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Research Article

DEVELOPMENT AND VALIDATION OF AN RP-HPLC METHOD FOR THE ESTIMATION OF SILDENAFIL, FLUOXETINE, AND LOVASTATIN

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ABSTRACT

Objective: Neuropathic pain (NP) arises from trauma to the somatosensory nervous system and can be managed using selective serotonin reuptake inhibitors, such as fluoxetine (FLX) and phosphodiesterase inhibitors, such as sildenafil (SD), and cholesterol-lowering agents such as lovastatin (LOVA). The present study aimed to develop and validate an analytical method for the simultaneous estimation of these drugs (SD, FLX, and LOVA [SFL]) using reverse-phase high-performance liquid chromatography (RP-HPLC).

Methods: An RP-HPLC method was developed and validated for the quantification of SFL. Chromatographic separation was achieved using a C-18 reverse-phase ODS column with a mobile phase consisting of acetonitrile and 0.2 M ammonium acetate buffer (55:45) in gradient elution mode. The flow rate was maintained at 1 mL/min, and detection was carried out at 228 nm. The method was validated following the ICH Q2 (R2) guidelines, assessing parameters such as linearity, accuracy, precision, limit of detection (LOD), and limit of quantification (LOQ).

Results: The developed method exhibited linearity within the concentration range of 20–100 µg/mL, with a regression coefficient (r^2) of 0.9992. Retention times for FLX, SD, and LOVA were recorded at 6.481, 4.238, and 19.778 min, respectively. Recovery studies demonstrated an accuracy range of 94.61–110.44%, with a relative standard deviation of 0.06–2.00%, confirming the precision of the method. The LOD values for FLX, SD, and LOVA were found to be 12.77 µg/mL, 14.81 µg/mL, and 13.28 µg/mL, respectively, while the LOQ values were 45.16 µg/mL, 42.33 µg/mL, and 38.71 µg/mL.

Conclusion: The validated RP-HPLC method met all required validation criteria and demonstrated suitability for the accurate quantification of FLX, SD, and LOVA in pharmaceutical formulations. These findings support the potential use of these drugs as an alternative therapeutic strategy for NP.

Keywords: RP-HPLC, Neuropathic pain, Elution, Analytical method, Validation.

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INTRODUCTION

Chemically, sildenafil (SD), fluoxetine (FLX), and lovastatin (LOVA) are 5-(2-ethoxy-5-((4-methylpiperazin-1-yl)sulfonyl)phenyl).-1-methyl-3-propyl-1,6-dihydro-7H-pyrazolo[4,3-d] pyrimidin-7-one 2-hydroxypropane-1,2,3-tricarboxylate [1], N-methyl-3-phenyl-3-(4-trifluoromethyl) phenox propane -1-aminene hydrochloride [2], (1S,3R,7S,8S,8aR)-8-(2-((2S,4R)-4-hydroxy-6-oxotetrahydro-2H-pyran-2-yl)ethyl) -3,7-dimethyl-1,2,3,7,8,8a-hexahydronaphthalen-1-yl (S2-methyl butanoate, respectively) [3] (Figs. 1 and 2.) SD, a drug for erectile dysfunction [4], alleviated neurotransmission and pain signaling [5] and treated the neuropathic pain (NP) [6]. FLX, an antidepressant [7,8], enhanced serotonin availability, prevented agonizing stimuli, and elevated mood. LOVA, a prodrug, is rendered inactive when administered orally. Statins have been shown in numerous studies to be beneficial in treating a range of neurological conditions. According to published research, statins may help treat neurodegenerative conditions, such as multiple sclerosis (MS) [9], Parkinson's disease [10], and Alzheimer's disease (AD) [11]. According to some research, statins can also lessen the effects of traumatic brain and spinal cord injuries [12,13].

Neuronal injury, direct nervous system damage, or somatosensory nervous system diseases are the causes of NP [6,14]. Paresthesia, hyperalgesia, and allodynia are its hallmarks [6,15]. The complicated process of NP development deteriorates as patients get inadequate treatment. Patients with HIV, rheumatoid arthritis, diabetes, cancer, MS, and brain and spinal cord traumas are at risk for a worsened state of NP [16-20]. As a result of numerous molecular, cellular, and systemic alterations throughout time, the nervous system developed maladaptive reactions and changed nerve function [21]. Neuronal hyperexcitability, cerebral sensitization, immunological activation, and modifications to neurotransmitter systems were among the factors behind this long-term development (Fig. 1a) [19,22]. The peripheral and central nervous systems were affected by these disorders, which resulted in abnormalities in the functioning of sodium and calcium channels as well as imbalances in substances, such as GABA, NMDA, NE/5HT, adrenoreceptors, cytokines, TRPV1, and AMPA/KA. These disturbances led to aberrant signaling pathways, which aided in the emergence of NP [23-27]. Over time, these changes affected neuronal excitability and pain modulation, perpetuating chronic pain and leading to sensory abnormalities (Fig. 1b) [22,28]. These disruptions affected the functioning of pain fibers and contributed to the degeneration of the peripheral and central nervous systems (Fig. 1c) [29]. Treating NP requires various medications, including anticonvulsants, antidepressants, and opioids [6,30,31]. A single medication alone could not effectively treat NP; rather, a combination of drugs was needed to manage the condition. In rats given a high-fat diet and a low dose of streptozotocin [32,33], as well as spinal nerve ligation [34,35], these NP symptoms are evident. Many research studies reported that the individual effect of a drug in treating the disease could not provide sufficient relief, hence the combination of these drugs offered a multi-faceted approach for treatment, potentially resolving the complications of the disease. Abdelshakour *et al*. developed a high-performance liquid

