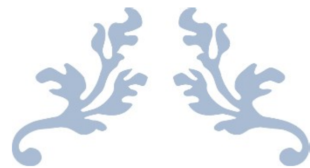

# Chapter 3

## Analytical and Bioanalytical Method Development and Validation

## 1. Introduction

Analytical method development constitutes one of the fundamental studies to be carried out at preformulation stage to quantify the drug accurately and specifically in both *in vitro* as well as *in vivo* samples. It is important to analyze the drug in formulation, in *in vitro* release study samples and to monitor plasma concentrations of the drug and in our case along with the molecule of interest, LSF, parent drug PTX (LSF and PTX undergo interconversion; **Chapter 2, Section 4**) was also required to be analyzed. Since the convenience and versatility of high-performance liquid chromatography (HPLC) has led to its acceptance as one of the most useful techniques available for the analysis of drugs in biological fluids, it was our obvious choice to develop analytical and bioanalytical method using HPLC with photodiode array detector (PDA) for the simultaneous determination of LSF and PTX in analytical samples and rat plasma. Our objective was to develop a simple, sensitive and reproducible analytical method that could be readily applied to quantification of pharmacokinetics (PK) study samples also. However, the methods reported in literature, although few, are based on either a complex sample preparation procedure or are based on use of sophisticated instruments like LC-MS/MS which hinders its application for routine analysis (**Table 3.1**). Chivers, D. A. *et al.* developed a HPLC-UV method for simultaneous quantification of LSF and PTX in plasma at a high flow rate (2 mL/min) using a sample volume of 1000 μL which also required more volume of extracting solvent (DCM, 10 mL).[1] Another reported HPLC-UV method in human plasma uses a complex and time consuming solid phase extraction (SPE) process requiring a large sample volume of 1000 μL.[2] Grasela, D. M. *et al.* developed a HPLC-UV method in whole blood based on a complex liquid-liquid extraction (LLE) process with multiple steps and very high injection volume (250 μL).[3] A LC-MS/MS method has also been reported which can quantify both LSF and PTX up to 1 ng/mL but it bears some major limitations such as a highly specific sample preparation method (lithium

precipitation using Seraprep™ reagent) and use of LC-MS/MS.[4] Nonetheless, use of LC-MS/MS for quantification of drugs is a recommended procedure and significantly enhances the sensitivity of the method; it is not available in every research laboratory, requires highly trained personnel and is associated with a high running and maintenance cost. Few HPLC-UV chiral chromatographic methods have also been studied for simultaneous quantification LSF and PTX in serum and tissues of mice and rats. In these studies, 200-500 μL of serum samples were used for analysis which increases the number of animals required for such studies. LSF has been administered at a high dose in most of the studies ranging from 25-50 mg/kg in animals [5,6] and 1-3 mg/kg in humans.[7] This high dose attributed to its extensive first pass metabolism and an extremely short half-life.[8,9] Thus, the need of detecting a concentration below 50 ng/mL may not arise for routine analysis and hence HPLC based methods might also be equally useful.

We developed HPLC based analytical and bioanalytical method for quantification of LSF and PTX in *in-vitro* study samples and rat plasma samples respectively within the range of 50-5000 ng/mL. The proposed bioanalytical method uses a simple LLE method using methylene chloride (2 mL) as an extracting solvent and a small sample volume (200 μL). 3-isobutyl 1-methyl xanthine (IBMX) was selected as internal standard (I.S). Method validation was carried out for selectivity, lower limit of quantification (LLOQ), limit of detection (LOD), precision, accuracy, carry over effect, dilution integrity and stability, using internationally accepted guidelines for bioanalytical method validation.[10,11] Stability studies were also performed to determine the stability of stock solutions and of plasma samples

**Table 3.1**
Methods for simultaneous quantification of LSF and PTX in different biological matrices available in literature.

| Author | System | Biological matrix (Source) | Column used | Mobile phase used | Linearity range (ng/mL) | Flow rate (mL/min) | Sample volume (µL) | Injection Volume (µL) | Extraction Procedure | Validation |
|---|---|---|---|---|---|---|---|---|---|---|
| Chivers, D. A. *et al.* [1] | HPLC-UV | Plasma | RP µBondPak C18 (300 × 3.9 mm, 10 µ) | Methanol - Phosphate buffer, 10mM, pH-7 (38:62 %v/v) | 50-1000 | 2.0 | 1000 | 100 | Liquid-liquid extraction (LLE) with DCM (10 mL) | †NR |
| Rieck, W. *et al.* [2] | HPLC-UV | Plasma (Human) | LiChrosorb RP C18 (250 × 4.9 mm, 10 µ) | Methanol –water (48:52 %v/v) | 150-6000 | 2.0 | 1000 | 20 | Solid phase extraction (SPE) | NR |
| Grasela, D. M. *et al.* [3] | HPLC-UV | Whole blood | Supelco, LC-8-DB RP (250 × 4.6 mm, 5 µ) | Acetonitrile-water (24:76 % v/v) | - | 1.0 | 250 | 250 | LLE with acetonitrile, Hexane (multiple steps) | NR |
| Kyle, P.B. *et al.* [4] | LC-MS/MS | Plasma (Human, Rabbit) | Phenomenex Luna C8(2), (150 × 1.0 mm, 5 µ) | Methanol-Ammonium acetate buffer, 0.5mM, pH-3.5 (50:50 % v/v) | 1-1000 | 0.05 | 500 | 15 | Lithium precipitation using Seraprep reagent | #R |
| Wyska, E. *et al.* [6] | HPLC-UV-Vis | Serum Tissues (Mice) | ChiralPak AD, (250 × 4.6 mm, 10 µ) | Hexane-2-propanol (78:22 % v/v) with 0.01 % diethyl amine | - | 1 | 200 (serum) 1000 (tissue homogenate) | 50 | LLE with methylene chloride | NR |
| Walczak, M. *et al.* [12] | HPLC-UV | Serum, Tissues (Rat) | ChiralPak AD, (250 × 4.6 mm, 5 µ) | Hexane-2-propanol (84:16 % v/v) with 0.01 % diethyl amine | - | 1.5 | 500 (serum) 200 mg(tissue) | 50 | LLE with methylene chloride : chloroform (1:1 % v/v) | R |

#R, Reported; †NR, Not reported

exposed to different storage conditions including repeated freeze-thaw cycles, autosampler, long term, and bench top storage. The developed method was applied to analysis of the PK study samples of LSF and PTX (25 mg/kg, i.v.) in Wistar rats.

## 2. Material and Methods

### *2.1. Chemicals, reagents and experimental animals*

LSF (purity ≥98%, HPLC) was synthesized *in house* as mentioned in *Chapter 2*. For reference standard, (±) LSF (purity ≥99%, HPLC) was purchased from Cayman Chemicals Inc. (Michigan, USA). PTX and IBMX (purity ≥99%, HPLC) were obtained from Sigma Aldrich (St. Louis, MO, USA). HPLC grade solvents, acetonitrile (ACN), methanol and methylene chloride were obtained from Merck Limited (Mumbai, India). Purified water used in our studies refers to the Mili-Q ultrapure water (Type 1, as described by ASTM®, ISO® 3696 and CLSI® norms) prepared using Milli-Q® Reference water purification system. Wistar rats (male; 8–10 weeks, 200–220 g) were procured from Central Animal Facility, BITS-PILANI (Pilani, India). Animal experiment protocol (IAEC/RES/19/07/Rev-1/21/8) was approved by Institutional Animal Ethics Committee (IAEC), BITS-PILANI, Pilani and experiments were conducted as per CPCSEA guidelines. Rats were housed in well ventilated cages at standard laboratory conditions with regular light/dark cycles for 12 h and fed with standard normal diet *ab libitum*. All other chemicals and reagents were of analytical grade and used as obtained.

