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Meloxicam quantification in rabbit plasma by RP-HPLC: optimization and application to pharmacokinetic study

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Abstract

Background: The goal of the proposed study was to validate a rapid, simple, an accurate, robust, and sensitive bioanalytical method for quantifying Meloxicam and Lornoxicam (as internal standard) in rabbit plasma.

Result: Limit of detection and limit of quantification for Meloxicam were found to be 0.0081 and 0.1035 $\mu\text{g mL}^{-1}$, respectively. The bioanalysis was continued according to standard guidelines and successfully used for bioavailability studies of meloxicam after single dose administration of pure drug and the formulation in rabbit plasma. Finally, obtained results proved its simplicity and an efficiency to be applied for the therapeutic drug monitoring and bioequivalence studies.

Conclusion: Therefore, the set RP-HPLC bioanalysis is simple, convenient, and acceptable to analyze meloxicam in bulk and pharmaceutical formulations in rabbit plasma.

Keywords: Bioanalysis, Meloxicam, Lornoxicam, Validation, Pharmacokinetic Study, Rabbit Plasma

Background

Chemically, Meloxicam (MLC) is 4-hydroxy-2-methyl-*N*-(5-methyl-2-thiazolyl)-2*H*-1,2-benzothiazine-3-carboxamide-1,1-dioxide (Fig. 1a) [1], a new nonsteroidal anti-inflammatory drug (NSAID) derived from enolic acid, which exhibits cyclooxygenase (COX)-2 selectivity over COX-1 as described in a randomized double-blind study [2]. Meloxicam is used in the treatment of rheumatoid arthritis, osteoarthritis, and other joint diseases [3].

Plentiful UV-VIS, HPLC, HPLC-MS/MS, LC-MS, LC-MS-MS, LC-ESI-MS/MS, LC-MS/TOF, and LC-MS analytical methods have been documented for quantifying the MLC from different formulation either alone or in combination with pharmacotherapeutic agents in biological fluid [1, 4–18]; a couple of them namely were employed liquid–liquid extraction or protein precipitation extraction (PPE) approach for quantification of

MLC from biological sample [5, 7, 14]. In spite of that, the reported approaches are time-consuming; some of them used organic solvents and hazardous solvents for the extraction process, and some of which reported much less recovery which may be due to the drug loss during the transfer. Moreover, most of which followed solid-phase and liquid-liquid extraction techniques which required a more complex process [19]. In addition, the strong chemical bonds between plasma proteins and pharmacotherapeutic agents lead to a decrease in the efficiency of extraction, thus completely hampering the removal of pharmacotherapeutic agents, and consequently, a lower recovery [20]. Though solid-phase extraction has been reported by Miyamoto et al. to quantify the MLC from biological sample, this method requires expensive equipment [1]. Couple of the bioanalytical methods used to quantify the MLC did not use the internal standard, which seemed to be a limitation of the reported method; some of which had been used but were complex in nature [8, 11, 12, 15, 17].

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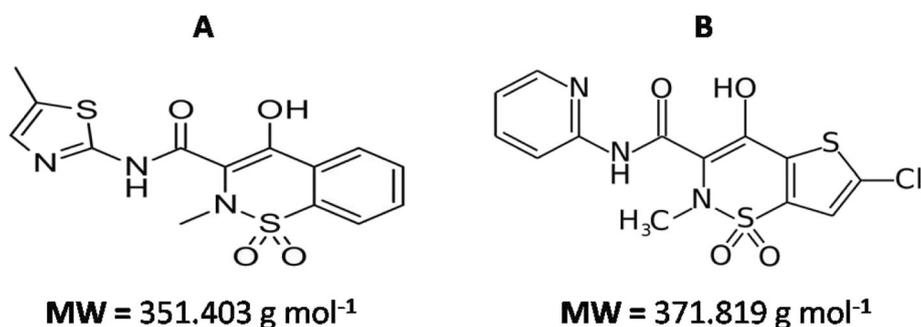


Fig. 1 Chemical structure of **a** Meloxicam and **b** Lornoxicam

Therefore, it would be very beneficial to find a specific simple and efficient bioanalytical method for the rapid estimation of MLC in biological samples and also to reduce the above mentioned deficiencies. Furthermore, the binding ability of MLC to plasma proteins requires determining whether other related drugs mediate MLC binding, and vice versa [20]. Precipitation of proteins is the simplest way to extract most of the protein from the plasma and also involves minimal processing steps, which is why protein precipitation approach is used in pharmacokinetics analysis [21]. Biochemical analysis of MLC in plasma should be investigated in accordance with the FDA standards to ensure the safety and efficacy of marketed formulations [22].

The present research was carried out by considering the observations for estimating MLC in rabbit blood plasma using a simple extraction technique with specific and appropriate internal standards (Fig. 1b) to establish and validate a fast, simple bioanalytical reversed-phase HPLC-UV process along with randomized single-dose pilot study to test the bioavailability of MLC in a formulation.

Methods

Chemicals and reagents

HPLC grade methanol and acetonitrile (ACN) were purchased from MERCK India Pvt. Ltd. Meloxicam (MLC) and Lornoxicam (LRC) were obtained from Macleods Pharmaceuticals Ltd. (Mumbai—400093, Maharashtra, India), and all other chemicals were of analytical grade. Double-distilled water was used for preparing mobile phase solutions. The 0.45 μm nylon filters were purchased from Sigma-Aldrich, India.

Instrumentation

Systronics-HPLC (Isocratic mode) equipped with plus pump (Liquid Chromatography—100), UV/Vis detector (Liquid Chromatography-UV 100), and installed Shanghai Wufeng operating software. The separation was carried out on C8 Capcell pack DDS5 column along with 5 μm particle size and a length of 0.46 cm \times 25 cm.

Chromatographic condition

Fifteen millimolar phosphate buffer having pH 4.7: methanol in the proportion of 40:60 v/v was prepared as a mobile phase, and then the mobile phase was degassed using sonicator for continuous 30 min. The final solution was filtered by using Millipore vacuum filter system with 0.45 μm membrane filter paper. The prepared mobile phase was run to maintain flow rate at 1.0 mL min^{-1} . UV 100 detector was set at 357.0 nm for the column effluence.

