Research Paper

Development and Validation of a HPLC-based Bioanalytical Method for Lorcaserin using Solid Phase Extraction and Application to a Pharmacokinetic Study in Rats

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A rapid, precise, accurate, specific and simple high performance liquid chromatography method for estimation of lorcaserin hydrochloric in human plasma, using metoprolol as an internal standard, was developed and validated as per the regulatory requirements. Sample preparation included solid phase extraction and chromatographic separation was performed using a Phenomenex Luna C18 column (250×4.6 mm i.d, 5 µ particle size), with phosphate buffer (pH 3):acetonitrile:methanol (65:20:15) as the mobile phase at a flow rate of 1.0 ml/min. Wavelength of detection was 222 nm. Retention times of internal standard and lorcaserin HCl were found to be 5.15 and 7.19 min, respectively. The method was developed and tested in the linearity range of 500 to 3000 ng/ml. The method was validated for accuracy, precision, linearity, recovery and stability in compliance to international regulatory guidelines.

Key words: Lorcaserin hydrochloride, metoprolol, bioanalytical method, HPLC

Lorcaserin hydrochloride (LOR) is an antiobesity (selective serotonin 2c receptor agonist), off-white to white powder, which is freely soluble in water, methanol, acetonitrile and dimethyl sulphoxide having log P value of 2.56 and pKa of 9.53. LOR is not official in IP, BP and USP and is available as 10 mg tablets.

A number of analytical methods were reported in literature to analyse LOR in tablet formulation. A liquid chromatography-electro spray ionization-tandem mass spectrometry (LC-ESI-MS/MS)[1] method was reported for *in vivo* and *in vitro* pharmacological characterization of LOR. Also a chiral LC-MS/MS method for the separation and quantitation of lorcaserin and its S-enantiomer has been reported[2]. Despite of the fact that various analytical methods are available for the estimation of lorcaserin, no solid phase extraction based bioanalytical high-performance liquid chromatography (SPE HPLC) method was available in the literature to the best of our knowledge. Thus, the main aim of the study was to develop a SPE HPLC method for the determination of LOR in plasma and its application to rat pharmacokinetic study. Metoprolol (MET) was used as internal standard (IS) for bioanalytical method development. MET is an antihypertensive agent and having solubility in water and official in IP, BP and USP having log P value of 1.88 and pKa of 9.67.

MATERIALS AND METHODS

LOR was purchased from Swapnaroop Drugs Pvt. Ltd., Aurangabad and was certified to contain 99.70 % (w/w) on dried basis whereas MET of pharmaceutical grade was obtained as a gift sample from Torrent Pharmaceuticals Pvt. Ltd., Ahmedabad and was certified to contain 99.30 % (w/w) on dried basis. Methanol and acetonitrile used were of HPLC grade and were purchased from Rankem, Ankleshwar. Potassium hydrogen orthophosphate of HPLC grade was purchased from Qualigens, Ahmedabad and orthophosphoric acid was purchased from Spectrochem, Vadodara. Drug free EDTA human

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plasma was procured from Suraktam Blood Bank, Vadodara. The liquid chromatographic system was of Shimadzu, Mumbai and consisting of following components an isocratic pump, variable wavelength programmable UV/Vis detector, a manual injection facility with 20 µl fixed loop. The chromatographic analysis was performed using Spinchrom software on a Phenomenax-RP-18 column (250×4.6 mm, 5 µm particle size). In addition, an electronic balance (Shimadzu AX120ELB300), a pH meter (Lab India Pico+), sonicator (Spectra Lab, Selec XT 543), hot air oven (SK Industries), solid phase extractor (Orochem, Ezypress HT48), vortex shaker (SPINIX), membrane filter 0.22 micron (Pall Lifesciences, Ultipor Nylon), deep freezer (EIE Instruments), micropipette (Tarsons, accupipete), SPE cartridges (Phenomenex, Oasis HLB Cartridges), refrigerated centrifuge (Remi), refrigerator (Godrej, Pantacool) were used in this study. The pharmacokinetic study was carried out in Male Sprague Dawley rats. The experimental procedure was approved by Institutional Animal Ethics Committee, Pharmacy department under protocol number (MSU/IAEC/2014-15/1419) on 22nd Sep 2014.

