Research Article

DEVELOPMENT AND VALIDATION OF A REVERSE PHASE-HPLC METHOD FOR DETERMINATION OF MELOXICAM IN PHARMACEUTICAL DOSAGE FORMS AND HUMAN PLASMA

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ABSTRACT

Developing and validating a simple, economic, sensitive and selective HPLC method with UV detection (362nm) for the quantitative determination of meloxicam (MLX) in bulk drug, pharmaceutical dosage form and human plasma. Reversed phase chromatographic analysis was performed on a C18 HI-Q-Sil column with acetonitrile: water: 1% aqueous (aq.) glacial acetic acid [56:34:10 % v/v/v] as the mobile phase system. Mobile flow rate was 1ml/min. Piroxicam was used as the internal standard (IS). The method was validated as per International Conference on Harmonization (ICH) guidelines. The developed method demonstrated good resolution between MLX and IS. It was selective to MLX and was able to resolve the drug peak from IS and formulation excipients. The retention time for MLX and IS were approximately 6.9 and 5.8 min, respectively. The polynomial regression for the calibration plots showed good linear relationship with coefficient of correlation, r = 0.9995 ± 0.0002; slope = 28729.04 ± 274.17 and intercept = 20725.38 ± 3191.08 (n=3) over the concentration range studied. The range of reliable quantification was set at 0.3-20 µg/ml, LOD and LOQ were found to be 0.39µg/ml and 1.19µg/ml respectively. Accuracy ranged from 99.96-103.75% and the % relative standard deviation (RSD) for both intra-day and inter-day precision was less than 2%. MLX showed minor degradation in acidic and basic conditions. There was no degradation of MLX in the presence of oxidative, neutral, photolytic, dry heat and wet heat stress conditions. In plasma studies, following a single-step liquid- liquid extraction (LLE) with methanol: 0.1N HCl (1:1), the analyte and IS were separated using the isocratic mobile phase system. The percent recovery of MLX was found to be 90.46 ± 0.53. A linear range of 0.3-5µg/ml was established (r^2=0.9982). LOD and LOQ were 0.28µg/ml and 0.85µg/ml, respectively. The mean accuracy was 86.86-109.66%. The HPLC method was validated with inter- and intra-day precision of 0.71-1.58% and 0.83-2.1% respectively. The proposed method was validated statistically by performing recovery studies by standard addition method, good recoveries from 96.57%-97.68% were found. The stability of MLX in plasma was confirmed by short term and long term stock stability, bench top stability and freeze thaw stability. The proposed methods are simple, economic, precise, reproducible and specific. The method can be extended to quantify MLX in biological fluids, in bulk drug and pharmaceutical dosage forms.

Keywords: Meloxicam, RP-HPLC, Human plasma, Liquid-liquid extraction, Validation.

INTRODUCTION

Meloxicam (MLX) [4-hydroxy-2 methyl-N-(5-methyl-2-thiaolyl)-2H-1,2 benzothiazine-3-carbaxamide 1,1-dioxide], is a highly effective non-steroidal anti-inflammatory drug (NSAID) used to treat rheumatoid arthritis, osteoarthritis, ankylosing spondilitis and other joint pains. It is a favoured cyclooxygenase (COX)-II inhibitor with a superior gastro intestinal tolerability[1]. On oral consumption, it is absorbed slowly but more or less completely with elimination half life of 20h[2]. The chemical structure of MLX is shown in Fig.1.

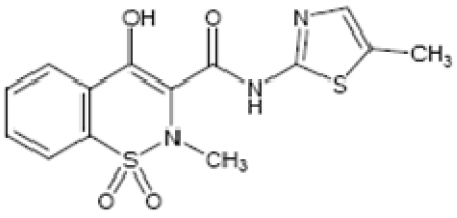

Figure 1: Chemical Structure of MLX

Although MLX is one of the most widely used anti-inflammatory drugs, no reference procedure exists for its determination in pharmaceutical formulations in International Pharmacopoeias. Effectively, there is only one monograph for MLX in the British Pharmacopoeia, based on non aqueous titration, which is not applicable to tablets due to interference from excipients used in pharmaceutical formulations[3]. Few methods have been reported for the determination of MLX including non aqueous titration[4], spectrophotometric methods[5,6], high performance liquid chromatography (HPLC) methods[7-10], fluorimetric methods[11,12], turbidimetric method[13], densitometric method[14], electrochemical method[15], voltammetric method[16], chemiluminometric[17] and electrophoretic method[18]. However most of these analytical methods have some limitations for routine analysis such as tedious and time consuming sample preparation, constant dependency on operator, long sample analysis time and use of expensive solvents and apparatus.

The primary objective of the present study was thus to develop and validate a stability-indicating method which could be employed for the routine analysis of the drug in bulk and pharmaceutical dosage forms. The method was validated as per ICH guidelines[19]. A sensitive, specific, simple and economic RP-HPLC bioanalytical method for MLX quantification in human plasma was developed and validated. A simplified protein precipitation and

extraction procedure was selected for extraction of MLX from the chosen biological matrix. Minimized sample handling and chromatographic run times provided fast quantitative results while maintaining the specificity, accuracy and precision required for the quantification of MLX.