Preparation of stock solution

One hundred milligrams of MLC and LRC were transferred into a separate 100 mL volumetric flask, and then the 20 mL mobile phase was added and mixed with continuous shaking. The clear solution was ultra-sonic for a further 15 min and the resulting solution was then diluted into the target with the same solvent in order to obtain a final solution with a concentration of 1000 $\mu\text{g mL}^{-1}$. Furthermore, concentration ranges of MLC from 0.5 to 25 mg mL^{-1} were prepared using stock solution of MLC further diluted with mobile phase and stored under standard condition. Additionally, the working standard solution of LRC (internal standard) was prepared a final concentration of 40 $\mu\text{g mL}^{-1}$ with an addition of LRC stock solution.

Extraction procedure

One hundred microliters of the plasma and 50 μL of respective concentrations (0.50, 1.00, 2.00, 5.00, 10.00, 15.00, 20.00, and 25.00 $\mu\text{g mL}^{-1}$) of MLC solutions were dropped into eight labeled microcentrifuge tubes (1500 μL capacity), and then all prepared samples were vortexed for a minute. Subsequently, 50 μL of LRC (40 $\mu\text{g mL}^{-1}$) as an internal standard was added into resultant each concentration solution. Repeatedly, the all resultant solutions were vortexed for a minute with following medium speed. Further, 100 μL of protein precipitating agent namely ACN was dropped in each labeled tube and the all resultant solution were vortex for 2 min under medium speed. Finally, the all prepared samples

were precipitated at room temperature, followed by centrifugation at 5000 rpm for continuous 15 min, and the supernatant was filtered through a 0.45 μm filter paper. At last, 20 μL of all samples were assessed under selected chromatographic condition.

Methods

In order to estimating MLC in rabbit plasma with precise, accurate, and reproducible bioanalytical reverse-phase HPLC-UV method, various solvent combination for mobile phases, stationary phases, and MLC preparation techniques have been assessed; however, the proposed mobile phase, stationary phase, MLC preparation techniques, λ_{max} , etc. have been found to be appropriate for quantitative determination of MLC and internal standard. Finally, the proposed optimized and developed method for MLC was validated for selected parameters according to standard guidelines [22, 23].

System suitability test

Prior to the analysis of each batch of samples, tests were carried out to confirm the reproducibility of the chromatographic system according to USP24/NF19 [24]. However, selected criteria namely retention time (RT), theoretical plate number (N), and tailing factor (T) are based on the actual performance of the method, as specified during its validation [25]. Selected parameters for the MLC peak were assessed to determine the percent coefficient of variation (% RSD) for the system suitability test.

Linearity

The linearity was confirmed using eight concentrations that range from 0.50 to 25.00 $\mu\text{g mL}^{-1}$ of MLC solution. The linearity experiment was tested to detect the response of the detector in linear with different concentrations of MLC solutions spiked in rabbit plasma, i.e., 0.50, 1.00, 2.00, 5.00, 10.00, 15.00, 20.00, and 25.00 $\mu\text{g mL}^{-1}$ with fixed concentration of LRC solution as an internal standard using the abovementioned extraction method. Finally, the prepared concentrations were injecting into the HPLC system and the calibration curves were constructed by plotting peak area of MLC versus concentrations of MLC, and the correlation coefficient, slope, and intercept were obtained from regression equations. Signal-to-noise ratios have been used to determine the limit of detection (LOD) and limit of quantification (LOQ) of MLC [26].

LLOQ (sensitivity)

The LLOQ is the lowest sample analysis concentration, can be accurately and precisely quantified, can be considered the lowest calibration curve point, and should be considered at least 5 times the blank sample [27–29].

Precision

The quality control (QC) sample having a concentration of 0.50, 5.00, and 25.00 $\mu\text{g mL}^{-1}$ are represented in the calibration curves of the MLC between the low, medium, and high ranges in order to determine the accuracy and precision of the system being developed. Six injections of each concentration (0.50, 5.00, and 25.00 $\mu\text{g mL}^{-1}$) were tested for the intraday study on the same day, while nine injection of each concentration (0.50, 5.00, and 25.00 $\mu\text{g mL}^{-1}$) were tested for an interday variation over a period of 15 day and finally observed results were used to calculate % RSD in precision, whereas accuracy was expressed as a percentage of the theoretical concentration [30].

% Recovery (accuracy)

In order to confirm the accuracy of the proposed method, three different concentrations of linearity curve covering the lower, medium, and higher ranges with a concentration of 0.50, 5, and 25 $\mu\text{g mL}^{-1}$ respectively were assessed. Absolute and relative recovery of the same nominal concentration for MLC was calculated by adding known quantities of MLC to the MLC free rabbit plasma. At the same nominal concentrations, absolute recoveries were determined with the peak area of the extracted QC samples compared to the peak area of the recovery standards [30–33].

Robustness

In proposed study, robustness is used to determine the effect of a small but deliberate variance in the flow rate of the pump at 0.9 and 1.1 mL min^{-1} ; pH of the phosphate buffer at 4.6 and 4.7; and percent of the phosphate buffer on 38 and 42 % in the mobile phase and were noted in % RSD.

Specificity

Checking any interference of an endogenous compound or corresponding placebo in the retention time of the MLC and LRC.

Stability

Stock solutions stability study was evaluated in three ways viz: (1) the freshly prepared plasma rabbit sample was accessed and then held overnight at a temperature of 15–20 $^{\circ}\text{C}$ for 24 h and then re-injected into the HPLC device; (2) rabbit plasma samples were stored overnight in the freezer at -20°C , collected at room temperature, and injected within 1 h after thawing thoroughly; and (3) standard stock solutions stored for 1 week at -20°C in freezer, brought to room temperature, and injected within 1 h after thawing.

Application to bioavailability studies

In a single dose pilot study with randomized, single dose, crossover protocol, bioavailability and bioequivalence of bulk MLC (as a reference) and its tablet formulation (MELFLAM tab, Cipla Ltd, Mumbai) were assessed via simple extraction procedures namely protein precipitation technique. The bioavailability and bioequivalence study of pure MLC and MELFLAM tabs was checked using albino rabbits (Protocol No. BVCPK/CPCSEA/IAEC/1/5) of either sex weighing between 2.100 and 3.200 kg and sustained under standard conditions as defined by IAEC BVCP, Kolhapur-416013, MS, INDIA. The selected six rabbits were equally divided into standard and test group; the standard and test group received suspension of pure MLC drugs and MELFLAM tab respectively. Subsequently, an aliquot of 1 mL of blood sample was drawn from the marginal ear rabbit vein before and after administration of the MLC test sample and the MELFLAM tab (15 mg) sample at intervals 0, 0.5, 1, 2, 3, 4, 6, 8, 12, 24, 36, and 48 ho, as shown by the Sherman process [34]. The collected rabbit blood samples were then placed in Vacutainer Heparin Tubes and followed the centrifugation for 10 min at 4 °C at a speed of 10,000 rpm, and then the supernatant was collected and stored in brown tubes at -30 °C until analysis time. The pharmacokinetic parameters namely maximum peak plasma concentration (C_{max}), T_{max} , area under curve (AUC), mean residence time (MRT), half-life at the terminal phase ($T_{1/2}$), and elimination rate constant (K_e) were assessed under non-compartmental moment analysis model.