Preparation of mobile phase buffer:

About 10 mM phosphate buffer was prepared by dissolving 0.136 g of potassium dihydrogen orthophosphate in sufficient water to produce 100 ml. The pH was adjusted to 3 using orthophosphoric acid. This buffer was filtered through 0.22 µ membrane filter and stored at ambient temperature.

Preparation of mobile phase:

Appropriate volumes of phosphate buffer (pH 3, adjusted with orthophosphoric acid), acetonitrile and methanol were transferred into a reagent bottle, mixed thoroughly, sonicated for 5 min and filtered through 0.22 µm membrane filter and used as mobile phase. The HPLC analysis was performed on reversed-phase HPLC system with isocratic elution mode using a mobile phase of phosphate buffer:acetonitrile:methanol (65:20:15 v/v/v) on Phenomenex Luna C_{18} column (250×4.6 mm, 5 µm particle size) with 1 ml/min flow rate at 222 nm using UV detector.

Stock solutions of LOR and MET (1000 ppm):

About 10.8 mg of LOR hemihydrate (10.8 mg LOR hemihydrates is equivalent to 10 mg LOR) was weighed accurately, transferred into a 10 ml volumetric flask, dissolved in double distilled water and the volume made up to the mark to obtain the LOR stock solution.

About 10 mg of MET (IS) was weighed accurately, transferred to a 10 ml volumetric flask, dissolved in double distilled water and the volume was made up to the mark to obtain a MET stock solution.

Calibration standards for LOR:

Appropriate aliquots of LOR stock solution were taken in different 6 ml volumetric flasks and diluted up to the mark with mobile phase to obtain final concentrations of 10-60 µl/ml. To appropriate aliquots of calibration standards, 0.1 ml of 400 ppm IS was spiked and the final volume of 2 ml was made up with plasma to obtain final concentration of 500-3000 ng/ml. The linearity range was selected on the basis of reported peak plasma concentration (C_{max}= 789 ng/ml) from literature.

To appropriate aliquots of calibration standards, 0.1 ml of 400 ppm IS was spiked and the final volume of 2 ml was made up with plasma to obtain final concentration of 500 ng/ml as lower limit of quantification control, 1000 ng/ml as low quality control, 2000 ng/ml as medium quality control (MQC), 3000 ng/ml as high quality control (HQC).

Bioanalytical method[3-6]:

Mobile phase trials were taken on unextracted samples. Various mobile phases like water:methanol (50:50 and 20:80), water:acetonitrile (30:70), water:acetonitrile (pH 3; 60:40), phosphate buffer:acetonitrile (pH 3; 70:30), phosphate buffer:methanol:acetonitrile (60:15:25) and phosphate buffer:methanol:acetonitrile (65:15:20) were tried in which phosphate buffer:methanol:acetonitrile (65:15:20) at pH 3 gave the best peak.

Also trials for selection of appropriate IS were taken in which screening was done on the basis of structural resemblance, log P value, pKa value and availability. Chromatographic trials for bisoprolol fumarate and MET succinate were undertaken with conditions described earlier in which MET succinate gave good peak as shown in Table 1.

Method validation[7,8,9]:

The optimized method was validated as per the recommendations of USP[10,11] and ICH[12,13] for the parameters like accuracy, linearity, precision, detection limit, quantitation limit and robustness. Recovery of LOR in plasma was evaluated by comparing the mean peak responses of at least six injections of each low, medium and HQC sample, prepared in plasma, to

mean peak responses of non-spiked samples prepared in elution solvent and external spiked matrix extracted sample. Recovery of IS in plasma was evaluated by comparing the mean peak responses of at least six MQC samples, prepared in plasma, to mean peak responses of non-spiked samples prepared in elution solvent and external spiked matrix extracted sample, replicates of aqueous samples of LOR. The mean standard deviation and % coefficient of variation (CV) for the peak area ratio and for the retention time of analyte and IS were calculated. Specificity and selectivity was carried out using six plasma samples. Blank (without IS) and zero sample (with IS) were analysed. The linearity of the method was determined over calibration range of 500 to 3000 ng/ml (Table 2). Calibration standards were prepared by spiking known concentration of LOR working standard solution. A linearity curve containing six non-zero concentrations was analysed (fig. 1). Back-calculated the concentrations of each level and plot the graph of back-calculated concentration against drug area ratio. The slope, y-intercept and correlation coefficient curve were calculated by suitable linear regression analysis as stated in Table 3. Accuracy and precision was measured on the samples spiked with known amounts of the analyte. Accuracy and precision were determined by replicate analysis of six determinations of low, medium and HQC sample, which covers the calibration range. Precision is expressed as the % CV. The accuracy and precision were evaluated as within batch and between-batch[14].