### *2.2. Analytical method development and validation of LSF using HPLC*

#### *2.2.1. Analytical method development*

Method development was started with the optimization of chromatographic conditions including mobile phase composition and chromatographic column to provide an optimum balance between sensitivity, accuracy and reproducibility. A Shimadzu HPLC system (Kyoto,

Japan) equipped with a binary pump (LC-20AD), PDA detector (SPD-M20A) and auto sampler (SIL-HTC, Shimadzu, Japan) were used to develop the analytical method. The HPLC system was equilibrated for approximately 40 min before beginning the sample analysis. Eluents were monitored at a wavelength of 273 nm. Control of hardware and data handling was performed using LCsolution software version 1.22 SP1. (**Table 3.2**)

***Preparation of stock solutions, calibration curve standards and quality control (QC) samples***

Mobile phase was prepared in ratio of 65:35 %v/v. Milli Q water was filtered through 0.22 μm cellulose membrane under reduced pressure. HPLC grade methanol was used as received. Mobile phase was bath sonicated for 30 min before use. The stock solution of LSF (1 mg/mL) was prepared by dissolving 3 mg of drug in 3 mL of HPLC grade water (Mili-Q). From this stock solution, the working standards (WS) in the range of 0.5 or 0.1 (look into the CC sample range)-250 μg/mL were prepared by appropriately diluting the stock solution with mobile phase from high to low concentration. Calibration curve standards were prepared in the range of 0.1-50 μg/mL (0.1, 0.2, 0.5, 1, 2, 5, 10, 20 and 50 μg/mL) in mobile phase. The QC samples were prepared using a working standard solution at three concentration levels, low QC (LQC, 0.4 μg/mL), medium QC (MQC, 4 μg/mL) and high QC (HQC, 40 μg/mL). All the stock and working solutions were stored in the refrigerator at -20°C until used for analysis.

### ***2.2.2. Analytical method validation of LSF using HPLC***

The proposed method was validated as per ICH guidelines for analytical method validation.

***Specificity***

Specificity of analytical method was evaluated in the presence of formulation excipients. Specificity analysis was carried out by spiking the QC samples with expected excipients including linoleic acid, polymer, and tween 80. To establish the selectivity of

analytical method, excipients solution was prepared in mobile phase followed by sonication for 15 min. These solutions were used to spike QC samples of LSF followed by analysis using developed HPLC method. Any interference of formulation excipients at retention time of analytes was observed.

***Linearity and calibration curve***

Five calibration curves were prepared on five consecutive days and linearity of the analytical method was evaluated by least square linear regression analysis. Calibration curves were plotted with peak area of analyte against the analyte concentration.

***LOD) and LOQ***

LOD and LOQ were determined by signal to noise ratio method. LOD was defined as minimum concentration at which signal to noise ratio is greater than 3 whereas, LOQ was defined as minimum concentration at which signal to noise ratio is greater than 10. The LOD and LOQ were determined by following formula. (**Equation 3.1 and 3.2** respectively)

$$LOD = \frac{3.3\sigma}{S}$$ **Equation 3.1**

$$LOQ = \frac{10\sigma}{S}$$ **Equation 3.2**

Where, S is the slope of calibration curve of analyte and σ is standard deviation of response.

LOD was determined by preparing dilutions of known amount of the analytes at concentration lower than LOQ and LOD was selected as the minimum level at which the analytes could be reliably detected. LOQ concentration for both analytes were analysed in five replicates (n=5).

***Precision and accuracy***

The intra-day and inter-day assay precision and accuracy were determined by analyzing five replicates at three different QC levels (LQC, MQC, HQC). For intra-day assay

precision and accuracy, samples were analyzed on same day, while inter-day assay precision and accuracy were determined by analyzing samples on three consecutive days. Accuracy is expressed as percentage deviation (% bias) and precision is expressed as percentage coefficient of variation (% CV).

***System suitability***

System suitability checks are appropriately used for chromatographic methods to ensure that the system is sufficiently specific and reproducible for the current analytical run. It was performed by injecting six consecutive injections of the system suitability test sample (an aqueous solution of the LSF at 10 µg/mL) in the run.

## *2.3. Bioanalytical method development of LSF in rat plasma using HPLC*

### *2.3.1. Bioanalytical method development*

Due to *in-vivo* interconversion of LSF to PTX, it is necessary to simultaneous analyze PTX and LSF. Simultaneous determination of LSF and PTX in rat plasma was started with the optimization of chromatographic conditions including mobile phase composition and chromatographic column to provide an optimum balance between sensitivity, accuracy and reproducibility. Optimized chromatographic conditions are shown in **Table 3.2**. LSF, PTX and IBMX (IS) were resolved on Inertsil® ODS (C18) column (250 × 4.6 mm, 5µm) with a mobile phase consisting of methanol and water (50:50 v/v) run in isocratic mode at a flow rate of 1 mL/min and injection volume of 80 µL. Control of hardware and data handling was performed using LCsolution software version 1.22 SP1.

***Preparation of stock solutions, calibration curve standards and quality control (QC) samples***

Stock solutions of LSF (1 mg/mL) and PTX (1 mg/mL) were prepared by dissolving accurately weighed amount of each of these analytes in Milli-Q water. The stock solution of IBMX (2 µg/mL) was prepared in ACN. Further, same volumes of LSF and PTX stock

solutions (1 mg/mL) were combined in 1:1 ratio and diluted with water to obtain 200 µg/mL working solution; from this, dilutions of 1, 2, 5, 10, 20, 50 and 100 µg/mL were prepared by stepwise dilution using mobile phase. CS and QC samples were prepared by spiking 190 µL of drug free rat plasma with 10 µL of corresponding working standard solutions. The working QC standard solutions were prepared at concentrations of 1.6, 6 and 60 µg/mL. The final CS were prepared at 50, 100, 250, 500, 1000, 2500 and 5000 ng/mL in plasma matrix. The QC samples were prepared using a working standard solution at three concentration levels, LQC (80 ng/mL), MQC (300 ng/mL) and HQC (3000 ng/mL). All the stock and working solutions were stored in the refrigerator at -20°C until used for analysis (the stock solutions were tested for stability under these storage conditions and were found to be stable, as detailed in section **3.3**).

***Sample preparation***

A LLE method was used for extracting both LSF and PTX from the rat plasma (**Figure 3.1).** A 200 µL aliquot of plasma sample containing LSF and PTX was transferred in 5 mL glass tube, followed by the addition of 50 µL of I.S (IBMX, 2 µg/mL) solution. Samples were vortexed for 1 min and then 2 mL of DCM was added as extracting solvent. The samples were vortexed for 5 min and centrifuged at 3500 rpm for 15 min at 4°C. The lower organic layer was collected and evaporated to dryness at 40 ± 0.5 °C under a stream of nitrogen gas. The residue was reconstituted with 100 µL of mobile phase and vortexed for 30 s. Finally, 80 µL of sample was injected into HPLC for quantification.

### *2.3.2. Bioanalytical method validation in rat plasma using HPLC*

The proposed method was validated as per internationally accepted guidelines for bioanalytical method validation.[10,11] Weighted linear regression analysis was performed on the calibration data. All the different weighting factors were applied to the data obtained from

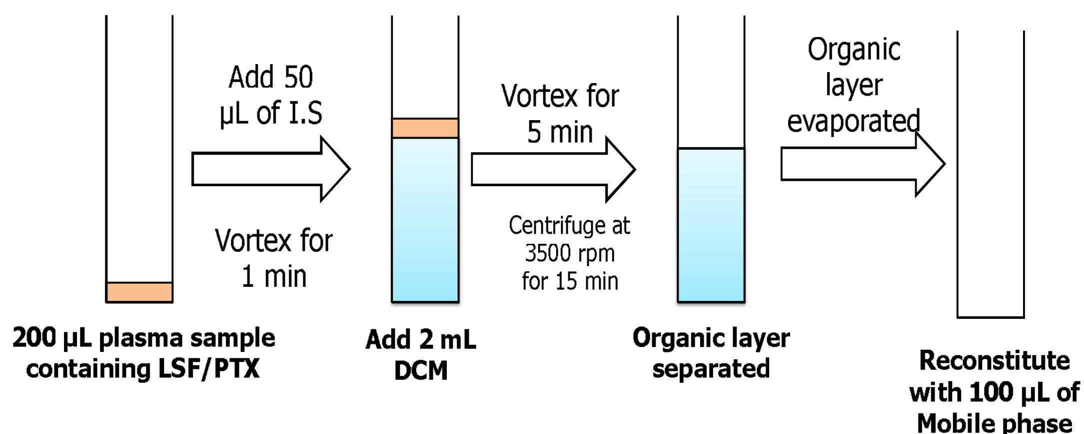


**Figure 3.1.** Sample preparation using LLE for bio analysis of LSF and PTX in rat plasma. (I.S is IBMX; 2 µg/mL)

least-squares regression analysis and the best weighting factors were chosen according the percentage relative error (% Σ RE).[13,14]

***Selectivity***

The selectivity of the method was determined to evaluate potential chromatographic interference from the rat plasma matrix. For this purpose, plasma samples were collected from six different randomly selected rats and analyzed as per the described chromatographic conditions (**Table 3.2**).