MATERIALS AND METHODS

Reagents and chemicals

Pharmaceutical grade of MLX was obtained as a gift sample from Zest Pharma Indore, India (Batch No. ALC/MLX/090302). It was certified to contain 99.78% w/w (on dried basis) and was used without further purification. The internal standard (IS) for MLX, piroxicam was obtained from Cipla Ltd., Mumbai, India (Batch No. PX 07/0708). HPLC grade LiChrosolv acetonitrile and methanol was from Merck Specialities Pvt. Ltd., Mumbai, India. Glacial acetic acid HPLC grade was obtained from Thermo Fisher Scientific India Pvt. Ltd. (Qualigens), Mumbai, India. Water of HPLC and Spectroscopic grade (J.K. labs, Mumbai, India) was used throughout the study. All other chemicals used were of analytical reagent grade. Commercial pharmaceutical preparation Muvera 15® (Sun Pharma, Sikkim) containing MLX 15mg was purchased from local pharmacy. Fresh frozen human plasma B.P. was obtained from Ashirwad Blood Bank, Mumbai and stored at -20^{o}C until analysis. Prior to the study the plasma was thawed at room temperature (30-37 ^{o}C).

Chromatographic conditions

The system comprised of a Jasco PU-2080 Plus HPLC Pump equipped with Jasco-2075 Plus UV/Vis Detector, LC-Net II ADC as the chromatography interface and a rheodyne injector with a 20-µl loop. Data integration was done using Borwin software package V 1.50. Specificity studies were conducted on Jasco PU-2080 Plus Intelligent HPLC Pump equipped with Jasco MD-2010 Plus multi wavelength detector (Photo Diode Array Detector), Jasco LC-Net II ADC as the chromatography interface and a rheodyne injector with a 20-µl loop. Data integration was done using Chrompass V. 2.1. In addition electronic balance (Mettler Toledo), microlitre syringe (Hamilton, 100 µl), micropipette (Labline Eco, 10-100 µl), refrigerated cooling centrifuge RC 4100 D (Eltek, Electrocraft India Pvt. Ltd., Mumbai), Cyclomixer (Remi Equipment Pvt. Ltd., Mumbai) and micropore filtration assembly was used in this study.

The separation of compounds was made on a HI-Q-Sil C18 column (4.6mm x 250mm, 5µm particle size) (Kya Tech Corporation, Japan). Detection was set at wavelength of 362nm. The mobile phase composed of acetonitrile: water: 1% aq. glacial acetic acid (56:34:10 % v/v/v,) pumped at a flow rate of 1ml/min. The mobile phase was filtered through a 0.45µ pore size membrane filter (Sartorius, Germany) and degassed ultrasonically after mixing. The run time was set at 10 min with the HPLC system operating at room temperature.

Preparation of stock solutions and working standard solution

Standard stock solution of MLX (100 µg/ml) and IS (100 µg/ml) were prepared in methanol. The working standards were obtained by diluting the standard stock solutions with methanol. MLX concentration in the working standard solutions chosen for the calibration curves were 0.3, 0.5, 1, 2, 3, 5, 10, 15 and 20µg/ml containing IS (10µg/ml). In plasma studies, the working standard solutions of MLX were produced by diluting the standard stock solutions with blank human plasma. The six calibration standards of MLX (concentrations: 0.3, 0.5, 1, 3, 5 µg/ml) were prepared independently. The working IS solution (10µg/ml) was prepared by diluting stock with methanol. The procedure for analysis followed is described earlier under the subsection, 'Chromatographic conditions'.

Extraction procedure

MLX solutions and the IS were added to blank plasma samples in a glass tube. Protein precipitation and extraction was carried out by a single-step liquid-liquid extraction (LLE) method. Extraction solvents methanol: 0.1N HCl (1:1 ratio, pH 2.60±0.02) was added to the tubes. The mixture was vortexed for 30 sec. The resultant was centrifuged at -20^{o}C, 5000 rpm for 5min. The supernatant was gently removed with micro-pipette and transferred to HPLC vials. The supernatants obtained were suitably diluted with the mobile phase and subsequently injected into the column for HPLC analysis.

Method development

Initial trial experiments were performed to select a suitable solvent system for estimation of MLX, and to attain good resolution between MLX, IS and the degradation products. The sensitivity of the assay, suitability for stability studies, time required for the analysis, ease of preparation, and use of readily available cost-effective solvents were the decisive factors for the suitability of mobile phase. Various mobile phase systems tried included: methanol: water (70:30, % v/v), acetonitrile: water (70:30, % v/v), methanol: water: acetic acid (55:45:5% v/v), methanol: water: glacial acetic acid (80:19.9:0.1, % v/v/v), methanol: water: 5 % aq. glacial acetic acid (56:34:10, % v/v/v), methanol: water: 0.2M disodium hydrogen phosphate (65:34:1, % v/v/v), methanol: acetonitrile: water: glacial acetic acid (40:40:19.9:0.1, % v/v/v/v), acetonitrile: water: glacial acetic acid (80:19.9:0.1, % v/v/v), acetonitrile: methanol: glacial acetic acid (80:19.9:0.1, % v/v/v), acetonitrile: methanol: 1 % aq. glacial acetic acid (56:34:10, % v/v/v), acetonitrile: water: 1 % aq. glacial acetic acid (50:30:20, % v/v/v) and acetonitrile: water: 1 % aq. glacial acetic acid (60:20:20, % v/v/v). A mobile phase system comprising of acetonitrile: water: 1% aqueous (aq.) glacial acetic acid (56:34:10, % v/v/v) was found to be optimum.