Statistical analysis

AUC_{0-t} , C_{max} , and $AUC_{0-\infty}$ were considered as crucial variables in bioequivalence study of MLC (in bulk and formulation) and were checked with the means of analysis of variance (ANOVA). 90% CI was calculated using Log transformed data [35, 36].

Results

The developed bioanalytical method was validated as per United State— Food & Drug Administration guidelines [20, 22, 28, 29].

System suitability test

The theoretical plate number (N) and tailing factors for MLC ($15 \mu\text{g mL}^{-1}$) were found to be 2251.50 ± 1.9992 and 1.69 ± 1.7553 respectively. The chromatographic conditions described ensured adequate retention and asymmetry for drug compounds [26, 37]. Therefore, the retention time of MLC was noted at 6.2398 ± 0.8608 min (Table 1). The theoretical plate were observed in the range of 2210 to 2339, whereas tailing factor of the MLC were noted in the range of 1.65 to 1.74. However, it was found that MLC retention time was within 6.1769 to 6.2914 min.

Specificity

According to the chromatogram results, no interference from impurity, excipients, or additives were found and the all present additives in tablets were practically insoluble in mobile phase (Fig. 2).

Linearity

Linear calibration curves was observed after plotting peak area versus concentration of MLC over the concentration range of $0.5\text{--}25 \mu\text{g mL}^{-1}$ and the correlation coefficients were found to be 0.9940, whereas intercept and slope were found to be -576.45 and 1324.25 respectively for MLC (Table 2). Limit of detection and limit of quantification for MLC in rabbit plasma samples were found to be 0.0081 and $0.1035 \mu\text{g mL}^{-1}$ respectively and proved the sensitivity of the proposed bioanalytical methods.

LLOQ (sensitivity)

Diluting known concentrations of MLC in rabbit plasma was used to check the LLOQ; however, the proposed assay method offered an LLOQ of $0.50 \mu\text{g mL}^{-1}$ for

Table 1 System suitability test for MLC ($n = 6$)

Obs. No.	Concentration ($\mu\text{g mL}^{-1}$)	Peak area		Area ratio	Retention time	Theoretical plate	Tailing factor
1	15	17686.2	20075.2	0.8809	6.2681	2235	1.65
2	15	17807.8	20496.7	0.8688	6.1769	2210	1.69
3	15	17864.2	20267.1	0.8814	6.2914	2250	1.74
4	15	17388.7	20705.5	0.8398	6.1985	2250	1.68
5	15	17773.5	20789.5	0.8549	6.3021	2225	1.68
6	15	18098.9	20550.5	0.8807	6.2021	2339	1.70
Mean		–	–	0.8677	6.2398	2251.5	1.69
S.D.		–	–	0.0171	0.0537	45.5137	0.0296
% RSD.		–	–	1.9804	0.8608	2.0214	1.7553

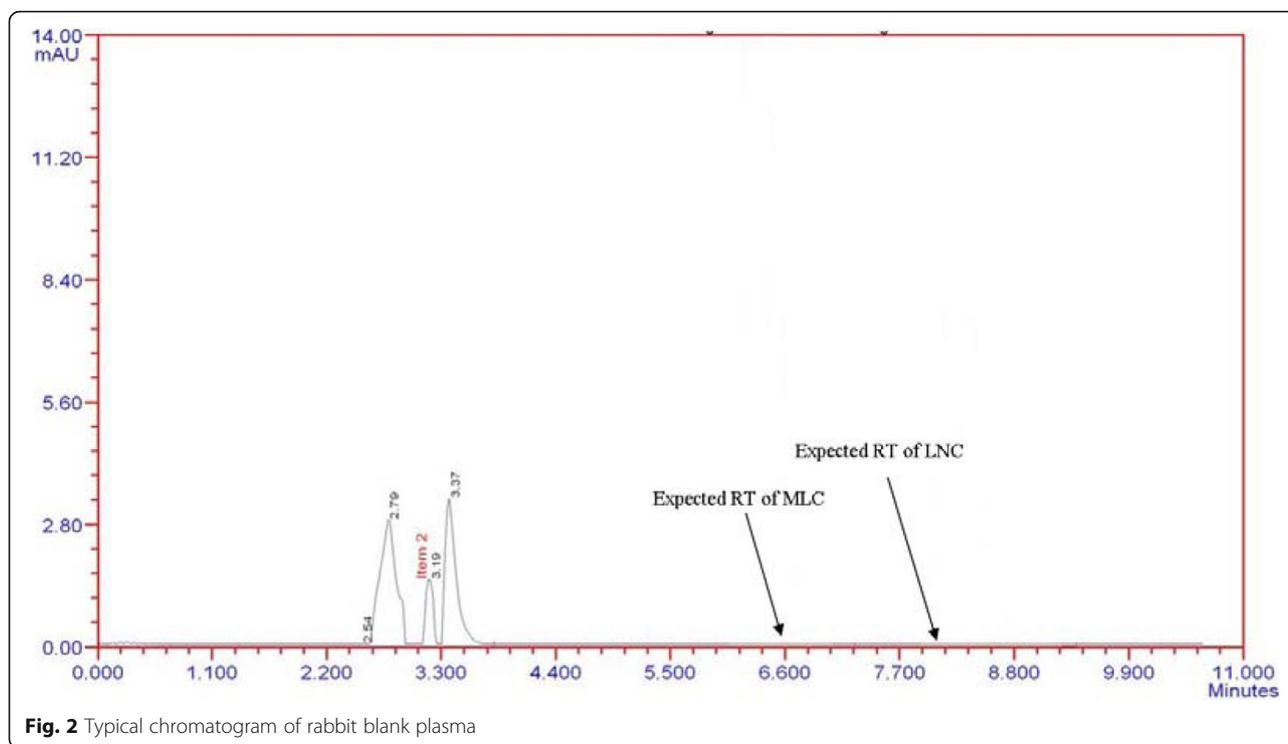


Fig. 2 Typical chromatogram of rabbit blank plasma

MLC in rabbit plasma. % RSD for the intraday precision and accuracy was noted to be 1.85% (in RSD) and 98.56% respectively for MLC. Whereas, an interdays precision of the method was found to be 1.95% in RSD and the accuracy of the method was found to be 95.98% for MLC.