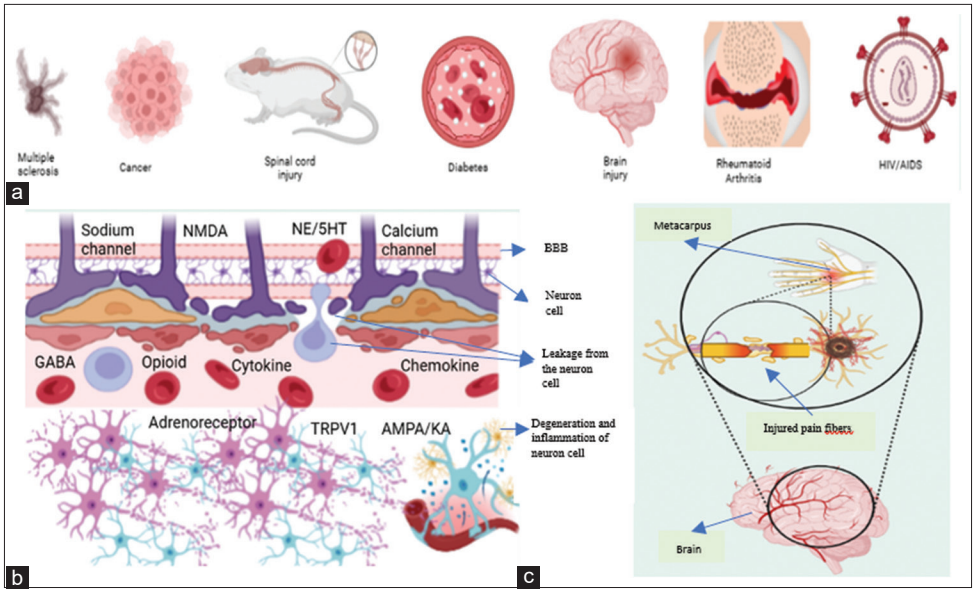

Fig. 1: Pathogenesis of NP (a) Causes of NP; Caused by diseases, such as multiple sclerosis, cancer, spinal cord injury, diabetes, brain injury, rheumatoid arthritis, and HIV/AIDS, (b) Alteration after the NP; Imbalanced receptors (NMDA; N-methyl-D-aspartate, NE/5HT; nor-epinephrine/5-hydroxytryptamine, GABA; Gamma-aminobutyric acid, Opioid, Cytokine, Chemokines, Adrenoreceptor, TRPV1; Transient Receptor Potential Vanilloid-1 and AMPA/KA; α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid/kainate-type glutamate receptors) and channel (Sodium Channel and Calcium Channel) function results in BBB (Blood-Brain Barrier) leakage due to inflammation and degeneration of the neuron, and (c) Inside the brain after NP; Loss of sensory function (in the Metacarpus) due to injury in pain fibers (Aδ and C fibers) resulting in degenerating the myelinated cells and loss of synaptic neuroplasticity

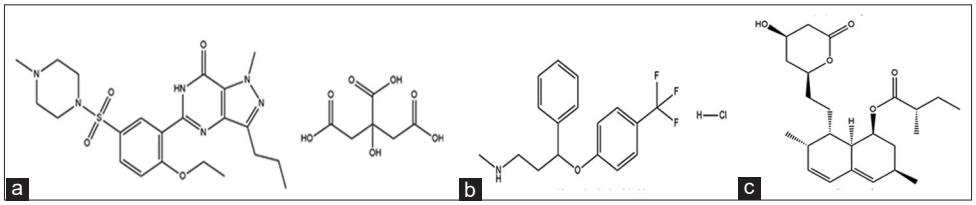

Fig. 2: Structure and IUPAC name of Chemicals (a) SD (Sildenafil Citrate); 5-(2-ethoxy-5-((4-methylpiperazin-1-yl)sulfonyl)phenyl)-1-methyl-3-propyl-1,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one 2-hydroxypropane-1,2,3-tricarboxylate (b) FLX (Fluoxetine hydrochloride); N-methyl-3-phenyl-3-(4-(trifluoromethyl)phenoxy)propan-1-amine hydrochloride (c) LOVA (Lovastatine); (1S,3R,7S,8S,8aR)-8-(2-((2S,4R)-4-hydroxy-6-oxotetrahydro-2H-pyran-2-yl)ethyl)-3,7-dimethyl-1,2,3,7,8,8a-hexahydronaphthalen-1-yl (S)-2-methylbutanoate

chromatography (HPLC) technique to analyze SD, vardenafil, and tadalafil simultaneously, along with their analogs such as dapoxetine, paroxetine, citalopram, tramadol, and yohimbine found in stimulated products. The mobile phase consisted of acetonitrile (ACN) and an aqueous 0.05% formic acid solution. The flow rate was 1 mL min^{-1}, and a gradient program was used. 230 nm was selected as the exact ultraviolet (UV) detection threshold. These eight medications were eluted in a total of 11 min. In addition, the development of a HPLC-MS/MS method allowed for the separation of compounds in just 6 min [36]. In a separate investigation, Aboul-Enein *et al*. employed a monolithic silica column and an isocratic elution of 60:40, v/v ACN/water. The 2.0 mL/min flow rate was used to monitor the analyte elution at 292 nm. Ambient temperature was employed for all analyses. The correlation coefficient (R 2) was >0.999 and linearity was observed in the concentration range of 50–3000 ng/mL. The detection limit was 25 μg/mL [37]. In one of the studies, Yang *et al*. employed a 70:30 mixture of ACN and 0.030 M ammonium formate (adjusted to pH 3.0 with formic acid). The drugs were detected using UV light at 230 nm. The total run time was 7 min, and the retention times for vardenafil, SD, and tadalafil were 1.654, 2.032, and 5.067 min, respectively [38]. An isocratically applied mobile phase by Emrah Dural, 10 mM phosphate buffer with 0.1% triethylamine (pH 3.5) and ACN (65:35, v/v), was used on a reverse phase C18 analytical (4.6×250 mm, 5 μm) column. A C18 reverse-phase analytical column with a 4.6 × 250 mm and a 5 μm particle size was used to achieve chromatographic separation. The mobile phase consisted of 10 mM phosphate buffer with 0.1% triethylamine (65:35, v/v, pH 3.5) and ACN. The temperature of the column was 35°C, and the mobile phase flow rate was 1 mL/min. 293 nm was the UV detector's set point [39]. In addition to these, the researchers employed ACN, buffer solution (pH 2.0), and a flow rate of 1.5 mL/min as additional analytical techniques for SD estimation [39,40]. Sheu *et al*. published an Analytical approach for liquid chromatography-based SD estimation in 2003. The mobile phase consisted of ACN and 30 mM potassium dihydrogen phosphate (phosphate buffer (pH 6.0 with 1N NaOH). Its flow rate was 0.5 mL/min [41,42]. In the same way, Tuchilă and Baconi set up an Analytical method for the FLX using HPLC-FL in human plasma. They used a flow rate of 1 mL/min and a mixture of 25:50:25 methanol, ACN, and formic acid. They stated a detention period of 3 min [43]. Mohamed A. developed the HPLC method to detect the presence of FLX in biological fluid, human plasma, and capsule dosage forms. they carry out the analysis using a reversed-phase C18 column with UV detection set to 228 nm. Acetonitrile and triethylamine buffer (48/52, V/V) make up the isocratic mobile phase (1.5 mL/min) [44]. They created a linear calibration model (correlation coefficient: 0.99863) using pyridoxine as an internal reference. Pyridoxine and FLX had retention durations of 2.10 and 3.20 min, respectively [45,46]. In a different work, Patel and Patel used reversed-phase liquid chromatography to quantify FLX HCl and olanzapine. The