Validation of method: quantitation of MLX in bulk and pharmaceutical dosage form

The proposed method was validated in compliance with ICH Guidelines. The method was validated for linearity and range, limit of detection (LOD), limit of quantitation (LOQ), precision, specificity, accuracy, repeatability and robustness.

Linearity

A calibration curve of MLX was constructed in the concentration range of 0.3, 0.5, 1, 2, 3, 5, 10, 15 and 20µg/ml containing IS (10µg/ml) to establish linearity of the proposed method. The linearity plot was obtained by plotting peak area against corresponding concentrations of MLX. Linear regression analysis was employed to calculate the regression equations and the correlation coefficients.

Limit of detection and limit of quantification

Based on the standard deviation of the response and the slope, LOD and LOQ were estimated using the formulae:

LOD= 3.3 σ/S

Where σ = the standard deviation of the response, S = the slope of the calibration curve

LOQ = 10 σ/S

Where σ = the standard deviation of the response, S = the slope of the calibration curve

LOD and LOQ were determined from the standard deviations of the responses for six replicate determinations.

Repeatability

Injection repeatability: Six injections of 10 µg/ml solution of MLX were analyzed and % RSD was calculated for injection repeatability.

Precision

Precision is a measure of the reproducibility of the analytical method under normal operating conditions. Precision is expressed as relative standard deviation (% RSD).

1. *Intra-day variation:* Measurement of intra-day variation of MLX solutions at three different concentrations (1, 10 and 15 µg/ml) was carried out by injecting the samples on the same day at different time intervals (n=3).
2. *Analysis repeatability:* It was obtained by determining the RSD of replicate samples (n=3) of the accuracy study.
3. *Intermediate precision (Inter-day variation):* Measurement of inter-day variation of MLX solutions at three different concentrations (1, 10 and 15 µg/ml) in triplicate on three consecutive days determined the intermediate precision.

Accuracy

Recovery studies by the standard addition method (n=2) were performed. Previously analyzed samples of MLX (6 µg/ml) were spiked with 50, 100, and 150% extra MLX standard and the mixtures were analyzed by the proposed method. Recovery (%) was calculated for each concentration.

Robustness

Robustness was determined by change in mobile phase composition (± 1 ml organic phase concentration) and flow rate (± 0.1 min).

Sample solution stability

The stability of the drug in solution during analysis were determined by repeated analysis of samples during the course of experimentation on the same day and also after storage of the drug solution for 72 h under laboratory bench conditions (25±2°C) and under refrigeration (8±1°C).

Specificity/Selectivity

The specificity of the method was determined by exposing the sample solution (1mg/ml) to acidic (1N HCl), basic (1N NaOH), neutral and oxidizing (30% H_2O_2), stress conditions. The samples were refluxed for 6 h at 60°C, filtered, suitably diluted and analyzed. MLX was stored in oven at 50°C for 72h to study dry heat degradation and for wet degradation MLX was stored at 50°C and 75% relative humidity for 3 months. The photochemical stability of the drug was studied by exposing the stock solution to direct sunlight for 7 days.

System suitability tests

The chromatographic systems used for analysis must pass the system suitability limits before sample analysis can commence. Injection repeatability, precision, tailing factor (T), theoretical plate number (N) and resolution (Rs) for the principal peak, internal standard and its degradation product were the parameters tested on a 10 µg/ml sample of MLX to assist the accuracy and precision of the developed HPLC method.

Analysis of MLX in marketed tablets (Assay)

Twenty tablets (strength: 15 mg/tablet) were crushed and triturated well in a mortar. A powder sample, equivalent to 15mg of MLX, was accurately weighed and transferred to a 25ml volumetric flask. The drug was extracted into methanol and mixed thoroughly for 10 min using a sonicator. The solution was filtered through 0.45 micron pore filter after making up the volume, adequately diluted with mobile phase and analyzed by the proposed HPLC method. The possibility of interference of excipients with the analysis was studied.

Bioanalytical method development and validation

Calibration curve, LOD and LOQ

The calibration samples were prepared by spiking 1ml of blank plasma with various concentrations of MLX. The extraction was done as per the method described earlier in section "extraction procedure''. The supernatants obtained were suitably diluted with mobile phase to attain a concentration range of 0.3-5 µg/ml in plasma. 20 µl of the samples were injected and peak area was obtained. A calibration curve was constructed by plotting peak areas versus concentrations. All solutions were stored at 4^{o}C and protected from light. Correlation coefficient (r^2) and % CV of the regression line of the standards were used to evaluate linearity, accuracy and precision. A generally accepted CV or 15% of the nominal concentration was taken as the acceptance criteria as per the guidelines by FDA.