***Linearity and calibration curve***

The calibration curves of two analytes (LSF and PTX) were prepared using seven calibration standards in a range of 50-5000 ng/mL. Five calibration curves were prepared by plotting peak area ratios of drug/IS on Y axis versus nominal plasma concentrations of LSF on X-axis.

***LOD and LLOQ***

LOD and LLOQ were determined by signal to noise (S/N) ratio method. The LOD and LLOQ obtained by S/N method were further confirmed by visual evaluation method

wherein, for LLOQ, analyte samples (n=6) at the concentration obtained from S/N ratio method and lower, were run in HPLC and LLOQ was determined as the concentration of the analyte which showed acceptable accuracy and precision (±20 %). Further, for LOD, both the analytes were analyzed at concentrations lower than LLOQ and the minimum level at which the analytes could be reliably detected was considered as LOD.

***Precision and accuracy***

The intra-day and inter-day assay precision and accuracy were determined by analyzing five replicates at three different QC levels (LQC, MQC, HQC) and LLOQ. For intra-day assay, precision and accuracy samples were analyzed on same day, while inter-day assay precision and accuracy were determined by analyzing samples on three consecutive days. The acceptance criteria for accuracy is recommended as within ±15% (expressed as percentage of deviation from nominal concentration, % bias) and for precision it should be within ±15% (expressed as percentage deviation, % CV) except for LLOQ, where it should not exceed ±20% for both accuracy and precision.[10]

***Recovery***

The percentage recoveries of LSF and PTX after LLE were determined by comparing the detector response obtained from known amount of analytes (at QC sample concentrations) added and extracted from plasma with that obtained from actual concentration of analytes in the mobile phase. The percentage recovery of I.S was also calculated at a single concentration of 400 ng/mL (n=6).

***Carry over effect and dilution integrity***

Carry over effect was determined by injecting upper LOQ calibration standard sample (ULOQ, 5000 ng/mL) followed by a blank sample. Dilution integrity was performed to assess the ability of the method to accurately quantify concentrations above 5000 ng/mL (which might be encountered in routine analysis). Dilution integrity was determined by 10 folds

dilution of plasma (n=6) containing 25000 ng/mL of LSF and PTX with blank (drug free) rat plasma to obtain 2500 ng/mL concentration (within calibration range) and accuracy value was calculated.

### *2.4. Stability studies*

Stability of analytes in both aqueous solution and in plasma matrix was evaluated before and after subjecting them to different conditions that could be encountered during regular analysis. Stock solution stability was assessed at -20 ± 0.5°C for 3 months.

Stability of analytes in plasma was evaluated in terms of freeze–thaw stability, bench top stability, long-term stability and autosampler stability. All the stability studies were conducted in three replicates (n=3) at each of the concentration of QC levels (LQC, MQC and HQC). **Freeze thaw stability** was performed after freezing (–80 ± 10°C) and thawing QC samples for three consecutive cycles. **Bench top stability** was analyzed at room temperature (RT) for 24 h and **long term stability** was evaluated after storing the samples at −80 ± 10°C for 45 days. Replicate injections of extracted plasma samples were analyzed after 48 h to estimate **auto sampler stability** at 4 ± 0.5°C.

All QC samples were extracted and quantified against fresh calibration curves. The acceptance criteria for accuracy and precision of all stability samples recommends that it should be within ±15%.

### *2.5. Analysis of PK study samples of LSF and PTX by developed bioanalytical method*

The intravenous (i.v.) PK study of LSF and PTX individually were performed on Wistar rats (200-220 g). LSF and PTX were separately dissolved in 0.9 %w/v saline (25 mg/mL) and administered intravenously at the dose of 25 mg/kg with maximum dosing volume of 250 µL to each rat without fasting (n=4). After i.v dosing, blood samples were collected in micro centrifuge tubes for each preset time point at 5, 15, 30, 45, 60, 120, 180, 240, 360, 480 min.

For blood sampling, rats were mildly anaesthetized and blood samples were collected from retro-orbital plexus into heparinized micro centrifuge tubes at pre-determined time intervals. Blood samples were centrifuged at 5000 rpm for 10 min to obtain plasma which was subsequently stored at -80 ± 10 °C until analyzed as per the developed method. LSF and PTX plasma concentration-time profiles were plotted separately and analyzed by non-compartmental model approach using Phoenix 2.1 WinNonlin (Pharsight corporation, USA) to determine $t_{1/2}$, elimination half-life; $C_0$, drug concentration in plasma at t=0; $AUC_{0-t}$, area under curve from zero to the last time point; $AUC_{0-\infty}$, area under curve from zero to infinity; MRT, mean residence time; CL, clearance and $V_z$, apparent volume of distribution.

## 3. Results and discussion

### *3.1. Analytical method development and validation of LSF using HPLC*

HPLC based analytical method for quantification of LSF was successfully developed (**Table 3.2**) wherein, LSF showed a retention time (Rt) of 4.6 min as shown in **Figure 3.2 A**. Different mobile phases viz. acetonitrile, methanol, water and buffers (sodium acetate and sodium formate pH 3.5, 5.0) were screened for analysis of LSF; among these methanol and water at 65:35 %v/v with 1.0 mL/min flow rate exhibited the best selectivity and highest sensitivity (LOQ-100 ng/mL) (**Figure 3.2B**).

### *3.2. Analytical method validation of LSF using HPLC*

#### *3.2.1. Specificity*

The method was found to be specific for LSF (in presence of formulation excipients) with a peak purity index of 0.99998 indicating a pure peak (**Figure 3.2C and D**).

#### *3.2.2. Linearity, calibration curve*

The least square linear regression analysis demonstrated that the analytical method exhibited good linearity with $R^2$ value of 0.9999 (**Figure 3.2B**).