mobile phase was ACN: methanol:0.032 M ammonium acetate buffer (45:05:50, v/v/v), and the flow rate was 1.5 mL/min. It was possible to measure concentrations between 0.2–4 and 0.1–2 μg/mL using UV detection at 235 nm. The average accuracy for FLX HCL was 101.16±0.59 and for olanzapine, it was 99.79±0.56%. The second approach involved separating the two medications using high-performance thin-layer chromatography and measuring their spots at 235 nm using densitometric analysis [47]. Patel *et al*. created an HPLC study for the Hypersil BDS C18 column. The best chromatographic separation was achieved at 19.9°C with ACN: sodium dihydrogen phosphate buffer (7.5 mM; pH 4, 60:40% v/v) as the mobile phase and a flow rate of 1.036 mL/min [47]. In 2010, Silva *et al*. developed and validated LOVA by HPLC in combination with Simvastatin and pravastatin. Ultraviolet–diode array detection at λ 238 nm and 1.5 mL/min flow for LOVA were performed in a solution of ACN and 0.1% phosphoric acid (65:35) at 30°C [48]. Anwar A Wassel and Heba El-agezy performed analysis on a Thermo Hypersil BDS C18 column (250 mm×4.6 mm i.d, 5 μm particle size) using a mobile phase of 375 mL distilled water, 0.1 mL tetra-n-butylammonium hydroxide, 0.4 mL triethylamine (adjust pH to 3.5 with phosphoric acid), and 625 mL ACN (375:625, v/v) at a flow rate of 1 mL/min and UV detection at 220 nm [49]. The devised method was linear over the concentration range of 1–16 μg/mL (r=0.99999), with a limit of detection (LOD) of 0.028 and 0.065 μg/mL for atomoxetine and FLX, respectively, and a limit of quantitation 0.085 and 0.198 μg/mL for atomoxetine and FLX [49]. Marais *et al*. performed *in vitro* skin penetration tests to identify and quantify LOVA, mevastatin, rosuvastatin, and simvastatin. They utilized a Venusil XBP C18 column (Agela Technologies, Newark, DE) with gradient elution and HPLC grade ACN. Linearity was attained, and the average recovery varied from 95.9 to 100.6%. LOVA and simvastatin were found at comparable amounts but measured at greater levels [50]. Bayat and Bozorgi extracted LOVA from an acidic 15-mL sample solution with a pH of 2. They impregnated a hollow fiber's pores with an organic extracting solvent (n-octanol) impregnated in the pores of a hollow fiber. They then back-extracted the LOVA into an acidified aqueous solution within the hollow fiber's lumen. Following extraction, a volume of 10 μL from the acceptor phase was injected into the HPLC system. These factors encompassed the pH levels of both sample and extractant phases, the specific type of organic phase used, the ionic strength, the stirring rate, the extraction time, and the temperature. Using 1-octanol as the solvent and keeping the pH at 2 while stirring at 750 rpm for 45 min at 45°C was the best way to get the best results. The method had an impressive relative recovery percentage of 85.2–97. This clearly highlights the method's ability to accurately analyze the concentration of the analyte [51]. Ayothiraman *et al*., Acetonitrile has influenced on the overall analysis time, whereas the acidifier determines the retention time of the hydroxy acid form of LOVA and the retention time gap between the individual forms [52]. A combination of

ACN and 0.1% trifluoroacetic acid (60:40, v/v) in isocratic elution mode eluted both hydroxy acid and lactone forms of LOVA at 4.5 and 5.4 min, respectively [53]. There are numerous methods for determining the levels of SD, FLX, and LOVA using HPLC and UV measurements. In terms of application and mixture, the method was new, as no one had ever reported delivering and treating neuropathy wounds with a mix of SD, FLX, and LOVA (SFL). Similarly, it has never been stated that the method used to estimate them in SFL was similar. ICH Q2 (R1) rules say that these are "new drug products," so that's what they are. Therefore, it is necessary to develop a method that is specific, sensitive, exact, and precise for estimating SFL. As previously mentioned, the combination of the drugs could effectively treat inflammatory illnesses such as ulcers, diabetic wounds, and trauma-related wounds. Therefore, we have endeavored to develop and evaluate a simultaneous method for determining SFL. In addition, we tested the developed analytical method for its specificity, sensitivity, and usefulness in measuring SFL. This challenging condition requires multimodal pharmacological approaches, including SD, FLX, and LOVA which have shown potential therapeutic benefits. However, no validated analytical method exists for their simultaneous estimation, which is essential for quality control, pharmacokinetic studies, and clinical applications. This study introduces a novel, precise, and robust RP-HPLC method for the simultaneous quantification of SD, FLX, and LOVA optimizing separation efficiency and detection sensitivity. Unlike previous methods focusing on individual or binary drug analysis, this approach ensures accurate assessment in pharmaceutical formulations and biological matrices. By adhering to ICH Q2 (R2) guidelines and incorporating stability studies under varied conditions, this research fills a critical gap, providing a reliable tool for therapeutic monitoring and reinforcing the potential of these drugs as an alternative NP treatment.

MATERIALS AND METHODS

Materials

Chemical reagents and equipment

Crystalline extra-pure powders of SD (Batch No. P11650921V and CAS NO. 148553-50-8), FLX (Batch No. 10240817V and CAS NO. 56296-78-7), and LOVA (Batch No. TCI/XWJRB and CAS NO. 75330-75-5), each with a purity of ≥98%, were procured from Yarrow Chemicals Pvt. Ltd., Mumbai, Maharashtra, India. The purity of these compounds was confirmed using HPLC. All drug standards were stored at room temperature (below 30°C) under controlled conditions to prevent degradation, ensuring accuracy and reproducibility in the analyses. Acetonitrile (HPLC grade) and Ammonium acetate (HPLC grade) were obtained from Merck, Mumbai, India, along with Glacial Acetic Acid (HPLC grade) and double distilled water (HPLC grade) from Merck, Mumbai, India. The mobile phase consisted of ACN and 0.2 M ammonium acetate buffer in a 55:45 ratio, selected to achieve optimal separation, peak resolution, and system suitability [54,55]. Acetonitrile was chosen due to its excellent elution properties, low viscosity, and compatibility with UV detection at 228 nm. The ammonium acetate buffer helped maintain the desired pH range, improving peak sharpness and retention time consistency. The gradient elution approach further enhanced the separation of SD, FLX, and LOVA ensuring accurate quantification. Filtration was carried out using a Nylon 0.22 μm membrane filter from Pall Corporation, Mumbai, India. The analysis was performed using an RP-HPLC (Shimadzu, LC-20AD, Prominence, Japan) instrument equipped with a UV detector, an auto-sampler, and a reverse-phase C18 column (4.6 × 250 mm, 5 μm particle size), controlled by LC-solution software. An analytical balance (Shimadzu LC-20AD, Tokyo, Japan), a bath sonicator (PCI, Mumbai), and a vortex mixer along with a cooling centrifuge from REMI, India, were used in the study. Methanol also used in the study purchased from Deejay Corporation from Jalandhar, Punjab, India. Stability studies were conducted by storing drug samples under different conditions to assess their degradation patterns. The samples were stored at room temperature (25°C±2°C), refrigerated conditions (4°C±2°C), and accelerated conditions (40°C±2°C with 75%±5% relative humidity) for a period of 3 months [56]. Periodic analysis was performed to evaluate any changes in retention time, peak shape, and drug concentration. The findings indicated minimal degradation under refrigerated conditions, while slight degradation was observed under accelerated storage, confirming the stability and robustness of the developed RP-HPLC method.

Methods

Determination of UV absorption maxima (λmax)

The absorption maxima (λmax) of SD, FLX, and LOVA were determined using a double-beam UV-visible spectrophotometer, with methanol as the solvent and blank. Stock solutions of each drug were prepared in methanol at specific concentrations (SD: 15 μg/mL, FLX: 10 μg/mL, LOVA: 20 μg/mL) to measure absorbance at their respective wavelengths, providing essential λmax data for analytical calibration. Each drug solution in methanol was analyzed over a wavelength range of 200–400 nm.