A blank sample (matrix sample processed without internal standard), a zero sample (matrix sample processed with internal standard), and six non-zero samples covering the expected range, including LOQ was analyzed for calibration studies.

LOD and LOQ were based on the standard deviation of the response and the slope of the corresponding curve. The acceptance criterion for each back calculated standard concentration was 15% deviation from the nominal value except LLOQ, which was set at 20%. The deviation of the mean from the true value serves as the measure of accuracy.

Determination of accuracy, precision and recovery

The precision of the method was determined by intra-day and intermediate precision (inter-day). 3 concentrations {low concentration (LC), intermediate concentration (IC) and higher concentration (HC) - 1µg/ml, 3µg/ml and 5µg/ml respectively} were measured three times in a day and the same were measured in next three consecutive days. The % CV was calculated.

Accuracy was measured using a minimum of five determinations per concentration (1µg/ml, 3µg/ml and 5µg/ml).

The recovery of an analyte in an assay is the detector response obtained from an amount of the analyte added to and extracted from the biological matrix, compared to the detector response obtained for the true concentration of the pure authentic standard. Recovery pertains to the extraction efficiency of an analytical method within the limits of variability. Recovery of MLX (n=5) was evaluated by comparing the mean peak areas of five extracted low, medium and high samples (8µg/ml, 10µg/ml and 12µg/ml) to mean peak areas of unprocessed standards that represent 100% recovery.

Specificity and selectivity

Randomly selected six blank human plasma samples, carried through the extraction procedure were chromatographed to determine the interference from endogenous matrix compounds. Chromatograms of plasma were examined for potential interfering substances that may co-elute with MLX.

Stability studies

The stability of MLX solutions was assessed in analytical standard solutions, processed sample extracts and biological matrix by comparison to freshly spiked plasma samples.

1. *Short term stock stability:* A stock solution of MLX and IS was kept at room temperature for 8 hours.
2. *Long term stock stability:* A stock solution of MLX and IS was kept at room temperature for 45 days.
3. *Bench top stability:* The replicate concentrations of low and high standard samples were determined by comparing the mean area ratio of freshly thawed samples with samples kept at room temperature for 6 hours.
4. *Freeze thaw stability:* The stability of low and high standard samples was determined after three freeze thaw cycles, by thawing at room temperature for 2-3 h, refrozen for 12-24 h. The concentration of MLX was determined and % CV was calculated.

RESULTS AND DISCUSSION

Optimization of extraction solvent

MLX binds significantly to plasma proteins, thus it is necessary to extract it and then ensure that all MLX is quantified. Prior to HPLC analysis, several common protein precipitating solvents and their combinations were tested to determine the composition for optimal MLX recovery from the biological matrix. Each solvent or mixture was added in a 1:1 ratio into blank plasma samples spiked with equal amounts of MLX and IS and subjected to similar sample preparation procedure as described previously. The supernatants were suitably diluted with mobile phase and injected into the column. The MLX peak in chromatogram was evaluated in terms of height, broadness of peak base, symmetry and recovery. The composition of the extraction solvent that produced the highest, narrowest peak base and most symmetrical peak was selected. The extraction solvent composition which gave highest extraction efficiency was selected for method validation. Extraction efficiency of MLX from plasma using various extraction solvents is shown in Table 1. Combination of 0.1N HCl and methanol was found to be a good extracting solvent and produced a satisfactory chromatogram. It was observed that pH of the solvent used for sample processing and preparation of stock and standard affected the shape of the chromatogram peak and RT of the analyte. MLX has two pKa values and the MLX profile was most likely to be affected by the pH of the environment and thus the extent of ionization of MLX molecules. Therefore it was concluded that low pH would be more suitable for construction of MLX solutions.

Table 1: Extraction efficiency of MLX from different solvent compositions

Extraction solvent/composition	Ratio	Extraction Efficiency (%) ± SD
Ethyl acetate	-	62.06 ± 1.42
Acetonitrile	-	72 ± 1.97
Methanol	-	88.31 ± 0.58
0.1 N HCl and ethyl acetate	1:1	68.61 ± 2.34
0.1 N HCl and methanol	1:1	90.46 ± 0.53
0.1 N HCl and acetonitrile	1:1	24.78 ± 0.82
Perchloric acid 70% and methanol	1:1	57.53 ± 1.66
Perchloric acid 70% and ethyl acetate	1:1	65.67 ± 5.09

Chromatographic separation

The composition of mobile phase was optimized through several trials to achieve good resolution and symmetric peak shapes of analyte and IS as well as short run times. A mobile phase system comprising of acetonitrile: water: 1% aqueous (aq.) glacial acetic acid (56:34:10, % v/v/v) achieved our purpose (Fig.2). No band tailing was found and the run time was short requiring only 10min. Short analytical time is considered good for plasma samples.