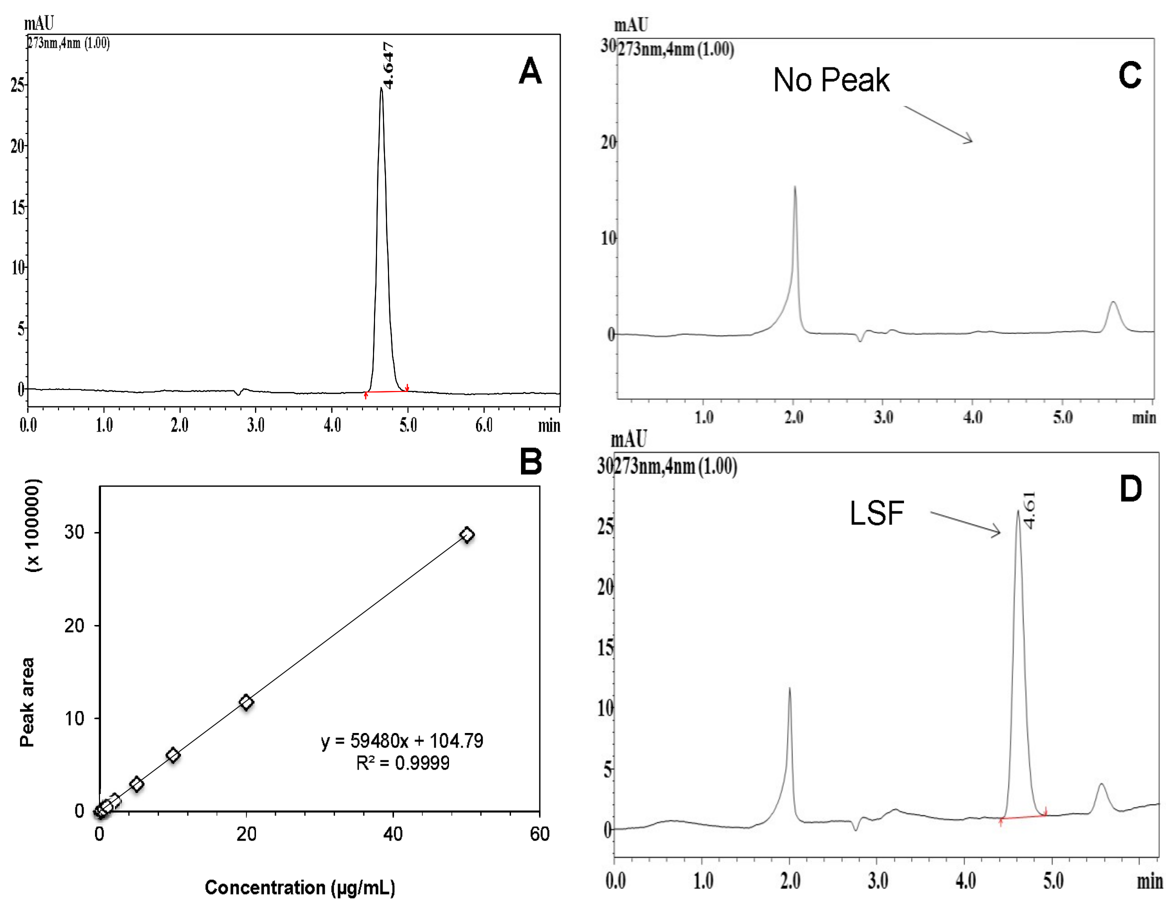


**Figure 3.2.** (A) Representative chromatogram of LSF using the optimized analytical method, (B) calibration curve for LSF in mobile phase, (C) and (D) representative chromatograms for specificity samples and LSF (4 µg/mL) respectively

### *3.2.3. Precision and accuracy*

Intra and inter day precision and accuracy for LSF were assessed at low, medium and high concentrations of the analyte as shown in **Table 3.3**. The values were found to be in acceptable limits (±5%).

### *3.2.4. LOD and LOQ*

The LOD and LOQ were determined by S/N ratio method and were found to be 45 and 100 ng/mL, respectively.

**Table 3.2**
Liquid chromatographic conditions for analytical and bioanalytical method of LSF

| | Analytical Method | Bioanalytical Method |
|---|---|---|
| **HPLC** | Shimadzu, Japan (Binary pump) | Shimadzu, Japan (Binary pump) |
| **Column (SP)** | Intersil® ODS(C18), 250× 4.6 mm, 5µ | Intersil® ODS(C18), 250× 4.6 mm, 5µ |
| **Mobile Phase** | Methanol : Water (65:35 % v/v) | Methanol : Water (50:50 % v/v) |
| **Retention time (RT)** | 4.6 min for LSF | 6.5, 7.67 and 9.97 min for PTX, LSF and IBMX (IS) respectively |
| **Run time** | 7 min | 15 min |
| **Flow rate** | 1 mL/min | 1 mL/min |
| **LSF λmax** | 273 nm | 273 nm |
| **Column temperature** | RT | RT |
| **Injection volume** | 20 µL | 80 µL |
| **Mode** | Reverse Phase | Reverse Phase |
| **Detector** | PDA | PDA |

**Table 3.3**
Precision (% CV) and accuracy (% bias) of the analytical method of LSF (n=9).

| QC | Conc. (µg/mL) | Precision (% CV) (n=9) | | Accuracy (% bias) (Mean±SD) | |
|---|---|---|---|---|---|
| | | Intra-day | Inter-day | Intra-day | Inter-day |
| **LQC** | 0.4 | 0.80 | 0.87 | 95.27±0.77 | 95.72±0.89 |
| **MQC** | 4 | 0.81 | 0.72 | 98.28±0.80 | 99.09.±0.71 |
| **HQC** | 40 | 1.92 | 1.51 | 99.64±1.92 | 101.09±1.59 |

## *3.3. Bioanalytical method development of LSF in rat plasma using HPLC*

### *3.3.1. Optimization of liquid chromatographic conditions*

A systematic approach was followed for the method development so as to obtain a method with suitable chromatographic conditions, along with simple and quick sample preparation technique while still ensuring an appropriate recovery, symmetry of peaks and high resolution of analytes and I.S. Initially liquid chromatographic conditions such as choice of mobile phase and its composition, column selection, flow rate and injection volume were optimized. Different system suitability parameters including retention time ($t_R$), peak tailing

(10%), resolution ($R_s$), height equivalent to theoretical plate (HETP), theoretical plate number (N) were assessed (**Table 3.4**). Best resolution was obtained using Inertsil® ODS column (250×4.6 mm, 5µm) using mobile phase comprising of methanol and water (50:50 v/v) in isocratic mode and 1 mL/min flow rate for the quantification of LSF and PTX; these conditions provided best peak shape and maximum peak intensities along with appropriate selectivity and speed of analysis. Using the optimized chromatographic conditions LSF, PTX and IBMX showed retention time of 6.50, 7.67 and 9.97 min respectively with an overall run time of 15 min (**Figure 3.3**).

### *3.3.2. Optimization of sample preparation procedure*

The plasma sample clean-up procedure dictates the sensitivity and selectivity of the method. Higher recoveries of analytes from plasma matrix can be obtained by minimizing sample preparation steps as well as appropriate selection of extraction solvent. The sample preparation technique was optimized for choice of extraction solvent, sample volume and time required (**Table 3.5**). We screened different solvents including methylene chloride, chloroform, methanol, ACN, chloroform- methylene chloride (1:1 v/v), methylene chloride - methanol (1:0.1 v/v), methylene chloride with 0.1 M hydrochloric acid; among these different solvents, methylene chloride was selected as the extraction solvent of choice because it provided good recovery of both the analytes, has a low boiling point (~40 °C) and could be easily evaporated after extraction using evaporator or simply by air drying. The extraction procedure required only a small volume of plasma for analysis (200 µL) and 2 mL of DCM which provided recovery ≥80 % for both LSF and PTX from plasma. The optimized extraction method exhibited good resolution factor for peaks of both the analytes and I.S ($R_s$ = 3.55 ± 0.07 for LSF and PTX peaks), appropriate retention times (6.5, 7.67 and 9.97 min for PTX, LSF and IBMX respectively) and no interference of plasma matrix (peak purity in all cases > 0.9999).