Chromatographic condition

SD, FLX, and LOVA were all simultaneously estimated using HPLC system (Shimadzu, LC-20AD, Prominence, Japan) with photodiode array detector (SPDM20A; Shimadzu, Japan) and Nucleodur C18 column with dimensions 250 mm×4.6 mm i.d., 5 μm was used for the simultaneous

estimation. The HPLC equipment included software for LC solutions. An analytical technique was developed using ICH Q2 (R2) standards to quantify drugs. Acetonitrile (A) and 0.2M ammonium acetate buffer (B) were used in the ratio 55:45 v/v as mobile phase using a flow rate of 1 mL/min and measured the chromatogram at 228 nm. Glacial acetic acid was used to maintain a pH of 7.2. ACN and % orthophosphoric acid, ACN and water 60:40, ACN-0.1% formic acid, methanol-water, ACN-water in different ratios were used to analyze SFL [57].

Validation of the method

The validation of the developed method was done according to ICH Q2 (R2) guidelines. In validation, various parameters were measured such as system suitability, linearity, the LOD, limit of quantification (LOQ), precision, and accuracy, which are discussed below.

Preparation of standard stock solution

Accurately weighed SFL (10 mg) was dissolved in 2 mL methanol in a 10 mL volumetric flask and filled with the distilled water. It gave a stock solution of 1000 µg/mL. Serial dilutions were performed by taking 1 mL of the above solution and making it up to 10 mL resulting in a solution of 100 µg/mL, which on further dilution yielded a solution of 10 µg/mL. From the prepared stock solution, serial dilutions were performed to get final concentrations of 20, 40, 60, and 80 µg/mL [58].

Stability of SD, FLX, and LOVA stock solution

10 mg/mL standard stock solution (discussed in Section 2.2.4) kept at 25°C room temperature and 40°C accelerated temperature for 1 week. Collections of samples were done at pre-set intervals as mentioned in the table. Then take 1 mL after every time interval from the stock solution and diluted by 10 mL to achieve a concentration of 1 mg/mL (to achieve a concentration of 1000 µg/mL). After injecting this diluted solution into the HPLC, compared the SD, FLX, and LOVA areas as a percentage relative to the starting area (i.e., 0 h) [59].

System suitability

In the system suitability study, various parameters were measured. These include retention time, peak area, tailing factor, theoretical plates/meter, and resolution. According to the ICH Q2 (R2) guidelines in the system suitability tailing factor, theoretical plates and resolution of the peaks must be under acceptance criteria such as the tailing factor must be <1.5, resolution between two peaks should be more than two, and theoretical plates should always be greater than two thousand [59,60].

LOD and LOQ

For SD, FLX, and LOVA, we used the calibration curve to determine the limits of detection and quantification. Their LOD and the minimum detectable concentration (LOQ) of an analyte is defined by the standards of acceptable accuracy and reproducibility in the field of analytical chemistry [61,62].

Below, you can find the formulas for both LOD and LOQ.

$$\text{LOD}=3.3\frac{\sigma}{S} \quad (1)$$

$$\text{LOD}=10\frac{\sigma}{S} \quad (2)$$

Accuracy

The quality and applicability of the developed method were checked by performing the recovery analysis of SFL at three levels, that is, LQC, MQC, and HQC of the medium concentration, which was 6 µg/mL. Standard solutions (LQC, MQC, and HQC) were injected 6 times, and the response mean values were recorded [59,63]. The percentage recovery was calculated from the following formula [59]. Percentage recovery can be calculated as actual concentration. The formula shown in the following equation (3) was used to estimate the absolute percentage of drug recovery: [61,64]

$$\text{Actual\% recovery}=\frac{\text{Actual concentration recovered}}{\text{Theoretical concentration}}\times 100 \quad (3)$$

Precision

Precision studies were performed in two parts: Repeatability and intermediate precision. In repeatability, standard solutions were injected 6 times each on the same day under the same conditions (intra-day). For the intermediate precision, an inter-day study was carried out by injecting 6 times of standard solution for three consecutive days and the inter-analyst study, three different analysts of the same laboratory injected 6 times of standard solution, which were prepared by other analysts by following the identical conditions of experiment. The mean of responses was noted, and the %RSD was calculated [65]. Following the collection of the mean data, the percentage relative standard deviation (RSD) was calculated using the formula shown in the equation (4): [66]

$$\text{\%Relative standard deviation}=\frac{\text{Standard deviation of peak area}}{\text{Average peak area}}\times 100 \quad (4)$$

Specificity

The goals were to determine the medication's specificity when combined and investigate any effects on each agent's retention duration [57].

Applications of validated method

The validated method was applied to SFL in the determination of drug solubility, drug loading, and drug release.

RESULTS AND DISCUSSION

Determination of UV absorption maxima (λmax)

The solutions of SD, FLX, and LOVA in methanol showed absorption peaks at 294 nm, 216 nm, and 237 nm, respectively, with an isosbestic point observed at 228 nm in the Fig. 3.

Development of chromatogram

The chromatogram was developed wherein the retention time of SD, FLX, and LOVA was found to be 6.4, 4.2, and 19.5, respectively (Fig.4.).

System suitability

The consistent application of chromatographic systems has an impact on their performance and the dependability of analytical results. The values of LOD and LOQ showed that, under certain chromatographic conditions, the approach was sufficiently sensitive to detect both substances. The system appropriateness results showed that the method is reliable and suitable for further research. Table 1 displays the system suitability results [67,68].

Stock solutions stability

The stability of the drug's standard stock solutions was examined at a concentration of 100 mg/mL for varied time intervals ranging from 4 to 168 h and corrected the area's mean, standard deviation, and percentage RSD. Throughout the trial, the results showed a change in both drug response areas. Determined the percentage RSD to be <2 in each case.

Table 2 presents the findings.

Table 1: Results of system suitability parameters

S. No.	Parameter	S	F	L
1.	Theoretical Plates	7563.56±12.01	7373±15.85	7931±20.08
2.	HETP	25.31±2.41	38.57±3.68	27.86±2.53
3.	Tailing factor	1.05±0.02	1.23±0.06	1.09±0.01