Bench top stability was performed at MQC level. Three replicates of MQC were withdrawn from deep freezer and were kept at room temperature for 12 h. These samples were preferred as stability samples after 12 h, prepared fresh samples of MQC concentrations of LOR in three replicates. These samples were referred as fresh or comparison samples. The freeze and thaw stability of analyte was determined after three freeze thaw (FT) cycles. The three sets of MQC samples were stored at –70±5 and subjected to three FT cycles at interval of 24 h. After the completion of three cycles of 12 to 24 h, the samples were analysed. Stability of samples was compared against freshly prepared samples.

The stability of LOR and IS in the stock solution were determined at room temperature for 7 h. Stock solution stability was performed by analysing three replicates of aqueous solutions prepared from freshly weighed stock solution against three replicates of aqueous solution prepared from aliquots of analyte and IS stored at room temperature for 7 h. The stability of LOR and IS in the stock solution were determined at 2-8° after 5 d. Refrigerated stock solution stability was performed by analysing three replicates of aqueous solution prepared from freshly weighed stock solution against three replicates of aqueous solution prepared from aliquots of analyte and IS stored at 2-8° after 5 d (stability samples; Table 4).

Pharmacokinetic studies:

The pharmacokinetic study was carried out in male Sprague Dawley rats. The six healthy animals were selected for the study. The animals were fasted overnight (~14 h) and had free access to water throughout the experimental period. LOR was administered by oral gavage at a dose of 10 mg/kg, as solution of drug in water. Blood samples (0.5 ml) were collected from the retro orbital plexus sinus at designated time points (0.25, 0.5, 1, 2, 3 and 4 h) into micro centrifuge tubes containing 100 µl of heparin. Plasma was harvested by centrifuging the blood using cold centrifuge compufuge at 3000 rpm for 10 min. Plasma (300 µl) samples were spiked with IS and processed same as standards as described above.

RESULTS AND DISCUSSION

Sample preparation technique used for the study plays a significant role with respect to bionalytical samples[15-20]. It is essential to reduce the effect of the biological and buffer matrix. Sample preparation is applied to remove interfering compounds. As a bonus, analytes can be concentrated during the extraction processes. Sample preparation procedure is tedious and time consuming. However, the cleanliness of the samples affects

TABLE 1: CHROMATOGRAPHY TRIALS FOR INTERNAL STANDARD SELECTION

Drug	Mobile phase	Column used	Flow rate	RT (min)	Peak shape and asymmetry
Bisoprolol fumarate	Phosphate buffer:methanol:acetonitrile (65:15:20) pH 3.0 adjusted with orthophosphoric acid	Phenomenex Luna C_{18} (250×4.5 mm, 5 µm)	1.0 ml/min	4.674	Bifurgated peak
Metoprolol succinate	Phosphate buffer:methanol:acetonitrile (65:15:20) pH 3.0 adjusted with orthophosphoric acid	Phenomenex Luna C_{18} (250×4.5 mm, 5 µm)	1.0 ml/min	5.130	Good and sharp peak