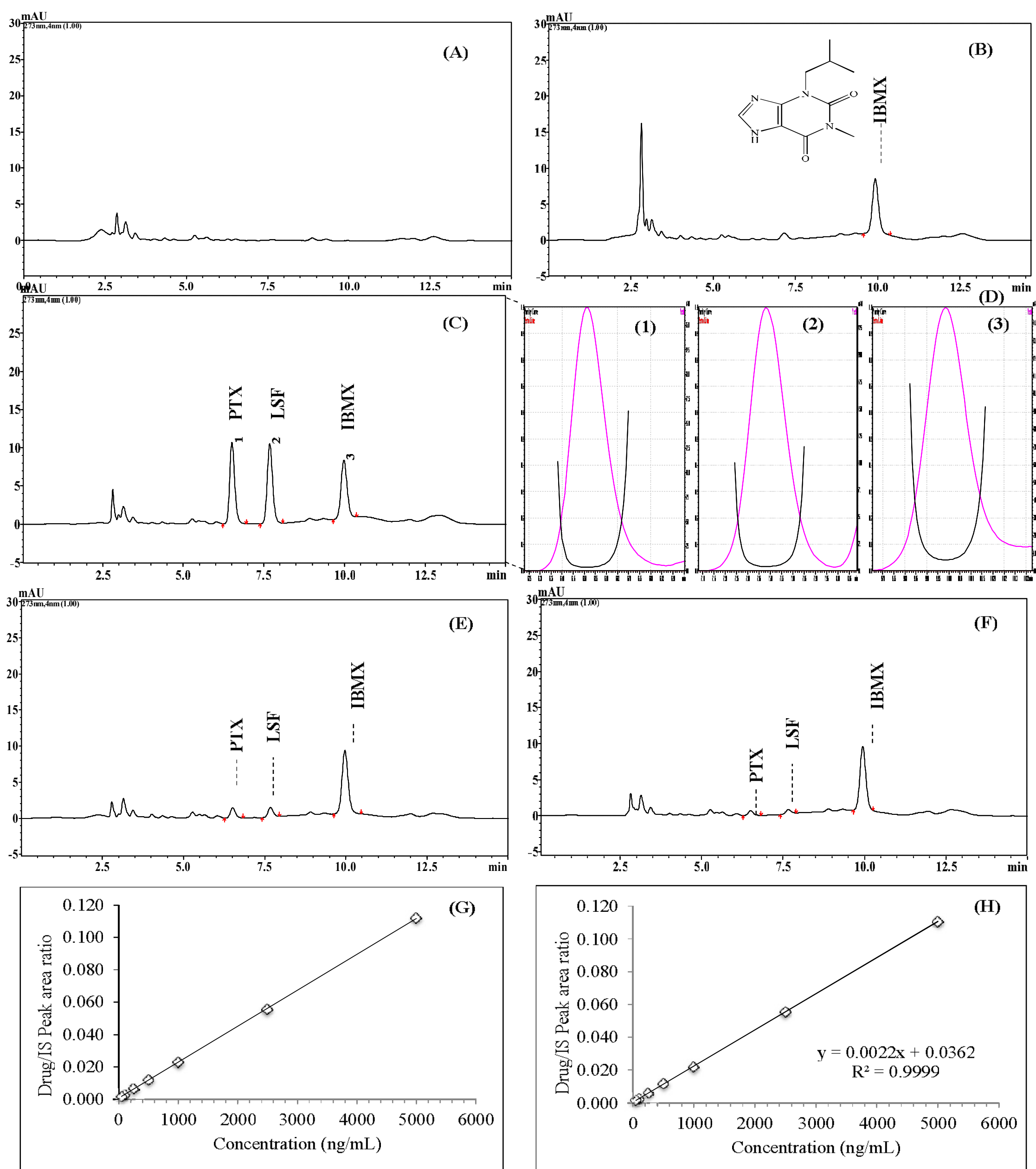


**Figure 3.3.** Representative HPLC-PDA chromatograms obtained after extraction and analysis of (**A**) Blank rat plasma; (**B**) Zero (blank rat plasma spiked with internal standard, IS) ;(**C**) LSF, PTX (both 500 ng/mL) and I.S (400 ng/mL) spiked in rat plasma; (**D**) Peak purity profiles of the analytes and I.S. (peak purity indices have also been indicated which show peak purity for all peaks ≥ 0.9999); (**E** and **F**) Representative HPLC-PDA chromatograms at LLOQ (50 ng/mL) and LOD (10 ng/mL) respectively of analytes in rat plasma and; (**G** and **H**) calibration curves for LSF and PTX respectively in rat plasma

### *3.4. Bioanalytical method validation of LSF in rat plasma using HPLC*

#### *3.4.1. Selectivity*

The analysis of blank plasma samples from six different healthy rats using the developed method confirmed the absence of matrix interference at the retention time of the analytes and I.S (**Figure 3.3A-D**).

#### *3.4.2. Linearity, calibration curve, LOD, LLOQ*

All the five calibration curves exhibited linearity and reproducibility in the range of 50-5000 ng/mL ($r^2$ > 0.9999). Weighted linear regression analysis was used to calculate $r^2$ slopes and intercepts using different weighing factors (1/var, 1/x, 1/$x^2$, 1/$\sqrt{x}$, 1/y, 1/$y^2$, 1/$\sqrt{y}$ ). 1/$y^2$ and 1/var were found to be the best weighing factors for LSF and PTX respectively as percentage relative error (% Σ RE) was minimum although, the difference observed was not significant from the un-weighted method (**Table 3.6**). The observed mean back calculated concentrations for calibration standards with accuracy (% bias) and precision (% CV) are presented in **Table 3.7 A**. At LLOQ, accuracy (% bias) was found to be -0.97 for LSF and -2.1 % for PTX with precision of ≤ 10.25 % for both the analytes. For S/N method, S/N ratio was found to be 4.16 and 14.28 respectively (acceptable limits ≥ 3 for LOD and ≥ 10 for LLOQ). LOD and LLOQ by S/N method and visual evaluation method were determined to be 10 ng/mL and 50 ng/mL respectively for both analytes as shown in **Figure 3.3E and F**. Representative calibration curves for LSF and PTX are shown in **Figure 3.3G and H.**

#### *3.4.3. Precision and accuracy*

As shown in **Table 3.7A**, inter and intra-day precision at all QC levels (LQC, MQC and HQC) and LLOQ were ≤ 5.92 for LSF and ≤ 7.25 for PTX. The % accuracy for both LSF and PTX was found in range of -4.43 to 5.01 %. Thus the obtained values for accuracy and precision for both the analytes were found to be within the recommended range (± 15 % except LLOQ ± 20%).

**Table 3.4.**

Optimization of bioanalytical method for LSF

| Variations in Chromatographic condition | Effect on chromatographic parameters of analytes (n=9) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | $t_R$ (min) | | Tailing factor (10%) | | HETP* | | N# | | £Resolution |
| | LSF | PTX | LSF | PTX | LSF | PTX | LSF | PTX | |
| **No variation (€Optimized)** | **7.75 ± 0.02** | **6.52 ± 0.02** | **1.05 ± 0.01** | **1.08 ± 0.01** | **29.10 ± 1.00** | **31.29 ± 1.32** | **5222.37 ± 200.29** | **5245.30 ± 219.67** | **3.55 ± 0.07** |
| Flow rate (0.9 mL/min) | 8.85 ± 0.09 | 7.25 ± 0.08 | 1.23 ± 0.04 | 1.22 ± 0.05 | 45.57 ± 0.50 | 50.32 ± 1.14 | 3299.45 ± 36.14 | 3034.30 ± 100.73 | 2.80 ± 0.05 |
| Flow rate (1.1 mL/min) | 8.05 ± 0.04 | 6.75 ± 0.08 | 1.16 ± 0.05 | 1.17 ± 0.04 | 13.75 ± 1.41 | 13.38 ± 1.07 | 11317.16 ± 281.90 | 10859.55 ± 250.21 | 4.14 ± 0.06 |
| Mobile phase composition (52:48 % v/v) | 6.60 ± 0.04 | 5.58 ± 0.03 | 1.14 ± 0.06 | 1.17 ± 0.06 | 44.25 ± 1.50 | 46.59 ± 1.05 | 3393.18 ± 112.40 | 3195.64 ± 70.55 | 2.39 ± 0.06 |
| Mobile phase composition (48:52 % v/v) | 9.12 ± 0.01 | 7.26 ± 0.01 | 1.05 ± 0.03 | 1.06 ± 0.02 | 41.59 ± 2.07 | 46.70 ± 1.54 | 3613.81 ± 171.23 | 3214.90 ± 106.02 | 3.32 ± 0.06 |
| Column temperature (35 °C) | 8.09 ± 0.08 | 6.57 ± 0.05 | 1.34 ± 0.01 | 1.36 ± 0.03 | 58.64 ± 1.42 | 60.92 ± 1.54 | 2621.37 ± 103.85 | 2609.44 ± 348.23 | 2.63 ± 0.05 |
| Column temperature (45 °C) | 7.63 ± 0.09 | 6.34 ± 0.04 | 1.20 ± 0.03 | 1.21 ± 0.03 | 45.71 ± 1.04 | 49.08 ± 1.61 | 3363.33 ± 187.40 | 2928.61 ± 273.80 | 2.68 ± 0.09 |

€Flow rate-1 mL/min, Mobile phase composition (50:50 % v/v) and Column temperature (40 °C)

£Resolution factor for LSF and PTX peaks

*HETP; Height equivalent to theoretical plate

#N; theoretical plate number

**Table 3.5.**

Optimization of Liquid-Liquid Extraction (LLE) method for sample preparation for bioanalytical method development of LSF.