Table 2: Results of precision study

Drug	Parameter	Level	Concentration (ng/mL)	Area 1	Area 2	Area 3	Area 4	Area 5	Area 6	Mean	S.D.	RSD (%)
Intra-day												
SD	1 h	LQC	120	5596	5652	5631	5598	5647	5687	5635.16	31.73	0.56
		MQC	150	7054	7082	7091	7128	7096	7167	7103.00	35.95	0.50
		HQC	180	8211	8185	8265	8174	8255	8197	8214.50	34.20	0.41
FLX		LQC	120	6081	6141	6084	6127	6073	6151	6109.50	31.13	0.50
		MQC	150	7381	7418	7356	7394	7429	7365	7390.50	26.39	0.35
		HQC	180	8211	8185	8265	8174	8255	8197	8214.50	34.20	0.41
LOVA		LQC	120	5368	5401	5382	5436	5347	5476	5401.66	43.23	0.80
		MQC	150	6785	6771	6803	6849	6895	6837	6823.33	42.03	0.61
		HQC	180	8053	8255	8291	8037	8096	8128	8143.33	96.79	1.18
SD	2 h	LQC	120	5674	5631	5673	5624	5651	5682	5655.83	22.22	0.39
		MQC	150	7106	7154	7184	7131	7125	7143	7140.50	24.50	0.34
		HQC	180	8412	8433	8437	8422	8472	8414	8431.66	20.20	0.23
FLX		LQC	120	6125	6173	6079	6092	6172	6085	6121.00	39.20	0.64
		MQC	150	7454	7493	7488	7386	7378	7357	7426.00	54.43	0.73
		HQC	180	8443	8373	8397	8462	8682	8444	8466.83	100.90	1.19
LOVA		LQC	120	5487	5469	5324	5397	5371	5422	5411.66	55.71	1.02
		MQC	150	6851	6899	6812	6785	6733	6855	6822.50	53.61	0.78
		HQC	180	8134	8046	8037	8127	8239	8178	8126.83	70.53	0.86
SD	3 h	LQC	120	5585	5625	5634	5597	5641	5671	5625.50	28.38	0.50
		MQC	150	7165	7123	7254	7204	7231	7268	7207.50	50.55	0.70
		HQC	180	8379	8483	8415	8434	8416	8438	8427.50	31.28	0.37
FLX		LQC	120	6278	6279	6157	6183	6295	6197	6231.50	54.07	0.86
		MQC	150	7415	7428	7368	7487	7391	7389	7413.00	38.27	0.51
		HQC	180	8371	8349	8411	8369	8456	8325	8380.16	42.67	0.50
LOVA		LQC	120	5431	5412	5497	5385	5475	5488	5448.00	41.40	0.75
		MQC	150	6824	6811	6754	6728	6735	6814	6777.66	39.63	0.58
		HQC	180	8034	8099	8145	8129	8179	8099	8114.16	45.20	0.55
Inter-day												
SD	Day 1	LQC	120	5615	5681	5617	5643	5594	5678	5638.00	32.60	0.57
		MQC	150	7238	7361	7296	7283	7379	7297	7309.00	47.66	0.65
		HQC	180	8388	8475	8451	8368	8392	8473	8424.50	43.17	0.51
FLX		LQC	120	6199	6185	6257	6236	6287	6168	6222.00	41.75	0.67
		MQC	150	7465	7514	7431	7461	7501	7436	7468.00	30.70	0.41
		HQC	180	8476	8367	8411	8501	8482	8386	8437.16	51.34	0.60
LOVA		LQC	120	5315	5381	5367	5492	5472	5401	5404.667	60.81	1.12
		MQC	150	6785	6837	6891	6758	6837	6876	6830.66	46.79	0.68
		HQC	180	8187	8138	8208	8261	8177	8269	8206.66	46.22	0.56
SD	Day 2	LQC	120	5674	5612	5584	5591	5573	5671	5617.50	40.59	0.72
		MQC	150	7132	7183	7167	7259	7267	7188	7199.33	48.49	0.67
		HQC	180	8371	8367	8383	8314	8354	8366	8359.16	21.91	0.26
FLX		LQC	120	6254	6179	6286	6197	6258	6178	6225.33	42.34	0.68
		MQC	150	7308	7468	7394	7387	7425	7419	7400.16	48.78	0.65
		HQC	180	8437	8367	8412	8467	8379	8411	8412.16	33.57	0.39
LOVA		LQC	120	5471	5322	5364	5391	5482	5491	5420.16	64.63	1.19
		MQC	150	6856	6887	6738	6814	6799	6834	6821.33	46.85	0.68
		HQC	180	8173	8176	8138	8241	8245	8166	8189.83	39.56	0.48
SD	Day 3	LQC	120	5579	5592	5576	5585	5549	5537	5569.66	19.81	0.35
		MQC	150	7243	7361	7358	7361	7454	7234	7335.16	76.05	1.03
		HQC	180	8382	8405	8437	8461	8411	8389	8414.16	27.31	0.32
FLX		LQC	120	6201	6158	6173	6254	6214	6278	6213.00	42.18	0.67
		MQC	150	7388	7314	7425	7451	7462	7381	7403.50	49.82	0.67
		HQC	180	8419	8437	8391	8475	8466	8386	8429.00	34.01	0.40
LOVA		LQC	120	5279	5314	5258	5381	5297	5337	5311.00	40.02	0.75
		MQC	150	6791	6752	6834	6861	6752	6814	6800.66	40.33	0.59
		HQC	180	8234	8267	8314	8236	8275	8341	8277.83	38.93	0.47
Intermediate precision (inter analyst)												
SD	Analyst 1	LQC	120	5587	5672	5641	5631	5597	5582	5618.33	32.43	0.57
		MQC	150	7354	7355	7431	7461	7425	7411	7406.16	39.45	0.53
		HQC	180	8299	8251	8267	8301	8360	8398	8312.66	51.20	0.61
FLX		LQC	120	6328	6349	6418	6411	6378	6389	6378.83	32.00	0.50
		MQC	150	7476	7425	7494	7482	7379	7399	7442.50	43.90	0.58
		HQC	180	8542	8567	8571	8485	8537	8516	8536.33	29.50	0.34
LOVA		LQC	120	5367	5385	5284	5267	5346	5284	5322.16	45.61	0.85
		MQC	150	6851	6893	6768	6725	6814	6758	6801.50	57.51	0.84
		HQC	180	8282	8146	8192	8274	8276	8351	8253.50	66.56	0.80

(Contd...)

Table 2: ***(Continued)***

Drug	Parameter	Level	Concentration (ng/mL)	Area 1	Area 2	Area 3	Area 4	Area 5	Area 6	Mean	S.D.	RSD (%)
SD	Analyst 2	LQC	120	5675	5648	5678	5634	5672	5679	5664.33	17.13	0.30
		MQC	150	7411	7434	7468	7308	7354	7391	7394.33	52.23	0.70
		HQC	180	8345	8361	8431	8425	8461	8472	8415.83	47.49	0.56
FLX		LQC	120	6315	6389	6412	6401	6459	6399	6395.83	42.56	0.66
		MQC	150	7501	7526	7518	7566	7537	7569	7536.16	24.62	0.32
		HQC	180	8514	8502	8469	8436	8496	8479	8482.66	25.53	0.30
LOVA		LQC	120	5257	5384	5287	5646	5276	5296	5357.66	135.04	2.00
		MQC	150	6741	6843	6868	6780	6854	6819	6817.50	44.33	0.65
		HQC	180	8154	8216	8184	8252	8216	8127	8191.50	41.80	0.51
SD	Analyst 3	LQC	120	5573	5605	5647	5587	5579	5675	5611.00	37.57	0.66
		MQC	150	7237	7298	7267	7345	7348	7401	7316.00	54.85	0.74
		HQC	180	8314	8405	8437	8481	8388	8279	8384.00	69.04	0.82
FLX		LQC	120	6201	6285	6299	6324	6315	6345	6294.83	45.99	0.73
		MQC	150	7493	7486	7491	7412	7502	7469	7475.50	30.09	0.40
		HQC	180	8496	8516	8512	8485	8468	8504	8496.83	16.43	0.19
LOVA		LQC	120	5237	5316	5274	5278	5397	5367	5311.50	55.51	1.04
		MQC	150	6873	6789	6767	6854	6743	6809	6805.83	45.79	0.67
		HQC	180	8134	8250	8173	8284	8183	8179	8200.50	50.61	0.61

