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Research Article

Comparative evaluation of moxifloxacin MDR-TB drug; as microspheres with respect to pure drug in lung tissue

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ABSTRACT

A novel moxifloxacin (MOX)-loaded poly (DL-lactide-co-glycolide) (PLGA) microspheres (MPs) suitable for oral administration was prepared by double emulsification solvent evaporation (w/o/w) method. To investigate the pharmacokinetic of MOX-MPs, a simple and rapid high performance liquid chromatographic method was developed for the quantification of MOX in plasma and lung tissue of rats treated with MOX-MPs. Gatifloxacin (0.2µg/ml) was used as an internal standard (IS). The chromatographic separation was achieved on a reversed phase C_{18} column using isocratic elution with (0.01% Triethanolamine in distilled water): Acetonitrile in the ratio 70:30 v/v pH 2 adjusted with ortho-phosphoric acid, at flow rate of 1 ml/min with a total run time of 6 min. The column effluent was monitored by UV detector at 290 nm. The assay was found to be linear and validated over the concentration range 0.025 to 3.2 µg/ml for MOX in plasma and 0.1 to 2.5 µg/g of lung tissue with correlation coefficient of r^2 0.9998 and r^2 0.9997 respectively. The system was found to construct sharp peaks for MOX and IS with retention times of 4.08 (±0.012) and 5.84 (±0.026) min for plasma, and 4.17 (±0.016) and 5.84 (±0.022) for lung tissue, respectively. The method exhibited accuracy, precision (inter-day relative standard deviation (RSD) and intra-day RSD values < 15.0 %. The method was applied for determining MOX concentration in plasma and lung after oral administration of 10mg/kg of free MOX and MOX MPs to rats. Results established selectivity and suitability of the method for pharmacokinetic studies of MOX from MOX-MPs.

Keywords: Moxifloxacin, HPLC, poly (DL-lactide-co-glycolide), Microparticles, Pharmacokinetic study.

INTRODUCTION:

Fluoroquinolones (FQs) are rapidly emerging as important drugs in the treatment of tuberculosis TB) worldwide (Lemos and Matos 2013). However, in multi-drug resistance tuberculosis (MDR-TB, the role of FQs is much better established (Falzon *et al.*, 2011). MOX (Figure (1a)) is much more effective as compared to other FQs such as levofloxacin and ofloxacin against rifampicin resistance strains (Souza 2006). MOX is even included in extensively drug resistance tuberculosis (XDR-TB) regimens since they have efficacy against ofloxacin resistant strains (Andriole 2005; Bolon 2009).

MOX has an excellent oral bioavailability, long half-life of 12 h in comparison with 1-2 h for the first-line anti TB drug, isoniazid (Souza 2006). Because of these properties, MOX has emerged as a new potential agent that can be used in combination with other anti-TB drugs. Although it can be used as monotherapy, it may cause resistance if used alone (Ginsburg *et al.*, 2005). long-term administration of MOX to MDR-TB patients showed hepatotoxicity and/or nephrotoxicity (Codecasa *et al.*, 2006). As the drug is used for long periods, MOX at higher

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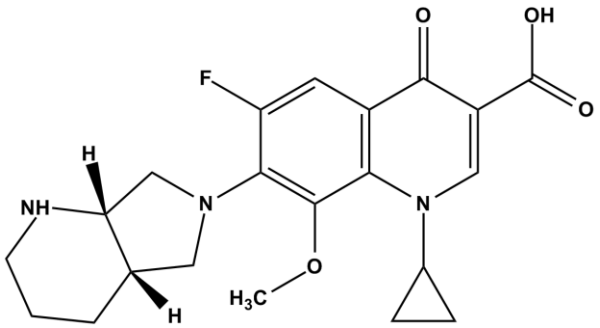
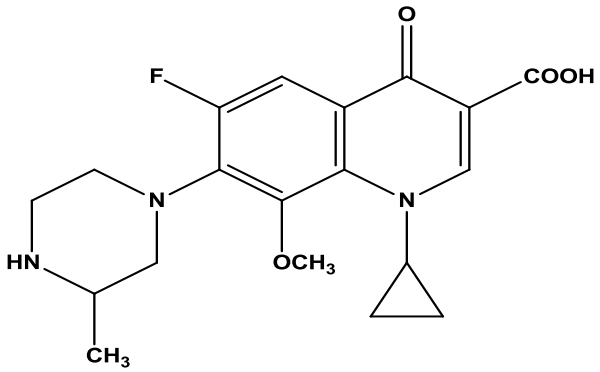