| Extracting solvent | Sample volume (µL) | Volume of extraction solvent added (mL) | Vortexing time (min) | Centrifugation [speed (rpm), time (min)] | % Recovery of LSF & PTX | Remarks |
|---|---|---|---|---|---|---|
| Acetonitrile | 100 | 5 | 10 | 5000, 15 | NC* | *Incomplete protein precipitation |
| Methanol | 100 | 5 | 10 | 5000, 15 | NC* | |
| Methylene chloride (DCM) | 100 | 5 | 10 | 3500, 15 | 79-88 % | Consistent and reproducible recovery for both analytes,<br>Faster solvent evaporation &<br>Less plasma matrix interference |
| Chloroform | 100 | 5 | 10 | 3500, 15 | 64-86 % | Inconsistent recovery for both analytes |
| Based on the above results, further optimization was carried out in solvent mixtures with DCM. | | | | | | |
| DCM: Chloroform (1:1 v/v) | 100 | 5 | 10 | 3500, 15 | 75-90 % | Inconsistent recovery for both analytes |
| DCM: Methanol (1:0.1 v/v) | 100 | 5 | 10 | 3500, 15 | 80-85% | Recovery of IS was drastically reduced (~40%) as compared to ~79% obtained with DCM alone |
| DCM with 0.1 N HCl | 100 | 5 | 10 | 3500, 15 | 72-74% | When plasma was acidified, no interference of plasma proteins observed but recovery was reduced |
| Based on the above results, DCM was selected as the solvent of choice for extraction and other extraction conditions (sample volume, vortexing time and volume of extracting solvent) were further optimized | | | | | | |
| DCM | 100 | 5 | 10 | 3500, 15 | 79-88 % | LLOQ was high (in µg/mL)<br>But |
| | 200 | 5 | 10 | 3500, 15 | 80-87 % | LLOQ was improved (in ng/mL) but increased injection volume (80 µL) was increased |
| | 200 | 2 | 5 | 3500 rpm, 15 min | **78-83%** | Consistent and reproducible recovery of both analytes and IS<br>LOD was 10 ng/mL. |

*NC; Not consistent

**Table 3.6.**

Regression parameters of the calibration curve generated for each weighting factor ($w_i$) and their respective sum of relative errors (Σ%RE).

| Analyte | Model | $w_i$ | *b* | *a* | $r^2$ | Σ%RE |
|---|---|---|---|---|---|---|
| | 0 | Unweighted | 0.002200 | 0.0682 | 0.9999 | 13.34 |
| | 1 | 1/var | 0.002235 | 0.0691 | 0.9998 | 16.28 |
| | 2 | $1/x^2$ | 0.002238 | 0.0646 | 0.9996 | 12.62 |
| | 3 | 1/x | 0.002220 | 0.0678 | 0.9999 | 13.25 |
| **LSF** | 4 | $1/x^{1/2}$ | 0.002218 | 0.0698 | 1.0000 | 15.39 |
| | **5** | $\mathbf{1/y^2}$ | **0.002235** | **0.0647** | **0.9997** | **12.42** |
| | 6 | 1/y | 0.002219 | 0.0685 | 0.9999 | 13.97 |
| | 7 | $1/y^{1/2}$ | 0.002218 | 0.07011 | 0.9999 | 15.77 |
| | 0 | Unweighted | 0.002200 | 0.03620 | 0.9999 | 12.67 |
| | **1** | **1/var** | **0.002211** | **0.03300** | **0.9999** | **9.97** |
| | 2 | $1/x^2$ | 0.002218 | 0.03248 | 0.9997 | 10.89 |
| | 3 | 1/x | 0.002206 | 0.03469 | 0.9999 | 11.52 |
| **PTX** | 4 | $1/x^{1/2}$ | 0.002205 | 0.03621 | 1.0000 | 12.25 |
| | 5 | $1/y^2$ | 0.002216 | 0.03254 | 0.9997 | 10.2 |
| | 6 | 1/y | 0.002206 | 0.03488 | 0.9999 | 11.11 |
| | 7 | $1/y^{1/2}$ | 0.002205 | 0.03626 | 1.0000 | 12.54 |

*wi;* Weighting factor, *b,* slope; *a,* constant, $r^2$, regression co-efficient

### *3.4.4. Recovery, carry over, dilution integrity*

The mean absolute recovery values for LSF and PTX were found to be > 80 % as shown in **Table 3.8**. The mean absolute recovery for I.S was found to be 78.87 ± 1.84 % (n=6). No carry over effect was observed as there was absence of any peaks of analytes and I.S in blank sample injected after ULOQ. Integrity of method upon dilution was also established by 10 times dilution of the plasma containing LSF and PTX (25000 ng/mL) wherein, accuracy values of 99.90 ± 4.58 and 100.35 ± 1.74 % respectively were obtained.

**Table 3.7A.**
Precision and accuracy of back calculated concentrations of calibration standard samples of LSF and PTX in rat plasma (n = 5)

| Analyte | Concentration (ng/mL) | Measured concentration (Mean ± SD, ng/mL) | Precision (% CV) | Accuracy (% bias) |
|---|---|---|---|---|
| | 5000 | 5055.08 ± 90.93 | 1.80 | 1.10 |
| | 2500 | 2501.52 ± 84.89 | 3.39 | 0.06 |
| | 1000 | 1001.92 ± 16.82 | 1.68 | 0.19 |
| **LSF** | 500 | 517.42 ± 07.61 | 1.47 | 3.48 |
| | 250 | 261.86 ± 11.37 | 4.34 | 4.74 |
| | 100 | 97.15 ± 07.48 | 7.70 | -2.85 |
| | 50 | 49.51 ± 05.07 | 10.25 | -0.97 |
| | 5000 | 5013.78 ± 127.95 | 2.55 | 0.28 |
| | 2500 | 2506.43 ± 61.93 | 2.47 | 0.26 |
| | 1000 | 982.22 ± 40.55 | 4.13 | -1.78 |
| **PTX** | 500 | 520.14 ± 22.51 | 4.33 | 4.03 |
| | 250 | 253.78 ± 13.46 | 5.31 | 1.51 |
| | 100 | 97.26 ± 5.70 | 5.86 | -2.74 |
| | 50 | 48.95 ± 3.73 | 7.61 | -2.10 |

**Table 3.7B.**
Precision (% CV) and accuracy (% bias) of the analytes in rat plasma samples at QC levels (n=5).

| Analyte | Level | Conc. (ng/mL) | Inter-day | | | Intra-day | | |
|---|---|---|---|---|---|---|---|---|
| | | | Measured Conc. (Mean ± SD, ng/mL) | Precision (% CV) | Accuracy (% bias) | Measured Conc. (Mean ± SD, ng/mL) | Precision (% CV) | Accuracy (% bias) |
| LSF | LLOQ | 50 | 49.24 ± 0.60 | 0.75 | -1.52 | 49.75 ± 0.54 | 0.67 | -0.49 |
| | LQC | 80 | 79.90 ± 6.57 | 5.92 | -0.13 | 76.46 ± 2.38 | 2.21 | -4.43 |
| | MQC | 300 | 309.74 ± 18.40 | 5.40 | 3.25 | 308.11 ± 14.20 | 4.19 | 2.70 |
| | HQC | 3000 | 3052.39 ± 68.50 | 2.22 | 1.75 | 3136.48 ± 119.57 | 3.78 | 4.55 |
| PTX | LLOQ | 50 | 48.23 ± 2.43 | 3.76 | -3.55 | 49.20 ± 4.76 | 7.25 | -1.60 |
| | LQC | 80 | 75.65 ± 1.16 | 1.26 | -5.44 | 79.10 ± 17.97 | 6.34 | -1.12 |
| | MQC | 300 | 312.67 ± 11.36 | 3.45 | 4.22 | 315.03 ± 17.99 | 5.43 | 5.01 |
| | HQC | 3000 | 3014.89 ± 31.37 | 1.03 | 0.50 | 3109.63 ± 158.84 | 5.08 | 3.65 |

Recommendation limits: ±15 % except LLOQ (±20 %)