FLX: Fluoxetine, SD: Sildenafil, LOVA: Lovastatin

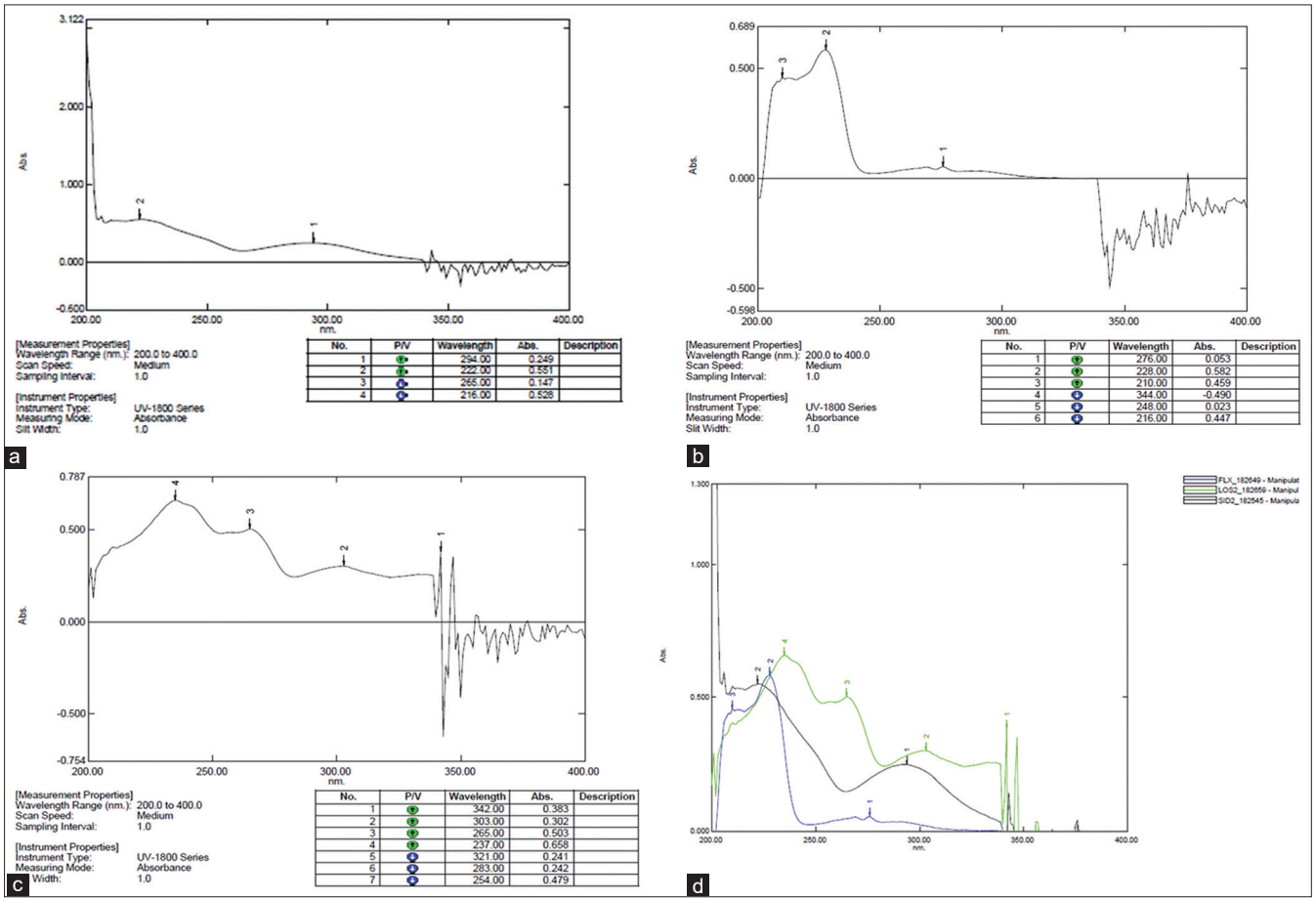

Fig. 3: Comparative UV Spectra of Compounds: (a) sildenafil, (b) fluoxetine, (c) lovastatin, and (d) The isosbestic point where spectra overlap

Linearity and range

The developed calibration curve was found to be linear in the range of 20–100 µg/mL. The value of the regression coefficient (R^2) for SD, FLX, and LOVA was found to be 0.9991, 0.9997, and 0.9994, respectively. These values were close to 1, indicating a high degree of correlation and good linearity in the procedure (Fig.5.).

LOD band LOQ

As per ICH guidelines, LOD and LOQ were calculated by the standard deviation of response and slope. The method has very low LOD and LOQ values that is, 12.77, 14.81, and 13.28 µg/mL and 38.71, 42.33, and 45.16 µg/mL, respectively, indicating that the presented method for estimation has a high sensitivity for the detection of the drug at lower concentrations.

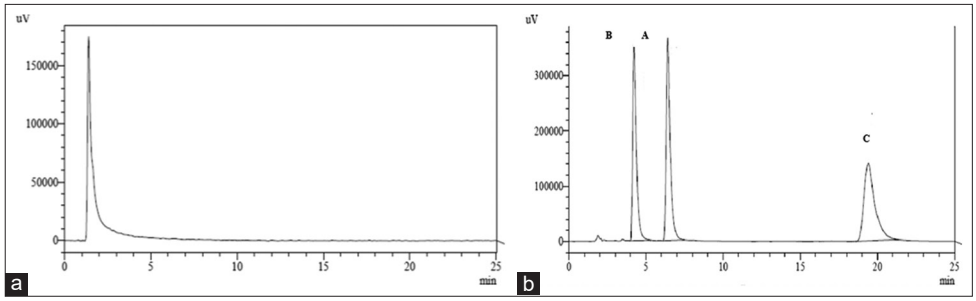

Fig. 4: (a) Chromatogram of blank of ACN and 5M Ammonium acetate, and (b) Chromatogram indication of the peaks A. Peak time for SD is 6.407 min. B. Peak time for FLX is 4.214 min. C. Peak time for LOVA is 19.408 min

Table 3: Results of the accuracy study

Drug	Level	Concentration of sample solution (ng/mL)	Total concentration of solution, actual (ng/mL)	Concentration of drug recovered (ng/mL), (n=6)	% Recovery	Mean % recovery
SD	LQC	50	120	119.93	99.94	102.01±2.811
	MQC	50	150	158.97	105.98	
	HQC	50	180	180.19	100.11	
FLX	LQC	50	120	132.22	110.18	102.92±5.59
	MQC	50	150	153.04	102.03	
	HQC	50	180	173.82	96.57	
LOVA	LQC	50	120	117.64	98.03	99.69±1.18
	MQC	50	150	150.49	100.33	
	HQC	50	180	181.29	100.72	