(a)



(b)

Figure 1: Chemical structures of (**a)** MOX and (**b)** gatifloxacin

concentration may increase the risks of cardiac arrhythmias, peripheral neuropathy, tendonitis, central nervous system stimulation and convulsions (Chiba *et al.*, 2004). MOX can be considered a class-I compound in the biopharmaceutical classification system, because it exhibits high water solubility and high membrane permeability. MOX is cleared from the body within 24 h, these drugs will require multiple or once daily administration (Pandey *et al.*, 2005).

Traditionally the plasma concentrations for an antibiotic and its relationship to the MIC (MIC_{90} for likely pathogens), have been used to predict its likely efficacy for a variety of infections. This approach is justifiable in the case of bacteraemia, but is less attractive where the infection is predominantly within an extravascular site such as lung tissue (Mustafa *et al.*, 2015). The rationale for measuring the concentrations of antimicrobial agents at potential sites of infections other than serum is that pathogens may be confined to sites which are separated from the blood by significant barriers to antimicrobial agent movement. Lung tissue comprises several potential sites of pulmonary infection including those involved in airway infections such as sputum and bronchial mucosa, and those in bronchi alveolar infections such as the epithelium lining fluid and alveolar macrophage (Lemoine *et al.*, 2000). In addition whole lung tissues contain lymphatics and elements of both the bronchial and pulmonary circulation.

To target a specific organ and maintain a drug concentration in the blood for long period of time, there is need to develop a modified release or controlled release or site-specific or targeted drug delivery system of MOX which would avoid hepatotoxicity and/or nephrotoxicity and assist in improving patient compliance. However, to evaluate their *in vivo* performance following administration of these formulations pharmacokinetic study is required. To determine the drug concentration in plasma and lung tissue, the preliminary steps are the development of a plasma and lung tissue sample preparation method of *in vivo* pharmacokinetic study. Hence, reliable plasma and lung tissue sample preparation and assay methods would facilitate such pharmacokinetic study.

Several chromatographic methods have been reported for the determination of MOX in biological fluids with UV and fluorescence detection) (Wua *et al.*, 2012; Djurdjevic *et al.*, 2006; Kumar *et al.*, 2009; Raju *et al.*, 2012; Chan *et al.*, 2006; Nguyena *et al.*, 2004; Liang et a;., 2002; Xu *et al.*, 2010). Mostly HPLC techniques

were either very exhaustive or applicable in the identification of MOX in biological fluids, nourishing animal products, feeds (supplements) and to a lesser extent, in pharmaceutical formulations. Majority of the described techniques involve troublesome mobile phase (buffers) and difficult detection methods (fluorescence or mass detectors (Moller *et al.,* 1998). These increase the cost and complexity of the method, even though sensitivity of the fluorescence or mass detector is much higher than that of a UV detector.

A response surface methodology (RSM) approach was used to identify the optimum conditions for analysis during method development Equation (1) represents a linear second-order model that describes a twisted plane with curvature, arising from the quadratic terms as follows (Singh *et al.,* 2012; Kumar *et al.,* 2015):

$$y = b_0 + bx_1 + bx_2 + b_{12}x_1x_2 + b_{11}x_1^2 + b_{22}\ x_2^2 \quad (1)$$

Where y is the experimental response to be optimized, b_0 is a constant term and b_1-b_5 are coefficients of the linear terms, x_1, x_2 represent the main effect, x_1^2, x_2^2 are the quadratic effect, and x_1x_2 are the interaction effect. Data were analyzed by nonlinear estimation using Design Expert software 7.0.