**Table 3.8.**
Absolute recoveries (%) of analytes in rat plasma samples from QC concentrations of the calibration ranges

| Analyte | Level | Nominal concentration (ng/mL) | n | Recovery (%) | |
|---|---|---|---|---|---|
| | | | | Mean ± SD | % CV |
| LSF | LLOQ | 50 | 5 | 82.50 ± 5.62 | 6.82 |
| | LQC | 80 | 5 | 79.52 ± 4.05 | 5.10 |
| | MQC | 300 | 5 | 80.60 ± 2.51 | 3.12 |
| | HQC | 3000 | 5 | 80.18 ± 2.37 | 2.96 |
| | | Mean | 20 | 80.47 ± 3.44 | 4.28 |
| PTX | LLOQ | 50 | 5 | 81.07 ± 5.08 | 6.27 |
| | LQC | 80 | 5 | 79.37 ± 3.63 | 4.57 |
| | MQC | 300 | 5 | 81.85 ± 1.49 | 1.83 |
| | HQC | 3000 | 5 | 81.27 ± 2.62 | 3.23 |
| | | Mean | 20 | 80.89 ± 3.73 | 4.61 |

n, number of samples
Recovery for I.S was 78.87 ± 1.84 % (n=6)

### *3.5. Stability studies*

Stability studies indicated that both the analytes were stable in aqueous solutions and in rat plasma under different storage conditions that may be encountered during routine sample analysis (**Table 3.9**).

**Table 3.9.**
**(a)** Stock solution (50 µg/mL) stability (-20 °C) for LSF and PTX

| Analyte | €Peak area (Mean ± SD) | | % CV | | % diff§ |
|---|---|---|---|---|---|
| | Old Stock (3 months) | Fresh Stock (0 h) | Old stock | Fresh stock | |
| LSF | 1993402 ± 14174 | 2003204 ± 6775 | 0.71 | 0.34 | 0.49 |
| PTX | 1925070 ± 14778 | 1934924 ± 8728 | 0.78 | 0.45 | 0.51 |

§ % difference determined by following equation: (mean test - mean control) / [(mean test + mean control)/2] × 100

**(b)** Stability of analytes in rat plasma at three QC levels

| Stability | Nominal concentration (ng/mL) | Measured concentration (Mean ± SD, ng/mL) | | Precision (% CV) | | Accuracy (% bias) | |
|---|---|---|---|---|---|---|---|
| | | LSF | PTX | LSF | PTX | LSF | PTX |
| 0 h (for all) | 3000 | 3070.13 ± 64.20 | 2999.66 ± 45.15 | 1.59 | 1.50 | 0.37 | -0.01 |
| | 300 | 303.58 ± 5.32 | 308.64 ± 9.77 | 1.75 | 3.17 | 1.19 | 2.88 |
| | 80 | 78.08 ± 1.18 | 78.64 ± 2.11 | 2.41 | 2.69 | -2.45 | -1.69 |
| Autosampler (48 h) | 3000 | 2969.36 ± 19.67 | 2931.44 ± 39.18 | 0.66 | 1.34 | -1.02 | -2.28 |
| | 300 | 281.20 ± 6.67 | 277.24 ± 3.37 | 2.37 | 1.22 | -6.27 | -7.59 |
| | 80 | 78.98 ± 1.29 | 82.40 ± 1.38 | 1.63 | 1.68 | -1.27 | 3.00 |
| Bench-top (24 h, RT) | 3000 | 2988.95 ± 99.20 | 2923.97 ± 93.13 | 3.31 | 3.19 | -0.03 | -2.53 |
| | 300 | 294.81 ± 7.89 | 283.60 ± 9.59 | 2.68 | 3.38 | -1.73 | -5.47 |
| | 80 | 75.88 ± 1.89 | 77.72 ± 3.83 | 2.49 | 4.93 | -5.15 | -2.85 |
| Freeze-thaw (-80 °C, 3 cycle) | 3000 | 2955.69 ± 35.58 | 2889.35 ± 27.81 | 1.20 | 0.96 | -1.48 | -3.69 |
| | 300 | 301.34 ± 1.97 | 287.41 ± 1.88 | 0.65 | 0.66 | 0.45 | -4.20 |
| | 80 | 78.85 ± 3.47 | 75.77 ± 2.71 | 4.41 | 3.58 | -1.44 | -5.28 |
| Short term (4 °C,48 h) | 3000 | 2970.80 ± 46.17 | 2910.85 ± 39.30 | 1.54 | 1.35 | -0.97 | -2.97 |
| | 300 | 292.04 ± 3.62 | 282.60 ± 2.69 | 1.12 | 0.95 | -2.65 | -5.80 |
| | 80 | 75.33 ± 2.77 | 78.18 ± 1.55 | 2.60 | 1.98 | -5.84 | -2.28 |
| Long term (-80 °C, 45 days) | 3000 | 2932.87 ± 67.28 | 2858.13 ± 45.48 | 2.29 | 1.59 | -2.24 | -4.73 |
| | 300 | 282.58 ± 14.00 | 275.67 ± 3.99 | 4.96 | 1.45 | -5.81 | -8.11 |
| | 80 | 73.14 ± 1.86 | 75.83 ± 2.44 | 2.54 | 3.22 | -8.57 | -5.22 |

### *3.6. Analysis of PK study samples of LSF and PTX by developed bioanalytical method*

The suitability of the method was demonstrated by analyzing the PK study samples wherein, LSF and PTX were administered at a single dose of 25 mg/kg (i.v.) individually in wistar rats. **Figure 3.4** shows representative chromatograms of PK study samples at 2 h and mean plasma concentration-time profiles of LSF and PTX. Different pharmacokinetic parameters were evaluated by non-compartmental model approach using Phoenix 2.1 WinNonlin software as shown in **Table 3.10**. In the pharmacokinetic studies of both LSF and PTX, peak of the other

analyte (LSF or PTX) was obtained which is attributed to the interconversion of both analytes *in vivo***.**