Accuracy (% recovery)

The absolute recovery of MLC was found to be 91.35 ± 1.55 , 93.31 ± 1.66 , and 94.71 ± 1.43 for the concentration of 0.50, 5.00, and 20.00 $\mu\text{g mL}^{-1}$ respectively, while the LRC was noted to be 89.85 ± 1.18 (Table 3). Moreover, relative recovery of the MLC was determined by comparing the strength of the drug-spiked plasma with the actual added concentration. Relative percent recoveries for 0.50, 5.00, and 20.00 $\mu\text{g mL}^{-1}$ MLC solution were found to be 101.00 ± 1.97 , 100.36 ± 1.85 , and 100.62 ± 0.88 respectively (Table 3).

Precision

The intraday precision of the proposed method was noted to be 1.97, 1.85, and 0.88 % RSD for MLC concentration of 0.50, 5.00, and 20.00 $\mu\text{g mL}^{-1}$ respectively, whereas interday precision was found to be 1.09, 1.63, and

1.60 $\mu\text{g mL}^{-1}$ % RSD for 0.50, 5.00, and 20.00 $\mu\text{g mL}^{-1}$ concentration of MLC, respectively. The above results indicated that the method is precise (Table 4). Intraday accuracy was noted to be 101.00, 100.36, and 100.62% for MLC concentration at 0.50, 5.00, and 20.00 $\mu\text{g mL}^{-1}$ respectively, while interday accuracy were observed to be 99.36, 98.18, and 98.95 for MLC at concentration 0.50, 5.00, and 20.00 $\mu\text{g mL}^{-1}$ respectively. The accuracy results also indicate that the proposed method is accurate.

Robustness

The intentional changes in the method have not been much affected in the selected parameter namely tailing factor, theoretical plates, and the % assay. Tailing factor, theoretical plate, and percent assay of the MLC were found to be 1.7983 ± 1.98 , 2396.93 ± 1.62 , and 94.59 ± 0.95 after adjustment of 74% phosphate buffer in the mobile phase respectively, whereas 74% phosphate buffer was reported to be 1.7033 ± 1.95 , 2356.50 ± 1.55 , and $94.15 \pm 1.08\%$ respectively. However, changing of flow rate at 0.9 mL min^{-1} , tailing factor, theoretical plate, and percent assay were noted to be 1.8233 ± 1.69 , 2410.83 ± 1.84 , and $93.57 \pm 0.89\%$ respectively, while the flow rate of the pump was adjusted at 1.1 mL min^{-1} , the tailing

Table 2 Linear regression analysis of calibration curves ($n = 6$)

Drug	Linearity range ($\mu\text{g mL}^{-1}$)	Intercept	Slope	Coefficient of determination (r^2)	LOD ($\mu\text{g mL}^{-1}$)	LOQ ($\mu\text{g mL}^{-1}$)
Meloxicam	0.5–25	-576.45	1324.25	0.9940	0.0081	0.1035

Table 3 Absolute and relative recovery of MLC and IS ($n = 6$)

Concentration added		Absolute recovery (mean* \pm % RSD)	Relative recovery (mean* \pm % RSD)
Meloxicam	0.50 ($\mu\text{g mL}^{-1}$)	91.35 \pm 1.55	101.00 \pm 1.97
	5.00 ($\mu\text{g mL}^{-1}$)	93.31 \pm 1.66	100.36 \pm 1.85
	20.00 ($\mu\text{g mL}^{-1}$)	94.71 \pm 1.43	100.62 \pm 0.88
IS	5.00 ($\mu\text{g mL}^{-1}$)	89.85 \pm 1.18	–

factor, theoretical plate, and percent assay noted to be 1.8383 ± 2.00 , 2426.83 ± 1.98 , and 92.37 ± 0.84 % respectively. Moreover, when pH of the phosphate buffer was changed from 4.7 to 4.6, the tailing factor, theoretical plate, and % assay of the MLC was observed to be 1.7350 ± 1.54 , 2274.66 ± 1.91 , and 92.45 ± 1.49 , respectively, whereas when pH was increased from 4.7 to 4.8, the tailing factor, theoretical plate, and % assay of the MLC noted to be 1.8433 ± 1.08 , 2418.50 ± 1.82 , and 90.78 ± 0.80 % respectively. All the results are presented in mean \pm % RSD.

Stability

First stability study indicated that even at an ambient temperature (25 ± 2 °C), MLC spiked rabbit plasma samples remained stable for 24 h and although at frozen conditions, MLC sample also remained stable for 8 days. Moreover, bench top stability study for MLC sample found to be 98.47 and 99.60% nominal for low- and high-quality control samples respectively, while 98.74 and 98.27% nominal for low- and high-quality control samples were reported in the freeze thaw stability study, respectively. In addition, an autosampler stability result of the proposed method found to be 96.57 and 98.44% nominal for MLC. All the results showed that the approach established is stable for the short term (Table 5).

Pharmacokinetic study

The developed protein precipitation extraction method was used to determine the bioavailability parameter after oral administration of the MLC bulk drug (reference) and MELFLAM tab (test) sample. Rabbit plasma (blank rabbit plasma), MELFLAM tab (after 1 h), and bulk drug suspension (after 1 h) were shown in Fig. 3a–c respectively.