the overall performance of the analysis. Different extraction techniques tried were protein precipitation, liquid-liquid extraction and SPE. For selection and optimization of particular extraction techniques various trials were taken as described below. Initially protein precipitation method was tried using acetonitrile, methanol and acetone as precipitating agents but it showed greater plasma interference, greater sample transfer and greater sample evaporation steps. Samples obtained were unclean, which can be harmful to life of analytical instrument in long run. So this technique was not preferred. Following it liquid-liquid extraction technique was tried in which interference due to plasma matrix was reduced. However for sample transfer and sample evaporation, tedious multiple extraction steps were involved, which produced less consistent results. Various extracting agents used for the study included chloroform, ethyl acetate and methyl tert-butyl ether (MTBE). Best recovery of about 70-75 % was obtained with MTBE. Finally SPE technique was tried in which interference due to plasma matrix was very less as compared to other techniques. In it, sample transfer and sample evaporation steps are not involved, leading to consistent results. In addition this technique required less biological material and less time. The C18 cartridges were used for the extraction procedure. Two brands of C18 cartridges were tried for the study. The brands of C18 cartridges used were Oasis of Waters and Orochem. Best recovery of 89 % was obtained by using Orochem brand C18 SPE cartridge.

TABLE 2: SUMMARY OF VALIDATION AND SYSTEM SUITABILITY TEST PARAMETERS

Parameter (units)	LOR
Linearity range (ng/l)	500-3000
Correlation coefficient	0.998±0.00038
Recovery of LOR (%)	86.856
Recovery of IS (%)	90.169
Precision (% RSD)	
Interday (n= 3)	1.23
Intraday (n= 3)	1.14
Robustness	Robust
Retention time (min) for LOR	7.19±0.2
Retention time (min) for MET	5.14±0.2

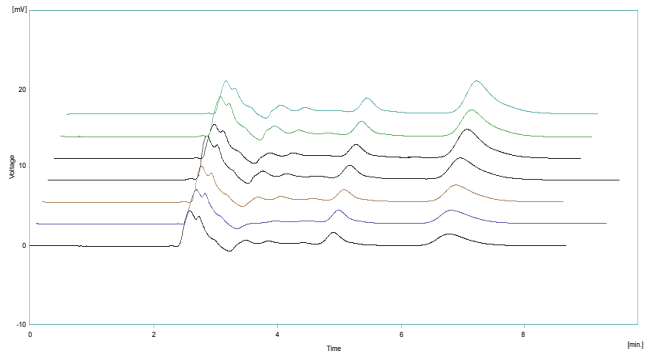

Fig. 1: Overlain chromatogram of mixture of LOR and MET (500-3000 ng/ml)

The optimized SPE method parameters used for the study included Orochem SPE cartridge, sample pre-treatment by 0.5 ml plasma sample+0.5 ml water (sample dilution, 1:2) conditioning with 0.5 ml methanol, equilibrating with 0.5 ml water, loading of 0.5 ml pre-treated sample, washing with 0.5 ml water, drying with nitrogen purging for 1-2 min and finally eluting with 0.5 ml methanol.

The extracted samples were retrieved from pre-labelled sample tubes stored in deep freezer at –20° and were pre-treated prior to extraction and then subjected to SPE. The extracted samples were subjected to HPLC. For development of analytical method for the estimation of LOR by RP HPLC, various chromatographic trials were taken. The various factors considered were flow rate, mobile phase composition, wavelength maxima and pH of mobile phase. The factors were varied on one factor at time basis[21-26]. In the HPLC method optimized on extracted samples, mobile phase consisted of phosphate buffer:methanol:acetonitrile

TABLE 3: BACK CALCULATED CONCENTRATIONS FOR THE STANDARD CALIBRATION GRAPHS

Batch ID	Back calculated concentrations for the standards						Slope	Intercept	R^2
	Standard 1	Standard 2	Standard 3	Standard 4	Standard 5	Standard 6			
Conc. (ng/ml)	500	1000	1500	2000	2500	3000	-	-	-
C1	445.168	844.276	1425.26	2063.28	2729.46	2948	0.023	6.418	0.996
C2	448.688	948.956	1401.517	2023.453	2658.78	2996.62	0.022	7.966	0.998
C3	475.74	958.35	1411	2031.47	2635.51	2961.56	0.021	9.336	0.999
Mean	456.532	917.194	1412.592	2039.401	2674.58	2968.727	0.022	7.906	0.997
SD	16.72746	63.32328	11.95132	21.06473	48.9281	25.08978	0.001	1.459	0.0015
% CV	3.664029	6.904023	0.846056	1.032888	1.82937	0.845136	4.5454	18.4657	0.1532
% Mean	91.306	91.72	94.172	101.968	106.983	98.718	-	-	-