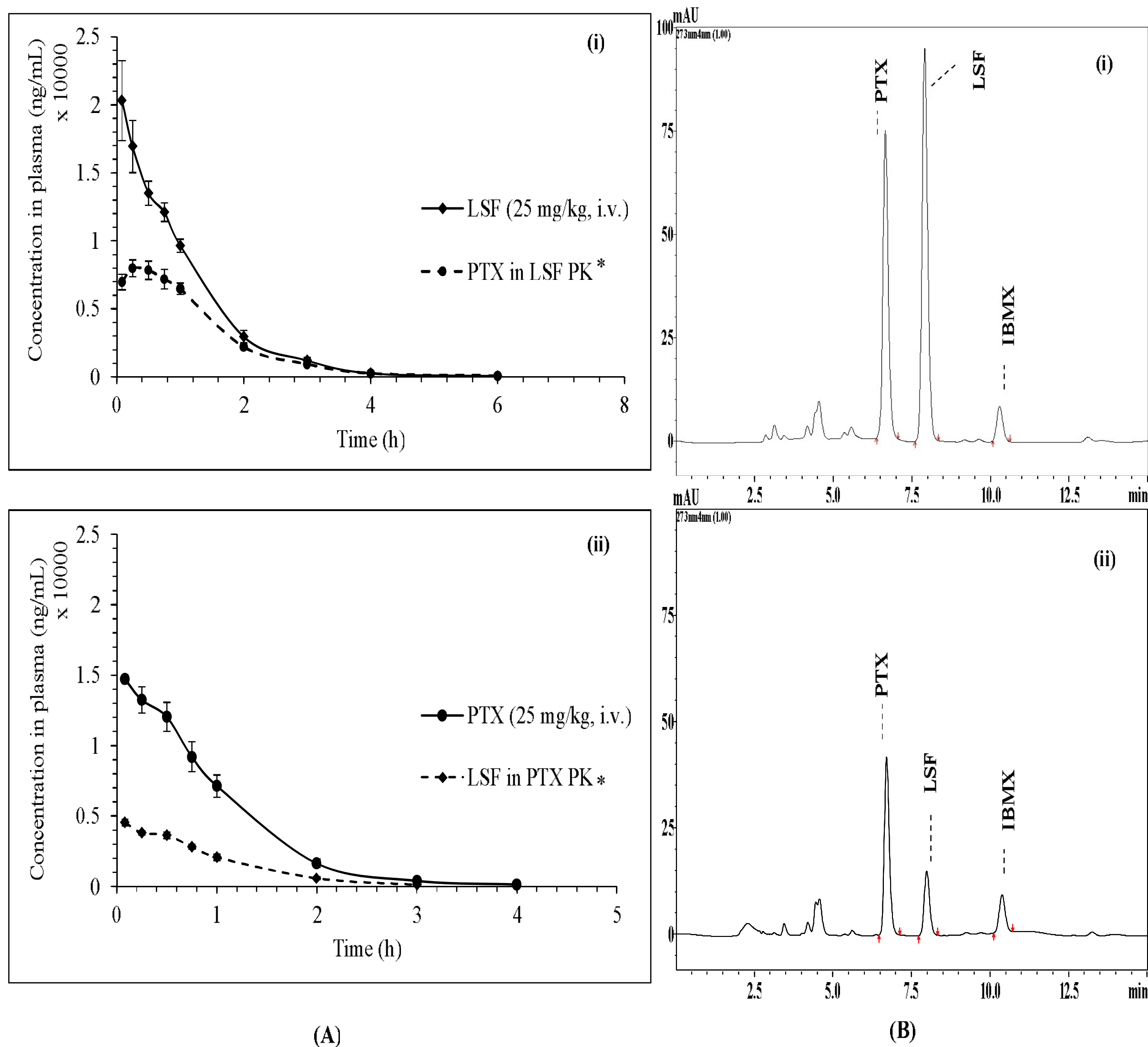


**Figure 3.4.** Analysis of PK study samples by the developed bioanalytical method, (A) Plasma concentration-time profiles for (i) LSF and (ii) PTX; (B) Representative HPLC-PDA chromatograms obtained for sample (i) LSF and (ii) PTX

*In both the chromatograms; a peak corresponding to the other analyte is also seen which is attributed to the *in vivo* interconversion of both these analytes

**Table 3.10.**
The non-compartmental PK parameters for LSF and PTX in rat plasma after i.v. bolus (25 mg/kg) administration to rat (n=4).

| Parameters | Mean ± SEM | |
|---|---|---|
| | **LSF** | **PTX** |
| $C_o$ (ng/mL) | 22295.204 ± 3691.39 | 15835.204 ± 711.96 |
| $t_{1/2}$ (h) | 0.661 ± 0.03 | 0.539 ± 0.06 |
| Ke (1/h) | 1.056 ± 0.05 | 1.317 ± 0.13 |
| $AUC_{0\text{-}last}$ (ng.h/mL) | 23944.589 ± 992.83 | 17092.707 ± 1008.34 |
| $AUC_{0\text{-}\infty}$ (ng.h/mL) | 24067.711 ± 995.38 | 17198.155 ± 959.69 |
| $AUMC_{0\text{-}last}$ (ng.h/mL) | 22441.435 ± 1592.68 | 13198.186 ± 382.86 |
| $AUMC_{0\text{-}\infty}$ (ng.h/mL) | 22789.458 ± 1692.67 | 13711.159 ± 502.03 |
| MRT (h) | 0.937 ± 0.07 | 0.778 ± 0.05 |
| $V_z$ (mL/kg) | 997.077 ± 71.35 | 1152.149 ± 201.24 |
| CL (mL/h/kg) | 1044.124 ± 43.53 | 1463.108 ± 84.87 |

## 4. Conclusion

The analytes quantified in the present study, LSF and PTX, carry immense therapeutic potential and the analytical method developed and validated by our lab could be extensively used for routine analysis of these analytes for studying the *in vivo* drug-drug interactions and PK-pharmacodynamic (PD) studies. It uses simple and rapid sample preparation process, offers freedom from matrix interference and requires a small plasma sample volume (200 µL), has application within a wide concentration range (50- 5000 ng/mL) and has a short overall run time (15 min) without using any sophisticated instruments like LC MS/MS. The study also highlighted complete stability of LSF and PTX in plasma when stored under different conditions; freezer storage (-80 °C), auto-sampler, bench top conditions and after three consecutive freeze-thaw cycles.

## Bibliography


1. Chivers, D.; Birkett, D.; Miners, J. Simultaneous determination of pentoxifylline and its hydroxy metabolite in plasma by high-performance liquid chromatography. *J Chromatogr.* **1981,** *225*, (1), 261-265.

2. Rieck, W.; Platt, D. Determination of 3, 7-dimethyl-1-(5-oxohexyl)-xanthine (pentoxifylline) and its 3, 7-dimethyl-1-(5-hydroxyhexyl)-xanthine metabolite in the plasma of patients with multiple diseases using high-performance liquid chromatography. *J Chromatogr.* **1984,** *305*, 419-427.

3. Grasela, D. M.; Rocci, M. L. High-performance liquid chromatographic analysis of pentoxifylline and 1-(5'-hydroxyhexyl)-3, 7-dimethylxanthine in whole blood. *J Chromatogr.* **1987,** *419*, 368-374.

4. Kyle, P. B.; Adcock, K. G.; Kramer, R. E.; Baker, R. C. Use of liquid chromatography–tandem mass spectrometry for the analysis of pentoxifylline and lisofylline in plasma. *Biomed Chromatogr.* **2005,** *19*, (3), 231-236.

5. Striffler, J. S.; Nadler, J. L. Lisofylline, a novel anti-inflammatory agent, enhances glucose-stimulated insulin secretion in vivo and in vitro: studies in prediabetic and normal rats. *Metabolism* **2004,** *53*, (3), 290-296.

6. Wyska, E.; Świerczek, A.; Pociecha, K.; Przejczowska-Pomierny, K. Physiologically based modeling of lisofylline pharmacokinetics following intravenous administration in mice. *Eur J Drug Metab Pharmacokinet.* **2016,** *41*, (4), 403-412.

7. Müller, R. Hemorheology and peripheral vascular diseases: a new therapeutic approach. *J Med.* **1981,** *12*, (4), 209-235.

8. Wyska, E.; Szymura-Oleksiak, J.; Pękala, E.; Obruśnik, A. Pharmacokinetic modelling of pentoxifylline and lisofylline after oral and intravenous administration in mice. *J Pharm Pharmacol.* **2007,** *59*, (4), 495-501.

9. Wyska, E. Pharmacokinetic-pharmacodynamic modeling of methylxanthine derivatives in mice challenged with high-dose lipopolysaccharide. *Pharmacology* **2010,** *85*, (5), 264-271.

10. Borrelli, S.; Christodoulou, M. S.; Ficarra, I.; Silvani, A.; Cappelletti, G.; Cartelli, D.; Damia, G.; Ricci, F.; Zucchetti, M.; Dosio, F. New class of squalene-based releasable nanoassemblies of paclitaxel, podophyllotoxin, camptothecin and epothilone A. *European journal of medicinal chemistry* **2014,** *85*, 179-190.

11. Kollipara, S.; Bende, G.; Agarwal, N.; Varshney, B.; Paliwal, J. International guidelines for bioanalytical method validation: a comparison and discussion on current scenario. *Chromatographia* **2011,** *73*, (3-4), 201-217.

12. Walczak, M.; Szymura-Oleksiak, J.; Pekala, E. Validation of a high performance liquid chromatography method for pharmacokinetic evaluation of pentoxifylline and lisofylline in rat serum and tissues. *Acta Pol. Pharm* **2009,** *66*, (3), 215-224.

13. Almeida, A. M.; Castel-Branco, M. M.; Falcao, A. Linear regression for calibration lines revisited: weighting schemes for bioanalytical methods. *J Chromatogr B Analyt Technol Biomed Life Sci.* **2002,** *774*, (2), 215-222.

14. Singtoroj, T.; Tarning, J.; Annerberg, A.; Ashton, M.; Bergqvist, Y.; White, N.; Lindegardh, N.; Day, N. A new approach to evaluate regression models during validation of bioanalytical assays. *J Pharm Biomed Anal.* **2006,** *41*, (1), 219-227.

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