Concentration time profiles of MLC in rabbit plasma following oral administration of the test and reference sample at interval 0, 0.5, 1, 2, 3, 4, 6, 8, 12, 24, 36, and 48 h were presented in Fig. 4. However, all pharmacokinetic parameters namely C_{max} , AUC_{0-t} , $AUC_{0-\infty}$, MRT, $T_{1/2}$, and Ke for MLC (test and formulation) were determined after administration of MELFLAM tab formulation and MLC bulk drug using a non-compartmental timing analysis model. C_{max} , AUC_{0-t} and $AUC_{0-\infty}$ were found to be 0.6577 ± 0.01 , 17.3070 ± 0.29 , and 25.2047 ± 0.67 $\mu\text{g mL}^{-1}$ respectively for reference sample, whereas for standard sample, C_{max} , AUC_{0-t} and $AUC_{0-\infty}$ were noted to be 0.7792 ± 0.01 , 19.7973 ± 1.06 , and 25.1649 ± 0.89 $\mu\text{g mL}^{-1}$ respectively. In addition, T_{max} , $T_{1/2}$, and MRT of the reference sample were observed to be 6.1 ± 0.36 , 21.5261 ± 0.57 , and 31.2453 ± 0.69 h respectively, while 4.0 ± 0.46 , 20.3629 ± 0.20 , and 29.3837 ± 0.55 h T_{max} , $T_{1/2}$, and MRT were found for the test sample. However, Ke for reference and test sample was observed to be 0.0320 ± 0.01 and 0.0340 ± 0.01 , respectively (Table 6), and the two-tailed unpaired student t test was also used to measure the statistical significance for MLC between groups. Geometric mean ratio of T/R for 90% CI of C_{max} , AUC_{0-t} and $AUC_{0-\infty}$ were noted to be 118.50 (115.21 and 121.78), 114.57 (106.66 and 122.47), and 100.09 (91.76 and 108.41) % respectively, which indicate test formulation/reference formulation lies between 80.00 and 125.00%. The p values obtained were noted to be 0.0002, 0.9853, 0.7709, 0.0146, 0.9493, 0.2436, and 0.4826 for the C_{max} , T_{max} , $T_{1/2}$, AUC_{0-t} , $AUC_{0-\infty}$, MRT, and Ke respectively, which suggested that the

Table 4 Result from study of intraday and interday precision and accuracy for MLC

Theoretical concentration ($\mu\text{g mL}^{-1}$)	Meloxicam					
	Intraday measured concentration			Interdays measured concentration		
	(mean ^a \pm S.D)	RSD %	Accuracy %	(mean ^b \pm S.D)	RSD %	Accuracy %
0.50	0.5050 \pm 0.01	1.97	101.00	0.4968 \pm 0.01	1.09	99.36
5.00	5.0183 \pm 0.09	1.85	100.36	4.9094 \pm 0.08	1.63	98.18
25.00	25.1564 \pm 0.22	0.88	100.62	24.6935 \pm 0.39	1.60	98.95

^a Values stands for 6 different plasma samples for each concentration

^b Values stands for 9 different runs over 2-week period

Table 5 Result from stability study for MLC ($n = 6$)

Sr. No.	Bench top		Freeze thaw		Autosampler	
	LQC	HQC	LQC	HQC	LQC	HQC
1	0.5021	25.1013	0.5014	24.9524	0.4895	25.0021
2	0.4982	24.4566	0.4982	24.5694	0.4789	24.1564
3	0.4856	25.0012	0.4892	24.5469	0.4625	24.9564
4	0.4761	24.9816	0.4985	24.9426	0.4892	24.5468
5	0.4921	24.8564	0.4856	24.1546	0.4911	24.4576
6	0.5001	25.0021	0.4892	24.2345	0.4856	24.5462
Mean	0.4923	24.8998	0.4936	24.5667	0.4828	24.6109
S.D.	0.009	0.230	0.006	0.340	0.009	0.320
RSD (%)	1.82	0.93	1.31	1.38	2.07	1.30
Nominal (%)	98.47	99.60	98.74	98.27	96.57	98.44

test formulation was quite similar to the reference formulation for pharmacokinetic parameters in MLC. Accordingly, the test and reference formulations were considered to be bioequivalent. All the findings are consistent with previous MLC interaction reports [1, 4, 5, 7, 10, 12, 14, 18]. In the final analysis, the results obtained for applying the proposed bioanalytical RP-HPLC approach have shown its efficacy in applying therapeutic drug monitoring and bioequivalence studies.