SD- standard deviation, % CV- percent coefficient of variation, R^2-regression correlation

(65:15:2, pH 3.0), at 1 ml/min flow rate, which gave two sharp, well-resolved peaks with minimum tailing factor for LOR and MET in human plasma as shown in fig. 2A. The retention times for LOR and MET were 7.19 and 5.14 min, respectively. UV overlain spectra of both LOR and MET showed that both drugs absorbed appreciably at 222 nm, so this wavelength was selected as the detection wavelength. The calibration curve for LOR was found to be linear over the range of 500-3000 ng/ml (fig. 1). The data of regression analysis of the calibration curves is shown in Table 3. The proposed method was successfully applied to the determination of LOR in biological matrix. The developed method was also found to be specific, since it was able to separate drug in the biological matrix.

The chromatogram presented in fig. 2B is of blank (unspiked) rat plasma sample extracted using SPE extraction procedure as optimized below. The chromatogram depicted in fig. 2C showed some small peaks, well separated from the drug peak, but in absence of standard metabolite or any definite chromatographic pattern, it was difficult to identify the metabolite peak. All these peaks did not interfere with the drug analysis. Fig. 3 depicted the changes in drug concentrations at various designated time points (0, 30, 60, 120, 180, 240, 300 min). The pharmacokinetic parameters were calculated with a non-compartmental model using Thermo Kinetica PK/PD analysis software (version 5.0 Thermo Fisher Scientific). The peak plasma concentration (C_{max}) and the corresponding time (T_{max})