Table 4: Short-term stability of SFL

Drug	Hour	Actual Concentration of drug (ng/mL)	Area 1	Area 2	Area 3	Mean	S.D.	RSD (%)	Amount of drug recovered in sample (ng/mL)	Recovery (%)
SD	1 h	120 (LQC)	5588	5627	5592	5602.33	17.51	0.31	120.52	100.43
		150 (MQC)	7285	7311	7458	7351.33	76.16	1.03	158.21	105.47
		180 (HQC)	8305	8378	8483	8388.66	73.05	0.87	180.57	100.31
FLX		120 (LQC)	6345	6412	6387	6381.33	27.64	0.43	129.75	108.12
		150 (MQC)	7512	7561	7589	7554.00	31.82	0.42	152.06	101.37
		180 (HQC)	8546	8573	8534	8551.00	16.30	0.19	171.03	95.01
LOVA		120 (LQC)	5276	5356	5295	5309.00	34.12	0.64	117.79	98.16
		150 (MQC)	6858	6781	6758	6799.00	42.76	0.62	150.30	100.20
		180 (HQC)	8138	8267	8294	8233.00	68.07	0.82	181.58	100.88
SD	2 h	120 (LQC)	5631	5662	5504	5599.00	68.35	1.22	120.44	100.37
		150 (MQC)	7328	7397	7461	7395.33	54.30	0.73	159.16	106.10
		180 (HQC)	8405	8463	8478	8448.66	31.47	0.37	181.86	101.03
FLX		120 (LQC)	6296	6325	6354	6325.00	23.67	0.37	128.67	107.23
		150 (MQC)	7489	7502	7587	7526.00	43.45	0.57	151.53	101.02
		180 (HQC)	8536	8579	8531	8548.66	21.54	0.25	170.98	94.99
LOVA		120 (LQC)	5213	5326	5240	5259.66	48.18	0.91	120.44	100.37
		150 (MQC)	6782	6897	6816	6831.66	48.23	0.70	151.01	100.67
		180 (HQC)	8205	8336	8277	8272.66	53.56	0.64	182.45	101.36
SD	3 h	120 (LQC)	5574	5591	5636	5600.33	26.15	0.46	120.47	100.39
		150 (MQC)	7287	7349	7480	7372.00	80.45	1.09	158.65	105.77
		180 (HQC)	8341	8326	8493	8386.66	75.43	0.89	180.52	100.29
FLX		120 (LQC)	6344	6387	6391	6374.00	21.27	0.33	129.61	108.00
		150 (MQC)	7538	7561	7517	7538.66	17.96	0.23	151.77	101.18
		180 (HQC)	8537	8539	8568	8548.00	14.16	0.16	170.97	94.98
LOVA		120 (LQC)	5247	5391	5263	5300.33	64.44	1.21	117.60	98.00
		150 (MQC)	6778	6794	6840	6804.00	26.28	0.38	150.41	100.27
		180 (HQC)	8213	8262	8139	8204.66	50.55	0.61	180.97	100.53

Accuracy

The result showed that the mean percentage recovery for all three levels was within the standard ranges, that is, 96.57–110.18%. This indicates that the developed method was accurate under the test conditions (Table 3).

Precision

The precision of the developed techniques was determined by calculating the % RSD for the six LQC, MQC, and HQC solution determinations at the intraday, inter-day, and inter-analyst levels under the same experimental studies. The percentage relative deviation was <0.19–2.00%, indicating that the developed technique was precise under the specified test conditions (Table 2).

Specificity

This method is used to confirm the separation potential of the system. A blank solution composed of ACN and water, which is also used as the mobile phase, is injected into the HPLC. The chromatograms are

Table 5: Freeze thaw stability of SFL

Drug	Cycle	Actual Concentration of drug (ng/mL)	Area 1 (cm^2)	Area 2 (cm^2)	Area 3 (cm^2)	Mean (cm^2)	S.D.	RSD (%)	Amount of drug recovered in sample (ng/mL)	Recovery (%)
SD	1 cycle	120 LQC	5632	5582	5567	5593.66	27.78	0.49	120.33	100.27
		150MQC	7363	7452	7342	7385.66	47.68	0.64	158.95	105.97
		180HQC	8347	8435	8388	8390.00	35.95	0.42	180.59	100.33
FLX		120 LQC	6279	6256	6320	6285.00	26.47	0.42	127.91	106.59
		150MQC	7591	7528	7543	7554.00	26.87	0.35	152.06	101.37
		180HQC	8534	8502	8516	8517.33	13.09	0.15	170.39	94.66
LOVA		5223	5328	5365	5223	5305.33	60.14	1.13	117.71	98.09
		6836	6745	6837	6836	6806.00	43.13	0.63	150.45	100.30
		8147	8134	8238	8147	8173.00	46.26	0.56	180.28	100.15
SD	2 cycle	120 LQC	5526	5574	5634	5578.00	44.18	0.79	119.99	99.99
		150MQC	7298	7267	7334	7299.66	27.37	0.37	157.10	104.73
		180HQC	8287	8374	8314	8325.00	36.35	0.43	179.19	99.55
FLX		120 LQC	6325	6341	6372	6346.00	19.51	0.30	129.07	107.56
		150MQC	7401	7468	7491	7453.33	38.17	0.51	152.06	101.37
		180HQC	8533	8574	8599	8568.66	27.20	0.31	171.36	95.20
LOVA		120 LQC	5156	5247	5253	5218.66	44.37	0.85	115.82	96.51
		150MQC	6889	6724	6743	6785.33	73.71	1.08	150.00	100.00
		180HQC	8175	8243	8241	8219.66	31.59	0.38	181.29	100.72
SD	3 cycle	120 LQC	5574	5531	5597	5567.33	27.35	0.49	119.76	99.80
		150MQC	7282	7264	7422	7322.66	70.62	0.96	157.59	105.06
		180HQC	8341	8401	8473	8405.00	53.96	0.64	180.92	100.51
FLX		120 LQC	6215	6345	6371	6310.33	68.24	1.08	128.40	107.00
		150MQC	7525	7585	7499	7536.33	36.01	0.47	151.72	101.15
		180HQC	8463	8515	8561	8513.00	40.03	0.47	170.30	94.61
LOVA		120 LQC	5247	5135	5179	5187.00	46.07	0.88	115.13	95.94
		150MQC	6828	6746	6824	6799.33	37.74	0.55	150.00	100.20
		180HQC	8137	8268	8279	8228.00	64.50	0.78	181.48	100.82

Table 6: Long-term stability of SFL

Drug	Week	Actual Concentration of drug (ng/mL)	Area 1 (cm^2)	Area 2 (cm^2)	Area 3 (cm^2)	Mean (cm^2)	S.D.	RSD (%)	Amount of drug recovered in sample (ng/mL)	Recovery (%)
SD	1 week	120	5634	5582	5601	5605.66	21.48	0.38	120.59	100.40
		150	7345	7364	7403	7370.66	24.14	0.32	158.63	105.75
		180	8234	8291	8354	8293.00	49.01	0.59	178.50	99.17
FLX		120	6482	6475	6472	6476.33	4.189	0.06	131.55	109.63
		150	7534	7564	7531	7543.00	14.89	0.19	151.85	101.23
		180	8534	8539	8568	8547.00	14.98	0.17	170.95	94.97
LOVA		120	5246	5359	5402	5335.66	65.78	1.23	118.37	98.64
		150	6785	6814	6713	6770.66	42.46	0.62	149.68	99.78
		180	8191	8254	8276	8240.33	36.02	0.43	181.74	100.97
SD	2 week	120	5534	5675	5692	5633.66	70.81	1.25	121.19	100.97
		150	7369	7483	7477	7443.00	52.38	0.70	160.18	106.79
		180	8311	8394	8474	8393.00	66.54	0.79	180.66	100.36
FLX		120	6474	6576	6521	6523.66	41.68	0.63	132.53	110.44
		150	7577	7596	7534	7569.00	25.93	0.34	152.34	101.56
		180	8531	8586	8657	8591.33	51.57	0.60	171.79	95.44
LOVA		120	5287	5243	5291	5273.66	21.74	0.41	117.02	97.51
		150	6805	6847	6769	6807.00	31.87	0.46	150.47	100.31
		180	8240	8197	8254	8230.33	24.25	0.29	181.53	100.85
SD	3 week	120	5594	5576	5673	5614.33	42.12	0.75	120.77	100.64
		150	7346	7281	7437	7354.66	63.98	0.86	158.28	105.52
		180	8279	8395	8437	8370.33	66.81	0.79	180.17	100.09
FLX		120	6551	6580	6572	6567.66	12.22	0.18	133.29	111.08
		150	7438	7482	7439	7453.00	20.51	0.27	150.14	100.09
		180	8579	8496	8512	8529.00	35.95	0.42	170.61	94.78
LOVA		120	5249	5194	5321	5254.67	52.00	0.98	116.60	97.17
		150	6795	6837	6875	6835.67	32.67	0.47	151.10	100.73
		180	8167	8238	8392	8265.67	93.91	1.13	182.30	101.27

