**SFB**	**LDV**
Linearity range [umg/ml]	1000-6000 ng/spot	100-600 ng/spot	5-60 umg/ml	2-12 umg/ml
Regression equation [Y = mX + C]	Y= 29653X - 49911	Y=5193X+1457 8	Y=24594X+1845 7	Y=24567X+24156
Correlation coefficient	0.999	0.998	0.9992	0.994
Limit of detection [ng]	0.298	0.238	0.348	0.279
Limit of quantitation [ng]	0.852	0.997	1.052	0.789
% Recovery [n = 3]	98.16-100.57	98.03-99.04	99.12-99.56	99.45-100.45
Ruggedness [%]				
Analyst I [n = 3]	99.88	100.19	99.68	99.98
Analyst II [n = 3]	99.85	100.69	99.95	100.11

Precision [% RSD]				
Repeatability [n = 6]	0.88	0.84	0.91	0.78
Inter-day [n = 3]	0.78-1.08	0.66-1.06	0.56-1.04	0.96-1.41
Intra-day [n = 3]	0.89-1.04	0.84-1.09	0.96-1.13	0.56-1.12
Robustness	Robust	Robust	Robust	Robust
Specificity	Specific	Specific	Specific	Specific

Table 5: Force degradation of SFB and LDV by HPTLC

Sample exposure condition	Number of degradation products [Rt values]		Recovery [%]	
	SFB	**LDV**	**SFB**	**LDV**
2 M HCl, 8h,RT[a]	2(0.32,0.38)	2(0.12,0.21)	89.56	85.95
2M NaOH,8h, RT[a]	2(0.21,0.38)	2(0.25,0.32)	86.88	90.91
30%H_2O_2,8h,RT[a]	1(0.23)	1(0.24)	95.96	96.78
Dry heat,3H, 55^0C	1(0.22)	1(0.22)	96.88	96.78
Photolight,8h	1(0.25)	1(0.27)	98.99	98.49

RT[a] Room temperature.

Table 6: Force degradation of SFB and LDV by HPLC

Sample exposure condition	Number of degradation products [Rt values]		Recovery [%]	
	SFB	**LDV**	**SFB**	**LDV**
2 M HCl, 8h,RT[a]	2(1.3,2.3)	2(1.12,1.21)	84.56	81.95
2M NaOH,8h, RT[a]	2(2.21,2.18)	2(1.25,1.62)	84.88	87.91
30%H_2O_2,8h,RT[a]	1(2.23)	1(1.45)	96.96	96.78
Dry heat,3H, 55^0C	1(1.12)	1(1.29)	97.88	97.78
Photolight,8h	1(1.15)	1(2.27)	98.19	98.19

RT[a] Room temperature.