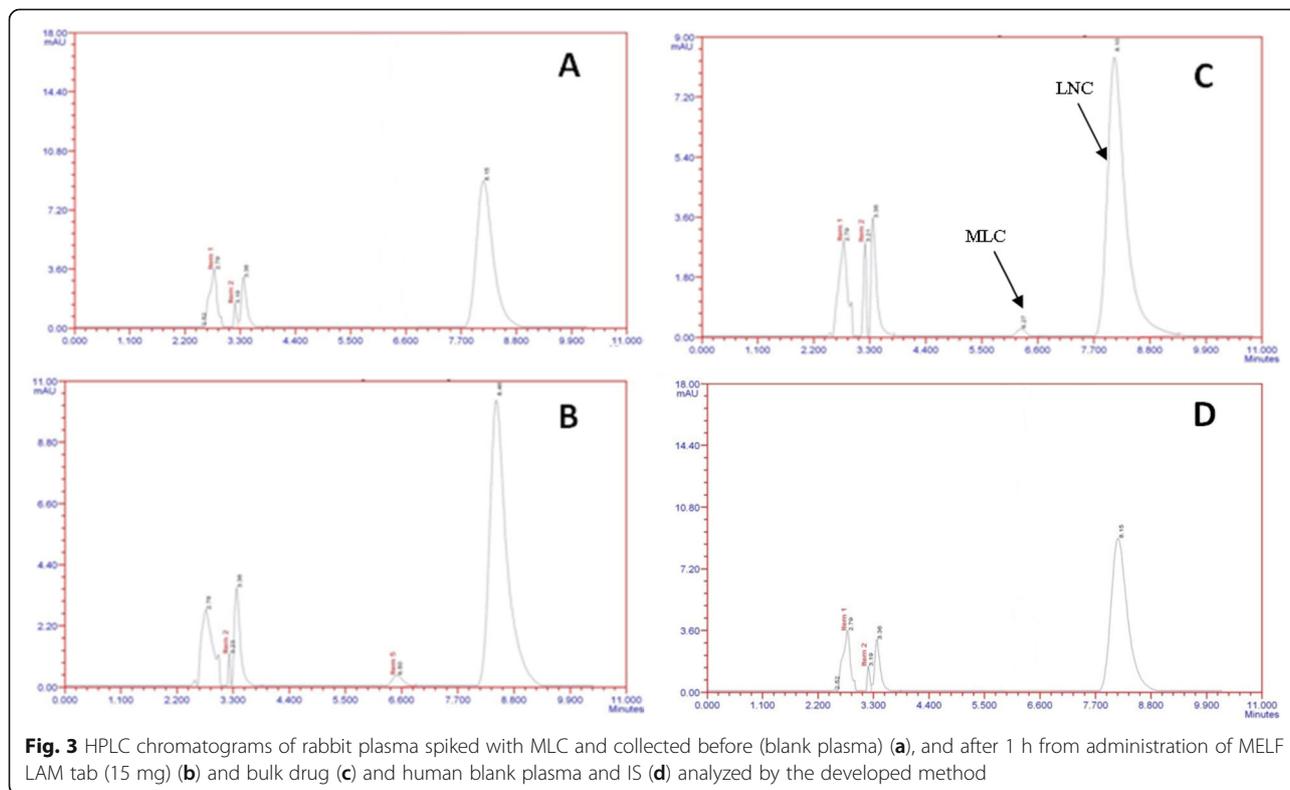
Discussion

Optimization of extraction method

The simple extraction method is important for bioanalysis if you want to progress in extraction efficiency. So, we studied various factors and got them together and the names are as follows, i.e., ion strength, dispersing solvents, pH, sample volume, and applied time. Solvents are very critical in the extraction process, so when selecting the solvent, it must be taken into account high densities, water immiscibility, and high solubility for analytes [38]; thus, the solvent is chosen according to the above. As a result, acetonitrile, diethyl ether, and methanol solvents have therefore been employed for detection of MLC and internal standard from rabbit plasma. In the ACN, the recovery of the MLC and internal standard was better than that of the solvents; in addition, the volume of ACN in the range of 0.05 to 0.5 mL should also be checked for better recovery. The best recoveries of the MLC and internal standard have been recorded with 0.1 mL of acetonitrile extraction solvent; henceforth, 0.1 mL ACN volume was continued for the experiment. As a result, an average recovery has been shown to be 98.83 ± 1.32 and $93.62 \pm 1.47\%$, respectively, for MLC and LRC.

Optimization chromatographic conditions

To achieve the greatest selectivity in separating and evaluating target products, optimization of chromatographic

**Fig. 3** HPLC chromatograms of rabbit plasma spiked with MLC and collected before (blank plasma) (a), and after 1 h from administration of MELF LAM tab (15 mg) (b) and bulk drug (c) and human blank plasma and IS (d) analyzed by the developed method

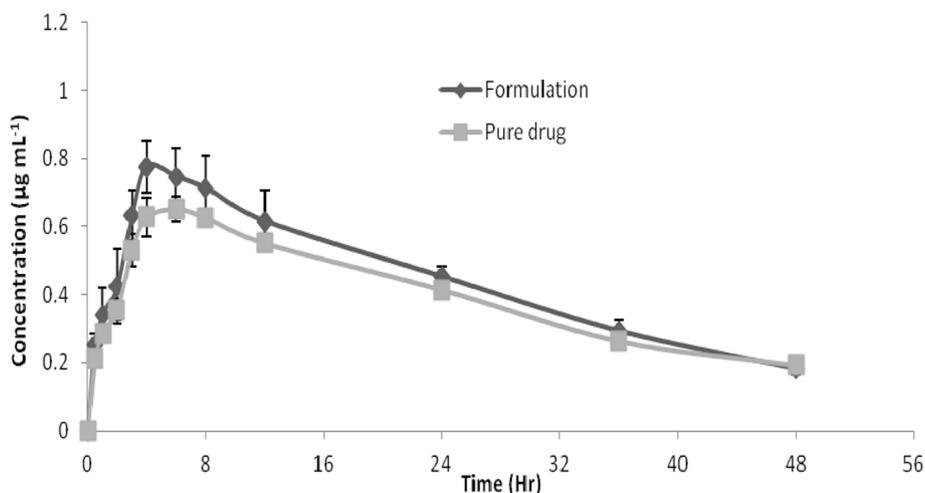


Fig. 4 Plasma concentration–time profiles of MLC in rabbit plasma after oral administration of test and reference sample

conditions is necessary [20]. A variety of chromatographic conditions namely flow rate, pH of the buffer, ratio of the mobile phase, and different solvents of the mobile phase have been tried to improve the resolution and shape of the peak for MLC and LRC. Nonetheless, various combinations of water, phosphate buffer, methanol, ACN, etc. were tried to set the mobile phase, among them the combination of methanol and phosphate buffer had a good peak shape and also an increased sensitivity over other, but as their volume ratio changed their runtime and resolution was changing. The ratio of methanol and phosphate buffer was therefore maintained at 60:40, so that the runtime and resolution of MLC and LRC would be better. Moreover, the extraction solvent and the LC mobile phase have difference in nature and strength. ACN is a very strong eluting solvent when injected into a LC system with the methanol and buffer as a mobile phase which have potentially impacted on the chromatogram and tailing factor of MLC.

In addition, variability of the phosphate buffer pH led to poor peak shape (especially at bottom). Finding the right pH for the phosphate buffer was difficult because pH of the buffer increased, broadening at MLC and LRC peak base were to be observed. The pH of the phosphate buffer has therefore been changed to precisely 4.7 by using *o*-phosphoric acid.

Moreover, flow rate parameter was assessed at interval 0.2 mL min^{-1} from 0.4 to 1.6 mL min^{-1} to get proper resolution and shape of MLC and LRC peak; consequently, MLC and LRC peak merging was observed at a higher range and also high expansion with high resolution of MLC and LRC peak were noted at a lower range; henceforth, 1.0 mL min^{-1} flow rate was part of optimized chromatographic parameter having better shape of peak. Eventually, the optimized mobile phase consisted of 15 mM phosphate buffer having pH 4.7: methanol (40:60 v/v), with 357.0 nm λ_{max} of UV

Table 6 Pharmacokinetic parameters of MLC in rabbit plasma after oral administration of MELFLAM tab (15 mg) tablets and Bulk drug ($n = 6$)