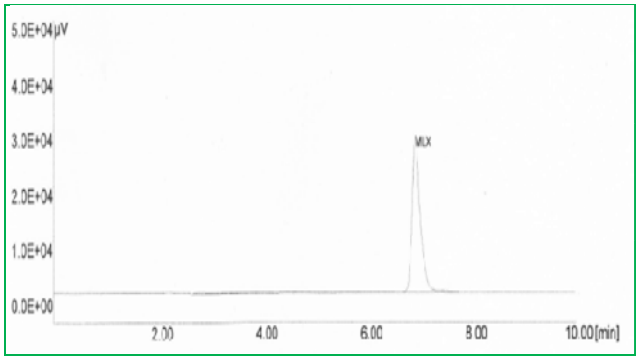

Figure 2: Acetonitrile: water: 1 % aq. glacial acetic acid (56:34:10, % v/v/v), RT=6.992 min

Fig.3 (a,b) shows the representative chromatograms of blank plasma, spiked plasma samples with MLX and IS. The analytes were well separated from endogenous matrix components under the described chromatographic conditions at retention times of 5.84±0.02 min for IS and 6.99± 0.02 min for MLX, respectively. The peak characteristics were satisfactory and completely resolved from one another. No endogenous interference from plasma matrix was observed.

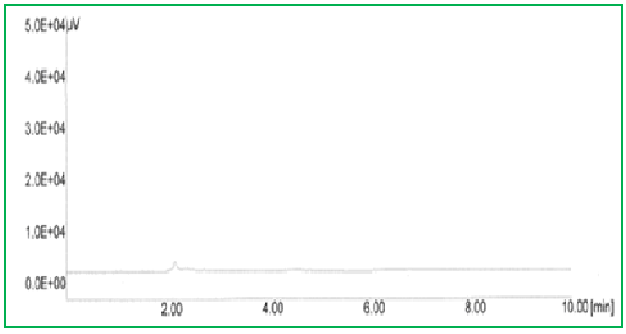

Figure 3a: Chromatogram of blank human plasma

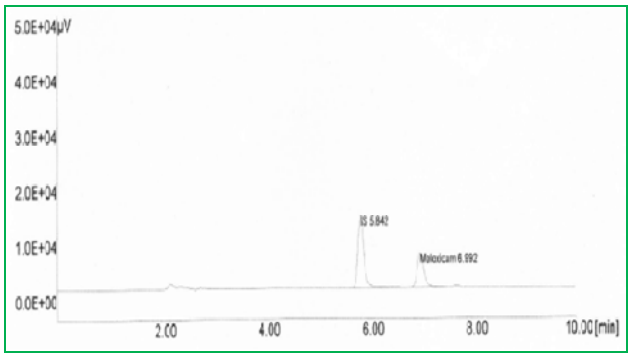

Figure 3b: Representative chromatogram of human plasma spiked with MLX and IS, Extraction solvent: 0.1N HCl and methanol (1:1)

Validation of method: quantitation of MLX in bulk and pharmaceutical dosage form

Calibration curves, precision, accuracy and linearity

Peak area versus drug concentration was plotted to construct a standard curve for MLX. The polynomial regression for the calibration plots showed good linear relationship with coefficient of correlation, r = 0.9995 ± 0.0002; slope = 28729.04 ± 274.17 and intercept = 20725.38 ± 3191.08 (n=3) over the concentration range studied. The range of reliable quantification was set at 0.3-20 µg/ml as no significant difference was observed in the slopes of the standard curves in this range. The correlation coefficient was indicative of high significance. The low values of the standard deviation, standard error of slope, and the intercept of the ordinate showed the calibration plot did not deviate from linearity. The calibration plot is shown in Fig.4. Chromatogram for linearity study is shown in Fig. 5. The LOD and LOQ were found to be 0.39µg/ml and 1.19µg/ml respectively. Precision was measured in accordance with ICH recommendations. The results of the determination of repeatability, intermediate precision and reproducibility are listed in Table 2 and 3. The low RSD values indicate the repeatability and reproducibility of the method. The recovery of the method, determined by spiking a previously analyzed test solution with additional drug standard solution, was found to be in the range of 99.96-103.75%. The values of recovery (%) listed in Table 4 indicate the accuracy of the method.

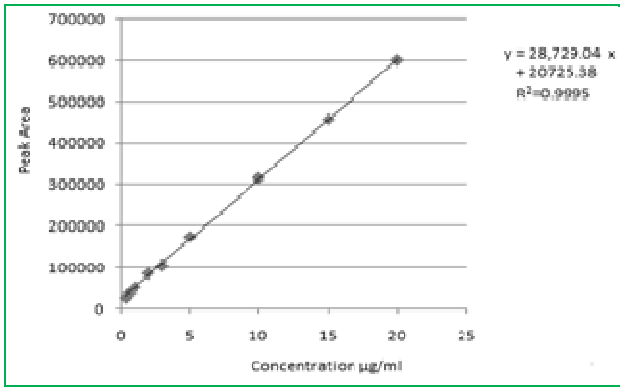

Figure 4: Calibration curve constructed for MLX

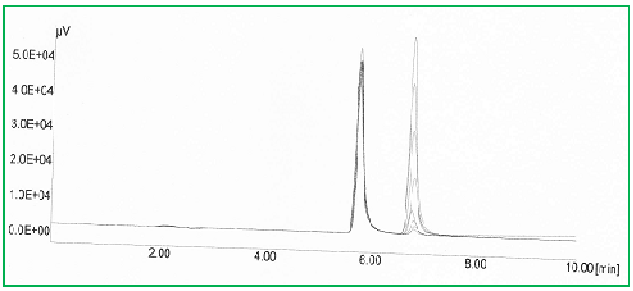

Figure 5: Chromatogram for linearity studies in the concentration range 0.3-20 µg/ml