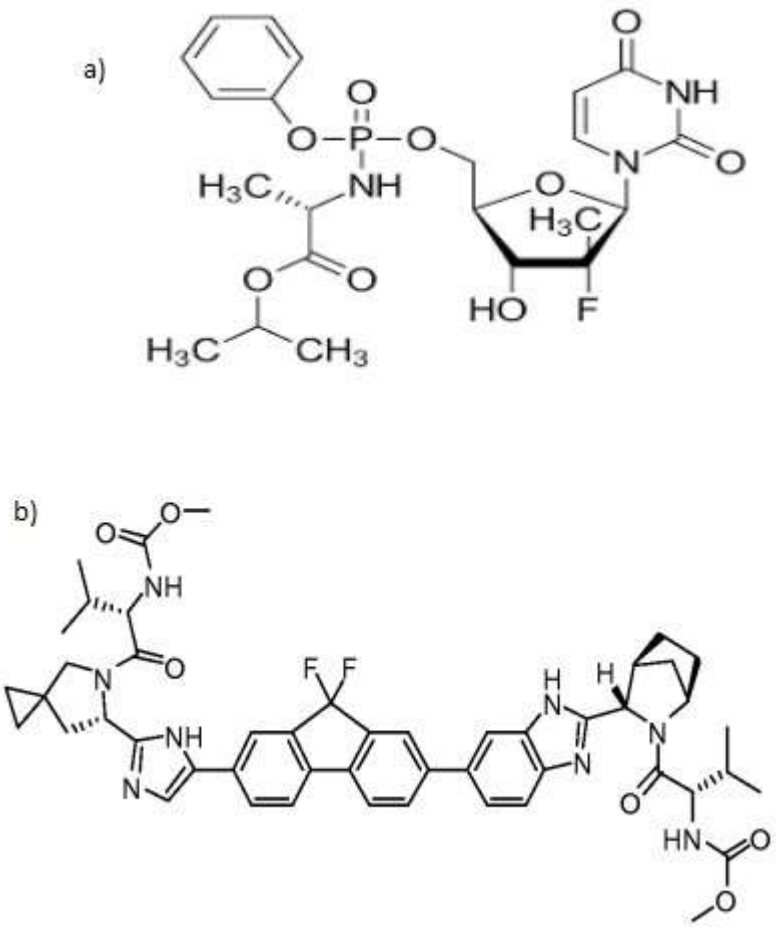

Figure 1: Chemical structure of a) SFB and b) LDV

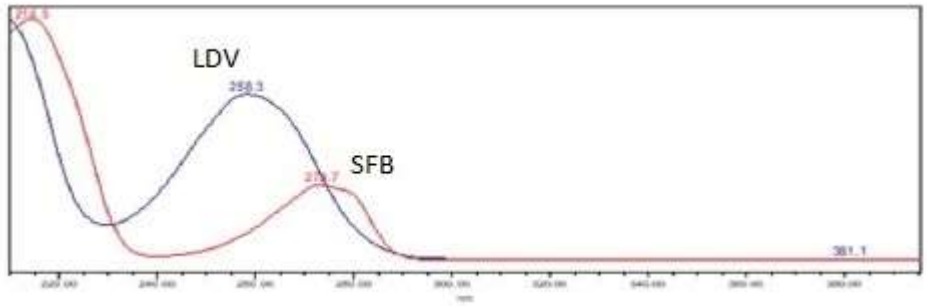

Figure 2: Overlain spectra of SFB and LDV

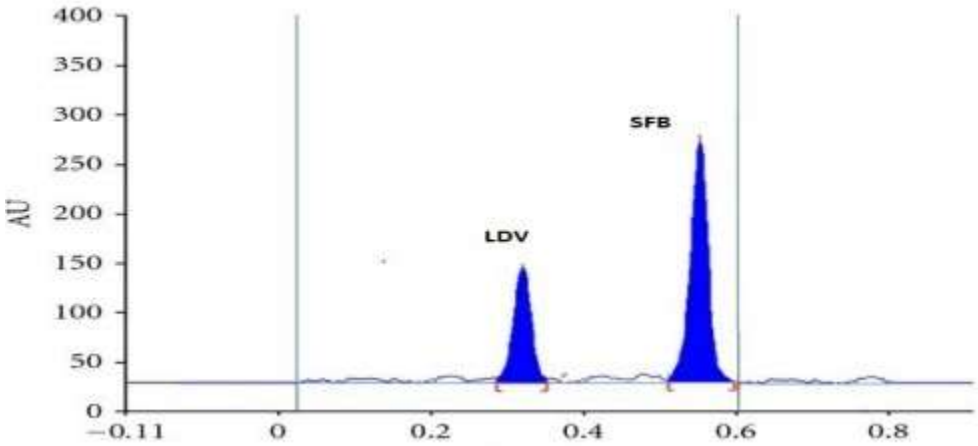

Figure 3 : HPTLC Chromatogram of LDV(Rf -0.37) and SFB (Rf -0.53)

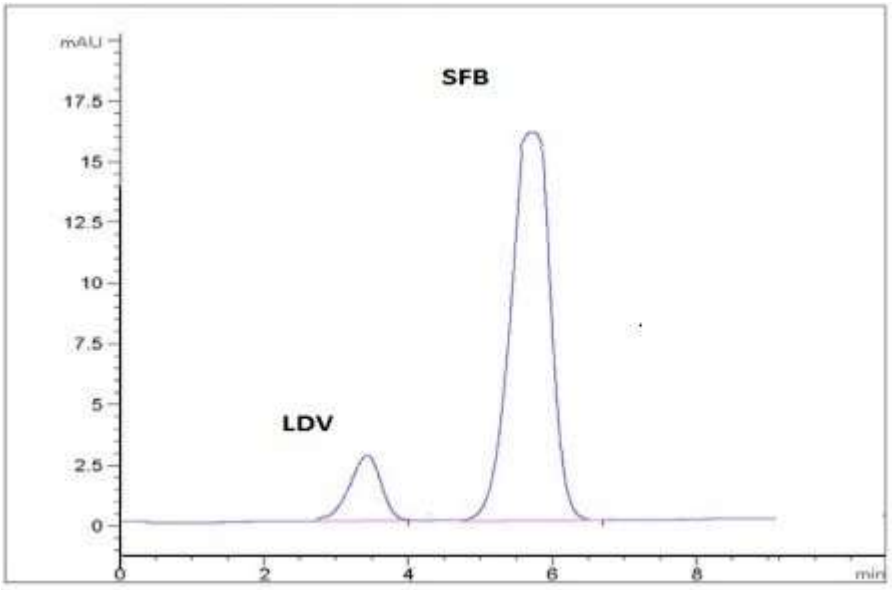

Figure 4 : HPLC Chromatogram of LDV(Rt-3.14 min) and SFB(Rt-5.732min)