examined for any interference at the retention times of the analytes. The absence of peaks in the ACN and water chromatograms indicates that the drugs are well-retained on the column, demonstrating the method's effectiveness.

Stability study of samples

For the LQC, MQC, and HQC samples, the stability study was conducted for spiking samples of SFL at three different levels, including short-term (Table 4), freeze-thaw cycles (Table 5, and long-term (Table 6). In all

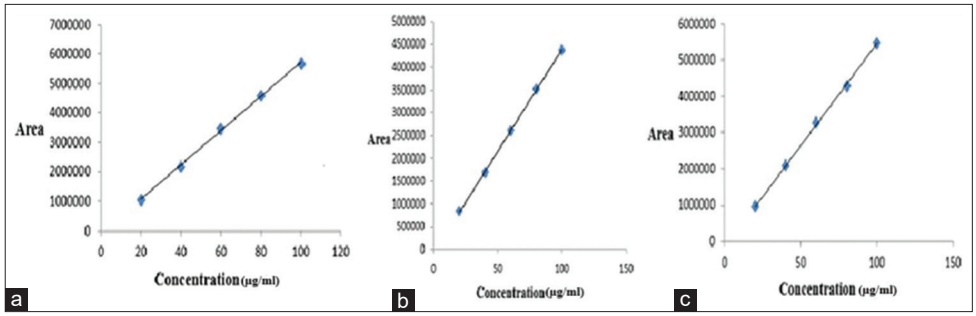

Fig. 5: Calibration curve (a) sildenafil; y=58310x−100654 and R^2=0.9991 (b) fluoxetine; y=44699x−85310 and R^2=0.9997 and (c) lovastatin; y=56213x−160335 and R^2=0.9994

cases, the results indicated that more than 95.99–105.77%, 94.61–101.56%, and 94.78–110.44% of the drug was recovered, with an RSD of <0.19–1.21%, 0.15–1.08%, and 0.06–1.25%, respectively.

CONCLUSION

Validation of an HPLC method for pharmaceutical applications is essential to confirm accuracy, precision, specificity, linearity, and stability, ensuring reliability in detecting and quantifying drug substances. This process follows regulatory guidelines, assessing factors, such as accuracy (through recovery studies), precision (repeatability and reproducibility under varying conditions), and specificity (analyte separation from impurities). Linearity establishes a proportional relationship between analyte concentration and response, while limits of detection (LOD) and quantitation (LOQ) define sensitivity. Stability studies confirm the analyte's integrity during handling. Comprehensive validation ensures the method's suitability for regulatory compliance, safeguarding product quality, and patient safety. We concluded the research by creating an Analytical method that is sensitive, accurate, precise, cost-effective, and simple to use for quantifying SFL. The recovery of SFL from samples was exceptional, with a range of 96.57–110.18%. System suitability studies verified the method's repeatability, and we determined that the percentage RSD of samples with varying concentrations used for intraday and intermediate precision studies was <2%. This method is significantly more effective than others in detecting SFL in any sample. In addition, it enhances the linearity, range, LOQ, and LOD values of samples as well as the recovery of drugs. The method that has been devised can be employed to investigate the pharmacokinetics and biodistribution of the drug in SFL or bulk form in a variety of pharmaceutical formulations.

Contribution of the paper

The primary contribution of this paper lies in the development and validation of a simple, reliable, and accurate RP-HPLC method for the simultaneous estimation of SD, FLX, and LOVA. These drugs, which target NP through different mechanisms, are assessed together in this novel analytical approach. This method addresses a significant gap by providing an effective analytical tool for the combined quantification of these agents, thereby supporting potential combination therapy development for NP management.

Clinical practice

NP remains a challenging condition to manage due to its complex etiology. Pharmacological management often requires the use of multiple drug classes, including selective serotonin reuptake inhibitors, PDE5 inhibitors, and statins. The developed RP-HPLC method facilitates precise and simultaneous estimation of SD, FLX, and LOVA providing a potential analytical foundation for combination formulations in clinical practice. Accurate quantification of these drugs ensures proper dosing and efficacy, ultimately contributing to better therapeutic outcomes for patients suffering from NP.

Research

The validated RP-HPLC method opens new avenues for further pharmaceutical and clinical research. Future studies may focus on formulating fixed-dose combinations of SD, FLX, and LOVA to evaluate their synergistic effects in NP treatment. n addition, pharmacokinetic and pharmacodynamic studies could be conducted using this validated method to understand drug interactions, bioavailability, and therapeutic efficacy. Research can also extend to applying this method for stability studies, routine quality control, and bioanalytical sample analysis in clinical trials.

Recommendations

- Pharmaceutical industries and quality control laboratories may adopt the validated RP-HPLC method for routine analysis of SD, FLX, and LOVA in combination formulations.
- Further preclinical and clinical studies are recommended to evaluate the combined therapeutic effect of these drugs in managing NP.
- Workshops and training sessions on analytical method validation techniques can be organized for researchers and analysts to enhance technical skills and regulatory compliance.
- Collaboration between pharmaceutical scientists and clinicians is encouraged to translate this analytical advancement into effective clinical applications.

CONCLUSION

The present study successfully developed and validated an RP-HPLC method for the simultaneous estimation of SD, FLX, and LOVA adhering to ICH Q2 (R2) guidelines. The method demonstrated excellent linearity, accuracy, and precision, with well-defined LOD and LOQ values for each drug. These results confirm the method's reliability and suitability for routine pharmaceutical analysis. Furthermore, this analytical advancement supports the future exploration of combination therapies, contributing to improved management strategies for NP.

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AUTHOR'S CONTRIBUTIONS

Indu mekani: Literature review, Methodology, Data curation, Writing-original draft, and Evaluation; Bimlesh Kumar: Idea, Writing original draft, Review and editing, Supervision, Evaluation, and Visualization; Shashi: Calculation and Writing original draft. Narendra Kumar Pandey: Data curation and Correction of manuscript; All authors have read and approved the final version of the manuscript and agree to be accountable for all aspects of the research.

DECLARATION OF CONFLICTS OF INTEREST

The authors express no conflicts of interest.

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