Table 2: Statistical evaluation of precision (repeatability) of developed method (n=6)

MLX	Repeatability	
Conc. (µg/ml)	Mean area ± SD	% RSD
10	320046.30 ± 2419.40	0.75

Table 3: Data for Intra-day and Inter-day precision (n=3)

MLX (µg/ml)	Intra-day precision			Inter-day precision		
	Mean area ± SD	% RSD	SEM	Mean area ± SD	% RSD	SEM
1	49320.27± 652.35	1.32	266.32	50833.486± 747.21	1.46	334.16
10	317521.55± 5481.58	1.72	2237.8	338795.16± 6399.21	1.88	2612.5
15	477141.55± 3304.01	0.69	987.72	509808.795± 9185.71	1.80	3750.1

Table 4: Recovery studies of MLX (n=2)

Recovery Level	Amount of Drug Analyzed/µg/ml	Amount of Drug Added/µg/ml	Theoretical concentration / µg/ml	Total Amount of Drug Recovered/ µg ± SD	% Recovery ± SD
0	6	0	6	5.97 ± 0.041	99.68 ± 0.69
50	6	3	9	9.34 ± 0.22	103.75 ± 2.47
100	6	6	12	12.09 ± 0.007	100.77 ± 0.03
150	6	9	15	14.99 ± 0.37	99.96 ± 2.49

Table 5: Results of Robustness studies (n=2)

Parameter	Level	Mean Area ± SD	%RSD
Mobile phase composition (±0.1ml)	-1	327968.60 ± 3795.33	1.15
	+1	330260.41 ± 5861.03	1.77
Flow rate (± 0.1 min)	-1	354635.59 ± 3544.73	0.99
	+1	353526.42 ± 898.74	0.25

Robustness

The % RSD of peak areas was calculated for each variable and was found to be less than 2%. The low values of % RSD as listed in Table 5 indicate that the method is robust.

Specificity

Chromatogram of blank sample did not show any peaks while the chromatogram of standard sample showed well resolved peaks of MLX and IS with a resolution of 4.26 ± 0.02 (Fig.6 and 7). The specificity of the method was determined by exposing 1mg/ml sample solutions of MLX to stress conditions, i.e., 1 N HCl, 1 N NaOH, 30% H_2O_2, neutral degradation, photo degradation, dry heat and wet heat degradation.

There was no degradation of MLX in the presence of oxidative, neutral, photolytic, dry heat and wet heat stress conditions. No significant change in peak area of MLX was observed but minor changes in RT were obtained. However, in presence of 1N HCl, a substantial change in the peak area of MLX was found. A degradation component had eluted at RT- 2.357 min and was well resolved with the peaks of MLX and IS. In case of 1N

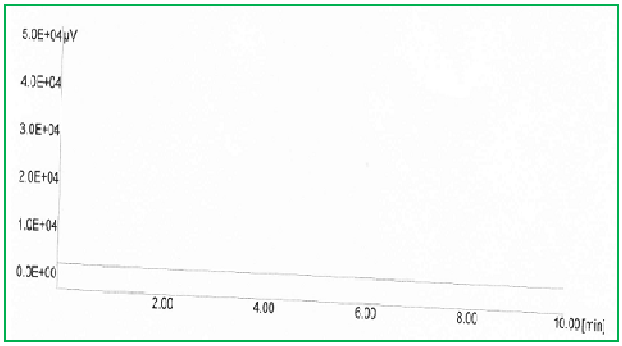

Figure 6: Chromatogram of blank sample

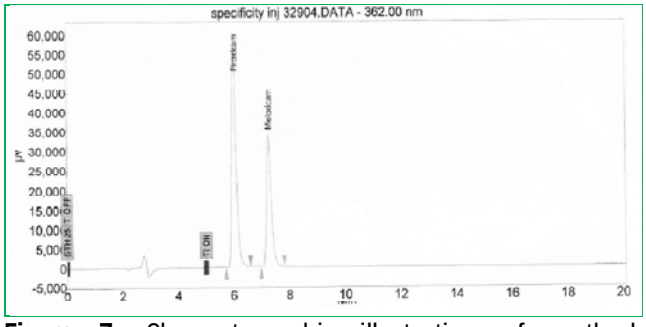

Figure 7: Chromatographic illustration of method specificity

NaOH degradation components were eluted at RT- 3.104 min and 12.309 min and were well resolved. Substantial change in the peak area of MLX was found. Chromatograms obtained from MLX after treatment with 1 N HCl, 1 N NaOH, 30% H_2O_2, neutral, photolytic, dry heat and wet conditions are shown in Fig.8 (A-G). The results from stress testing, including separation of the degradation product and quantification of MLX after exposure to stress conditions, show that the method is stability indicating.