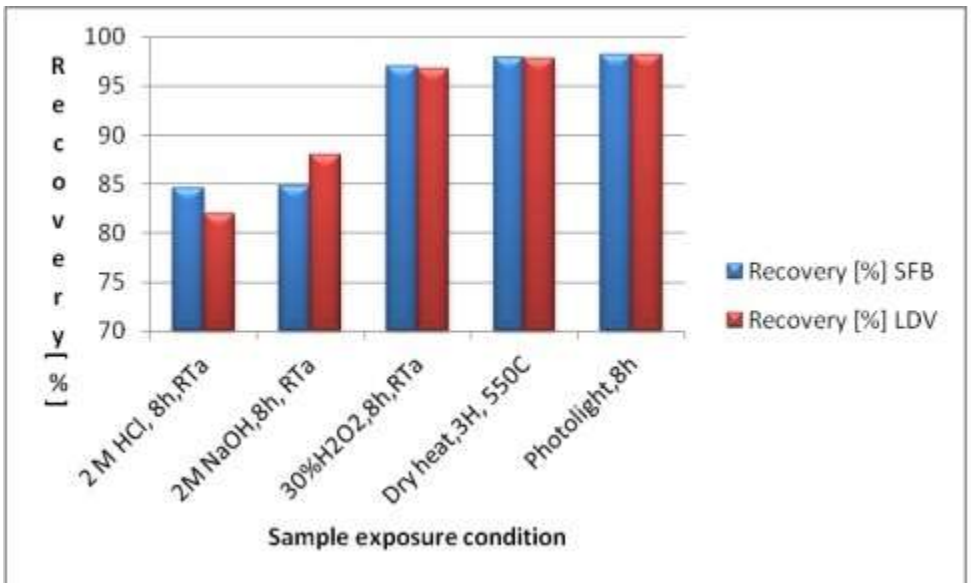

Figure 5: Force degradation of SFB and LDV by HPTLC

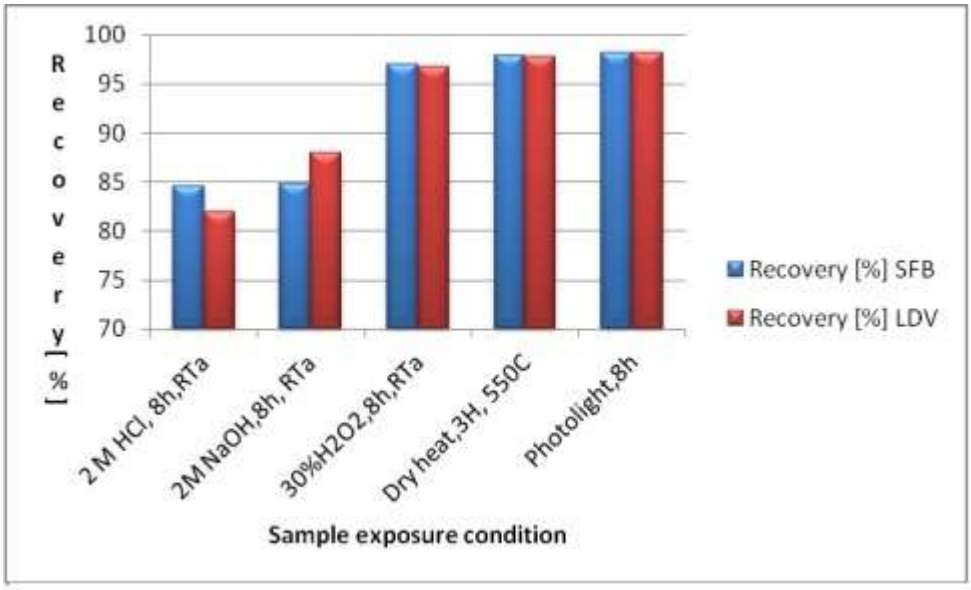

Figure 6: Force degradation of SFB and LDV by HPLC