Pharmacokinetic parameter	Reference (mean \pm SD)	Test (mean \pm SD)	Geometric mean ratio of T/R (90% CI) ^a	<i>P</i> value
C_{max}^a , $\mu\text{g mL}^{-1}$	0.6577 ± 0.01	0.7792 ± 0.01	118.50% (115.21, 121.78) ^b	0.0002
T_{max} , h	6.1 ± 0.36 (5.8–6.5)	4.0 ± 0.46 (3.5–4.4)	–	0.9853
$T_{1/2}$, h	21.5261 ± 0.57	20.3629 ± 0.20	–	0.7709
AUC_{0-t}^a , $\mu\text{g h mL}^{-1}$	17.3070 ± 0.29	19.7973 ± 1.06	114.57 (106.66, 122.47) ^b	0.0146
$\text{AUC}_{0-\infty}^a$, $\mu\text{g h mL}^{-1}$	25.2047 ± 0.67	25.1649 ± 0.89	100.09 (91.76, 108.41) ^b	0.9493
MRT, h	31.2453 ± 0.69	29.3837 ± 0.55	–	0.2436
K_e	0.0320 ± 0.01	0.0340 ± 0.01	–	0.4826

^a Bioequivalence criteria are defined as 90% CI of the geometric mean ratios of the test formulation/reference formulation lies between 80.00% and 125.00% for AUC_{0-t} , $\text{AUC}_{0-\infty}$, and C_{max} . ^bStatistical calculations for AUC and C_{max} were based on log-transformed data; c values in the bracket indicate with range (lowest T_{max} – highest T_{max})

detector. During all analytical processes, the retention period for MLC was continuously observed at about 6.2498 ± 1.4001 min (Fig. 5). The linear regression of the MLC showed a very significant non-zero intercept. The representative chromatogram rabbit plasma with

LRC is depicted in Fig. 3d. Whereas, Fig. 5a–h illustrates the chromatogram of rabbit blood plasma spiked with MLC and internal standard in the strength of 0.50, 1.00, 2.00, 5.00, 10.00, 15.00, 20.00, and 25.00 $\mu\text{g mL}^{-1}$ respectively.

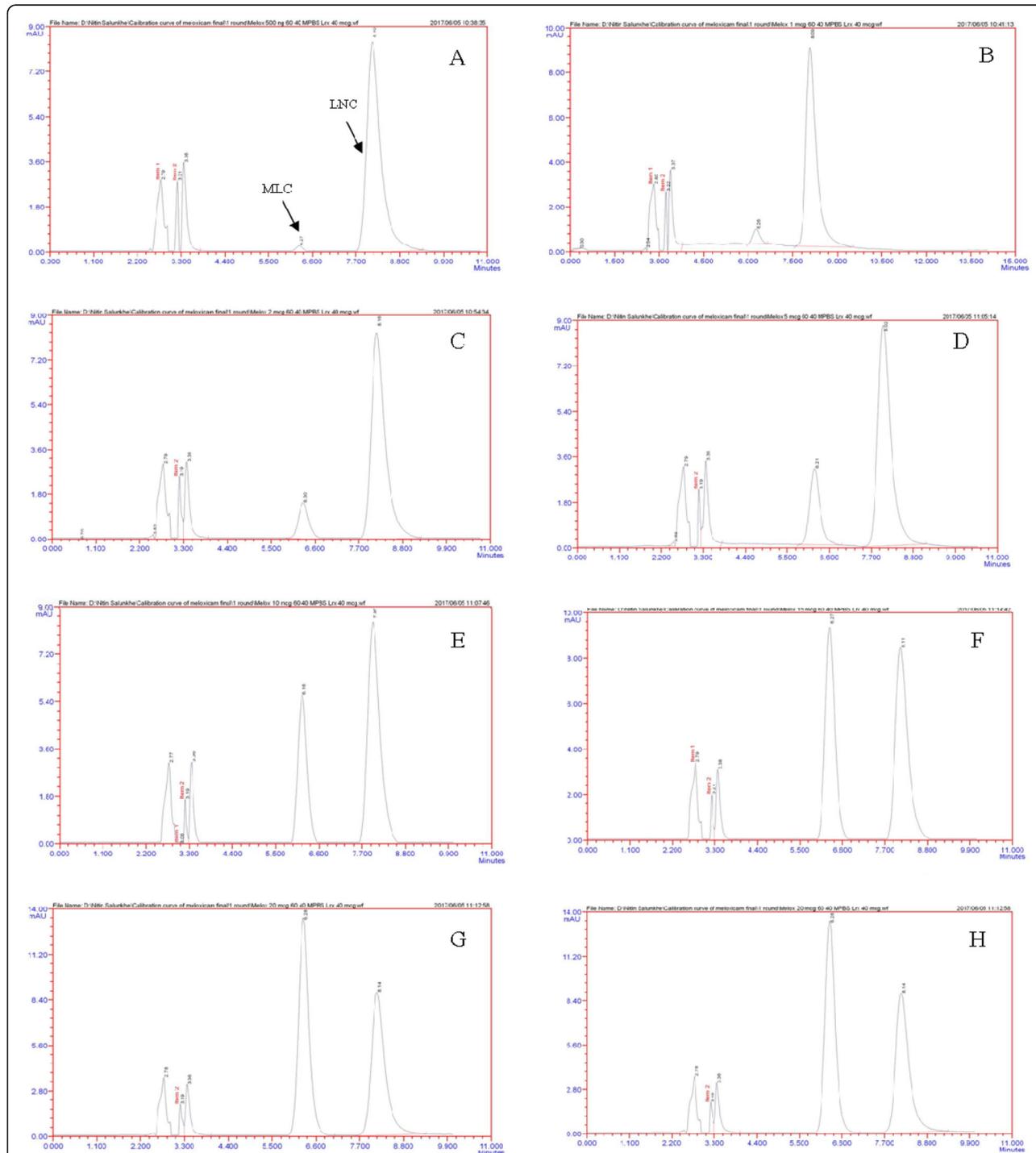


Fig. 5 Typical chromatogram obtained for rabbit plasma spiked with **a** 0.50, **b** 1.00, **c** 2.00, **d** 5.00, **e** 10.00, **f** 15.00, **g** 20.00, and **h** 25.00 $\mu\text{g mL}^{-1}$ MLC and LNC as IS

Table 7 Comparison of the developed HPLC method and the reported methods for analysis of MLC in plasma samples