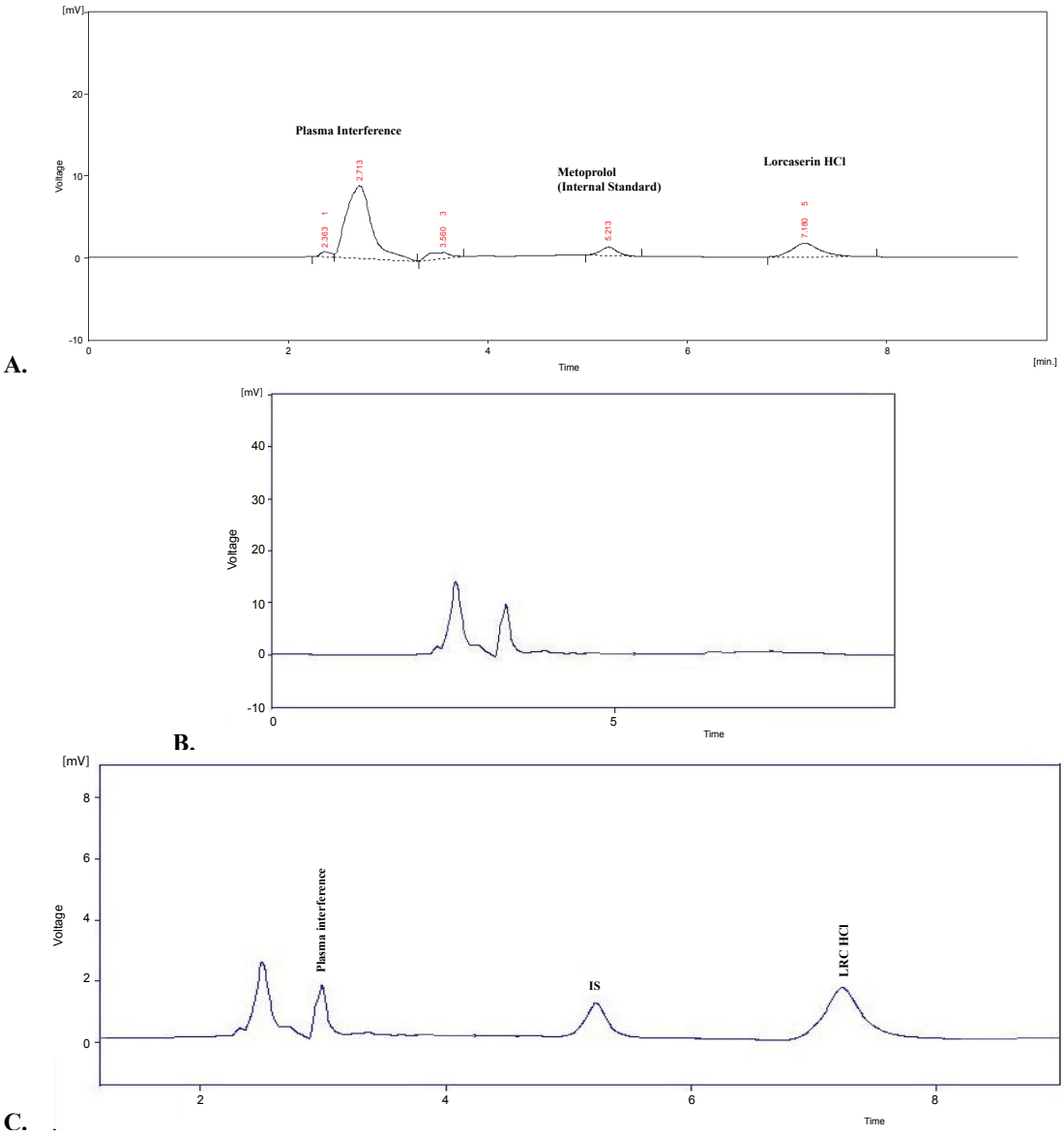

Fig. 2: HPLC chromatograms

Chromatogram of analyte and internal standard spiked in human plasma (A); chromatogram of blank unspiked rat plasma sample (B); chromatogram of extracted sample at time interval of 60 min after administration of the drug

TABLE 4: SUMMARY OF STABILITY STUDIES FOR LOR AND MET

Stability conditions		% Accuracy
Bench top stability at RT for 12 h		100.6736±1.60
Freeze thaw stability previously frozen at -70±5° and thawed at room temperature over three cycles		99.12±5.04
Stock solution stability		
Short term (at RT for 7 h)	LOR	99.52±3.541
	MET	101.5±2.835
Long term (at 2-8° for 5 d)	LOR	97.89±3.915
	MET	102.6±4.806

RT: room temperature; LOR: lorcaserin hydrochloride; MET: metoprolol

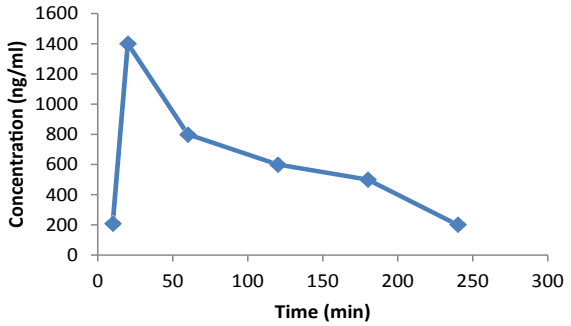

Fig. 3: Mean plasma concentration vs. time profile of LOR after oral administration

were directly obtained from the raw data. The other pharmacokinetic parameters were obtained using non compartment model. AUC_{total} was calculated using mixed log linear model. The pharmacokinetic data is presented in Table 5[27-29].

From the results and discussions, it could be concluded that the method developed for the analysis of LOR in rat plasma is specific, accurate, precise and reproducible. The use of this method could enable the characterization of LOR and its pharmacokinetics after single oral dose without any interference from the metabolite. According to pharmacology and toxicology review by CDER, based on plasma profiles, overall pattern of metabolism in humans is most closely approximated the metabolite pattern seen in rats[30,31]. The assay can therefore be easily extended to quantitate LOR in plasma for routine monitoring of plasma levels of LOR in laboratories.

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Conflict of interest:

The authors declare that there is no conflict of interests.

TABLE 5: PHARMACOKINETIC PARAMETERS FOR BIOANALYTICAL METHOD

Parameters	Observed value	Reported value[31]
C_{max} (ng/ml)	837.2	789
T_{max} (h)	0.5	0.25
AUC_{total} (µg/ml.h)	2.964	Dose dependent
$T^{½}$ (h)	1.539	Dose dependent

C_{max}- peak plasma concentration, T_{max}- time required to reach C_{max}, AUC_{total}- area under curve, $T^{1/2}$-half life

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