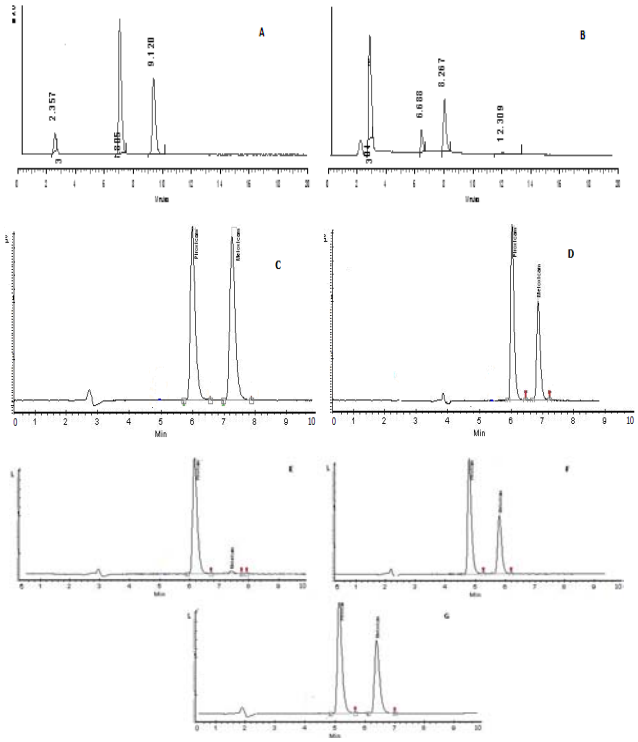

Figure 8: Chromatographic illustration of degradation products of MLX; (A) Acidic condition (B) Basic condition; (C) Dry heat condition; (D) Neutral condition (E) Oxidative condition (F) Photo degradation (G) Wet heat degradation

Stability

There was no significant change in analyte composition (sample concentration = 10 µg/ml) over a period of 72 h. The mean RSD between peak areas, for the samples stored under refrigeration (8±1°C) and at laboratory temperature (25±2°C) was found to be 0.92% and 0.65% respectively, suggesting that the drug solution can be stored without any degradation over the time interval studied.

Analysis of MLX from marketed tablets

A single peak was observed at the retention time of MLX when a suitably diluted solution of the tablet formulation was chromatographed. No interaction was observed between MLX and excipients present in the tablets. The MLX content was found to be 99.68% and the RSD was 0.04%. The low RSD indicated the suitability of this method for routine analysis of MLX in pharmaceutical dosage forms.

System suitability tests

The system suitability parameters for studied are listed in Table 6.

Table 6: System suitability parameters

Parameters (recommended values)	Observed values	Inference
T (<2.00)	1.45 ± 0.01	Complies
N (>2000)	13242.35 ± 53.22	Complies
% RSD (<2.0)	0.75	Complies
Rs (NLT 2)	4.80 ± 0.02	Complies

Bioanalytical method development and validation

Calibration curve, LOD and LOQ

The calibration curve for the determination of MLX in human plasma was linear over the concentration range of 0.3-5µg/ml with correlation coefficient r^2= 0.9982. The coefficient of variation (CV) values for each concentration was within the generally accepted range of 15%. The lower limit of quantification (LLOQ) was 0.85µg/ml, in which percent deviation was within 20% of the nominal concentration. LOD was established at 0.28µg/ml. The regression data of the calibration plots is given in Table 7. The calibration plot is shown in Fig.9 and representative chromatogram for linearity is shown in Fig. 10.

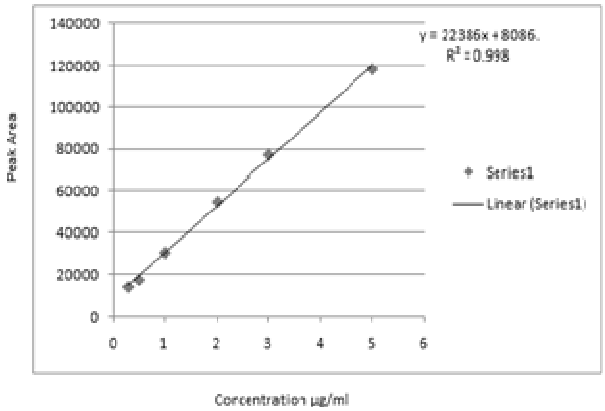

Figure 9: Calibration curve constructed for MLX in human plasma at 6 concentration levels in the range of 0.3-5 µg/ml