Author name(s)	Method	Linearity	LOD	Extraction method adopted	% Recovery	Mobile phase	Plasma	Reference number
Miyamoto A	HPLC-UV	20 to 1000 ng mL ⁻¹	Not performed	Solid phase extraction	92.80 %	50 mM phosphate buffer (pH 2.15, solvent A) and acetonitrile (solvent B) at a flow rate of 0.8 mL/min. Isocratic elution (solvent A:solvent B = 60:40)	Rat	1
Dasandi B, Shivaprakash, Saroj H, Bhat KM	HPLC	50 to 1500 ng mL ⁻¹	10 ng mL ⁻¹	Protein precipitation	More than 85%	Sodium acetate buffer (pH 3.3, 170 mmol): acetonitrile (62:38 v/v) mixture	Human	4
Bae JW, Kim MJ, Jang CG, Lee SY	HPLC	10–2400 ng/mL	–	Liquid-liquid extract	Maximum 86.7 %	Acetonitrile–20mM potassium hydrogen phosphate (40:60, v/v, pH 3.5)	Human	5
Hye YJ, Hye WL, Young HK, et al	LC-MS/MS	0.50–200 (ng/mL)	–	Liquid-liquid extraction	87.1 % maximum	Methanol:ammonium formate (15 mM, pH 3.0) (60:40, v/v)	Human	7
Pairis-Garcia MD, Johnson AK, Kukanich B, et al	HPLC-MS	–	0.005 µg/mL	Protein precipitation	–	A. 0.1% formic acid in water and B. 0.1% formic acid in an acetonitrile at a flow rate of 0.250 mL/min	Swine	10
Shukla M, Singh G, Sindhura BG, et al	HPLC	–	0.02 µg mL ⁻¹	Protein precipitation	89.00–93.00 %	65% water:acetic acid (99:1, v/v) and 35% acetonitrile	Human	12
Velpandian T, Jaiswal J, Bhardwaj RK, Gupta SK	HPLC	0.52–52 mg/mL	0.029 mg/mL	Liquid-liquid	102.30%	Methanol, acetonitrile and an aqueous solution of diammonium hydrogen orthophosphate (50 mM) in the ratio of 4:1:5	Human	14
Yuan Y, Chen X, Zhong D	LC-MS	0.10–50.0 ng/mL	–	Protein precipitation	97.20 %	Acetonitrile–water–formic acid (80:200.2, v/v/v)	Human	18
Salunkhe N, Jadhav N, Bhinge S.	UV-HPLC	0.5-25 µg mL ⁻¹	0.0081 µg mL ⁻¹	Protein precipitation	94.71 %	15 mM phosphate buffer (pH 4.7 adjusted with dilute ortho-phosphoric acid solution): methanol (40:60 v/v)	Rabbit Plasma	Proposed Method

Pharmacokinetic study

The proposed approach was effectively implemented in the pharmacokinetic and bioequivalence studies following a single oral dose of MLC bulk drugs and the formulation of MELFLAM tab (15 mg) tablets in rabbit. MELFLAM tab (15 mg) is the product of zydus cadila also prescribed regularly in the region of Karad taluka, Maharashtra—415110. We have served in our region, and most of the doctors prefer the MELFLAM tab to their patients, and therefore we have chosen the said brand for the pharmacokinetic study of the developed method.

Different parameters were verified for the bioequivalence study of bulk drug as reference and MELFLAM tab formulation as test sample of MLC. However, oral suspension of the formulation of MELFLAM tab and bulk drugs were well tolerated by the respective rabbit group and no adverse reactions were observed. Subsequently, rabbit blood plasma of respective groups was tested in HPLC until 48 h for the pharmacokinetic approach. Experimental pharmacokinetic parameters, test/reference ratio (T/R), confidence intervals (90 CIs), and their significant statistical numbers for MLC following oral administration of 15 mg of MELFLAM tablets ranged from 80 to 125% in accordance with the FDA Bioequivalence Guideline for AUC_{0-t} , $AUC_{0-\infty}$, and C_{max} [39, 40].

Figure of merits

Miyamoto et al. [1] have developed a solid-phase extraction approach for the estimation of MLC from biological sample, which have required extra assembly for the extraction process, while Bae et al. [5], Hye et al. [7], and Velpandian et al. [14] have developed a biological method for the estimation of MLC using a liquid-liquid extraction procedure due to the high plasma protein binding characters of MLC that make its complete extraction from plasma suspicious. Moreover Dasandi et al. [4], Pairis-Garcia et al. [10], Shukla et al. [12], and Yuan et al. [18] have used protein precipitation method which is a cost-effective method than abovementioned (Table 7). However, Pairis-Garcia et al. [10] and Shukla et al. [12] have not clearly mentioned the linearity range of meloxicam, while Yuan et al. [18] have used limited validation parameter (excluding LOD, LOQ, etc.). Moreover, Dasandi et al.'s method have followed all validation parameter but recovery of MLC is less than the proposed method (Table 7). Nonetheless, the results of recovery for MLC using 0.1 mL ACN proved that the protein precipitation approach is suitable for MLC quantification and also has high extraction efficiency. Method reproducibility at short times is of major concern in rabbit plasma determination. The process is non-tedious and substantially time consuming compared to the other

method, which takes approximately 13 min to extract the MLC and internal standard with high reproducibility absolutely. Therefore, the expense of testing diminished only was 100 μ L of rabbit plasma and ACN solvent. While the extraction processes have been performed in a simple step, manipulation in a short time is still easy. The validation and bioequivalence results proved the usefulness of the developed biological method in analysis of MLC with LRC.

Conclusion

In conclusion, an optimized and established bioanalytical method for quantification of Meloxicam is selective, precise, accurate, fast, and sensitive. Furthermore, Meloxicam analyte was extracted from biological sample of rabbit with simple extraction process and proved the bioequivalence of bulk drug sample and selected tablet formulation which could aid in therapeutic drug monitoring and bioavailability studies.

Abbreviations

ANOVA: Analysis of variance; AUC: Area under curve; C_{max} : Maximum peak plasma concentration; MRT: Mean residence time; LRC: Lornoxicam; MLC: Meloxicam; LOD: Limit of detection; LOQ: Limit of quantification; LQC: Lower quality control sample; MQC: Medium quality control; HQC: Higher quality control; ACN: Acetonitrile; RSD: Relative standard deviation

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Authors' contributions

NRJ, NS, and SDB carried out the study designs. NRJ, NS, and SDB execution of the proposed study and drafting of manuscript. NRJ, NS, and SDB participated in in vivo studies and performed this statistical analysis. All authors have read and approved the manuscript.

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Availability of data and materials

Data and material are available upon request.

Ethics approval and consent to participate

The research work was approved by Bharati Vidyapeeth College of Pharmacy, Kolhapur, Maharashtra, INDIA as per guidelines of CPCSEA; (Approval no. BVCPK/CPCSEA/IAEC/01/05).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interest.

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