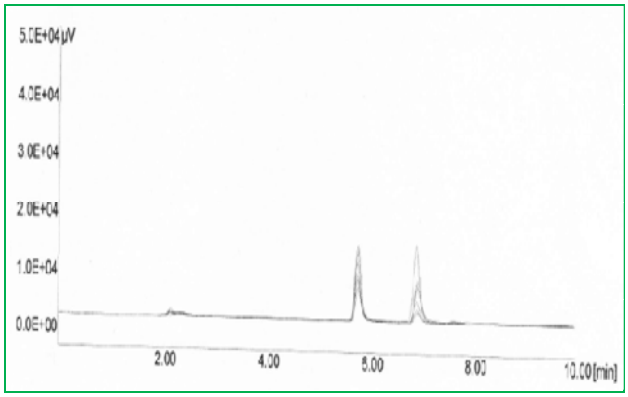

Figure 10: Representative chromatograms for linearity studies

Table 7: Statistical data of the regression equation and validation parameters for MLX

Measured wavelength (nm)	362
Linearity range	0.3-5 µg/ml
Slope	22386
Intercept	8086
Correlation coefficient	0.9982
LOD, µg/ml	0.28
LOQ, µg/ml	0.85
SD of residuals from line (Sy.x)	1919.2

Precision, Accuracy and Recovery studies

The intra-day and inter-day coefficients of variation were less than 2% (Table 8 and 9), over the range of concentrations from 1-5 µg/ml and accuracy was in the range of 86.86-109.66% (Table 10). The recovery of MLX was estimated at 8, 10 and 12 µg/ml. The recoveries ranged from 96.57-97.68% (Table 11).

Table 8: Intra-day precision data for the developed method, (n=3)

Level	Mean area ± SD	% CV	% Mean Recovery ± SD
LC (1 µg/ml)	32677.35 ± 380.08	1.16	109.6 ± 1.70
IC (3 µg/ml)	66488.22 ± 1053.79	1.58	86.95 ± 1.56
HC (5 µg/ml)	121666.56 ± 870.86	0.71	101.46 ± 0.76

Table 9: Inter-day precision data for the developed method, (n=3)

Level	Mean ± SD	% CV	% Mean Recovery ± SD
LC (1 µg/ml)	33205.55± 700.97	2.1	112.13 ± 3.14
IC (3 µg/ml)	66130.301 ± 841.22	1.27	86.42 ± 1.25
HC (5 µg/ml)	121300.55 ± 1012.25	0.83	101.14 ± 0.90

Table 10: Accuracy data (n=3)

Conc. µg/ml	Mean ± SD	% CV	Total drug found (±SD)	% Content (±SD)
1	32635.974 ± 194.78	0.59	1.09 ± 0.008	109.66 ± 0.87
3	66426.436 ± 251.07	0.37	2.6 ± 0.01	86.86 ± 0.37
5	121084.46 ± 545.44	0.45	5.04 ± 0.02	100.95 ± 0.48

Table 11: Data for recovery studies at 80, 100 and 120% (n=3)

Conc. µg/ml	Mean ± SD	% CV	% Recovery (±SD)
8	144168.83 ± 2932.37	2.03	96.57 ± 1.96
10	227814.29 ± 955.10	0.41	97.53 ± 0.41
12	236172.8 ± 3397.31	1.43	97.68 ± 1.40

Table 12: Stability data (n=3)

Conc. µg/ml	Stability type	Mean ± SD	CV
1	Short term	32913.58 ± 212.71	0.00646
	Long term	32341.126 ± 264.81	0.00818
	Bench top	33910.88 ± 670.55	0.01977
	Freeze thaw	32912.69 ± 722.60	0.02195
5	Short term	118636.88 ± 696.76	0.00587
	Long term	121489.46 ± 1471.70	0.01211
	Bench top	121370.53 ± 457.65	0.00377
	Freeze thaw	122281.06 ± 460.70	0.00376

Specificity

Chromatograms of blank human plasma and plasma spiked with IS were examined for interference from endogenous compounds. The method was specific for the determination of MLX and IS from the spiked samples without any potential interfering compounds. The chromatograms for blank plasma and plasma spiked with IS is shown in Fig. 3(a,b).

Stability

Stability of the method was carried out by performing short term, long term stock stability, bench top stability and freeze thaw stability. The studies were carried out in triplicate for both low and high concentration. CV for each set of data was calculated. The results are given in Table 12.

Table 13: System suitability parameters

Parameters (recommended values)	Observed values	Inference
T (<2.00)	1.32 ± 0.01	Complies
N (>2000)	14505.18 ± 3398.79	Complies
% RSD (<2.0)	0.57	Complies
Rs (NLT 2)	5.61 ± 0.01	Complies

CONCLUSION

HPLC method for quantification of MLX in bulk drugs and in pharmaceutical dosage forms has been developed and validated. System suitability tests and statistical analysis performed proved the method to be precise, accurate, reproducible, specific and stability-indicating and hence can be employed for routine analysis of MLX in bulk and commercial formulations.

The method validated for determination of MLX in human plasma by RP-HPLC is simple, sensitive, economic, and reliable. The retention time and in-turn run time was very short, hence making it more economical and rapid. The method may be applicable for pharmacokinetic studies of MLX as a part of in-vivo studies of the developed formulations.

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