In order to fully characterize the MOX MPs formulation, a suitable and validated method is required for a critical assessment of pharmaceutical parameters such as drug content, encapsulation efficiency, and *in vitro* and *in vivo* release performance. Literature review reveals that HPLC methods have been reported for the quantitation of MOX in combination with other drugs (Wua *et al.,* 2012; Smet *et al.,* 2009), and a few bioanalytical methods are also reported ((Lemoine *et al.,* 2000; Sousa *et al.,* 2013).

To the best of our knowledge, the use of liquid chromatographic technique for the determination of MOX MPs *in vivo*, after oral administration in rat plasma and lung tissue has not been demonstrated. Presently, we report an optimized HPLC method and its application for determining pharmacokinetic parameters, after oral administration of free MOX and MOX MPs at a dose of 10 mg/kg. The method was validated according to Food and Drug Administration (FDA) (Food and Drug Administration, 2001) and International Conference on Harmonization (ICH) guidelines (ICH Harmonized Tripartite Guideline and Methodology Q2 (R1), 2005).

MATERIALS AND METHODS

Materials and Reagents

MOX and Gatifloxacin (IS, Figure (1b)) (99.8% w/w and 99.7% w/w, HPLC) were provided ex-gratis by M/S Cipla Sikkim, Sikkim, India. Other chemicals such as, acetonitrile, triethanolamine and ortho-phosphoric used were of HPLC grade purchased from Sigma Aldrich. Deionized water used in all the experiments was passed through a Milli-Q water purification system (18.2MΩ/cm) Millipore (Bangalore, Karnataka, India).

Instrumentation and Chromatographic Conditions

The HPLC (Shimadzu, Japan) instrument was equipped with a model series with two LC-10 ATVP pumps, SPD-10 AVP UV-Vis detector, Rheodyne injector with a 50 μL loop and a computer running Varian workstation version 6.42 software for data acquisition and processing. The chromatographic separation was performed on a Phenomenex C_{18} column (250 × 4.6 mm I.D., 5 μm particle size) at 30ºC. The mobile phase composed

of (0.01% Triethanolamine in distilled water): Acetonitrile in the ratio 70:30 v/v, pH 2 adjusted with ortho-phosphoric acid, run under isocratic elution and pumped at a flow rate of 1.0 ml/min. Mobile phase was filtered through a 0.22 µm nylon membrane filter and degassed ultrasonically prior to use. The volume of injection was 20 μl. The column effluent was monitored by UV detector at 290nm.

Experimental Design for HPLC Separation Optimization

Statistical evaluation and experimental design are two major tools for optimization techniques. It is beneficial to evaluate and identify the most imperative parameters with a minimum number of runs, while using an appropriate model. The choice of the proper parameter levels through trial-and error experiments is a time-consuming process, from which the optimal parameter settings may not readily be obtained. There were three steps of the optimization of HPLC method: (A) preliminary experiments to choose essential requirements of the method, (B) screening to select important variables, (C) response surfacing to locate the optimal point. During the optimization steps, retention time, peak resolution, and peak asymmetry responses were screened in order to minimize the analysis time and maximize the peak resolution and optimal peak asymmetry of the developed method.

Taguchi A orthogonal array design was employed in preliminary experiments to screen the most appropriate parameters. Furthermore, Central composite design was selected to determine the best experimental conditions in RP-HPLC. Thirteen experiments were conducted using the levels described in Table 1 and conditions described in Table 2. Minimum and maximum values for concentration of acetonitrile (A) were selected as 20% and 40%, respectively. Likewise, minimum and maximum contents of buffer pH (B) were fixed as 1.5 and 2.5, respectively. Retention times ($Y1$), peak resolution ($Y2$), and peak asymmetry ($Y3$) were the responses for these studies.

Preparation of Plasma Calibration Curve (CC) and Quality Control Samples (QC)

Eight point calibration curve (CC) was prepared by serial dilution of MOX stock solution (100µg/ml) in the range of 0.025-3.2µg/ml. The concentrations were corrected for potency and amount weighed. Calibration standards were prepared daily by spiking 0.1 ml of blank plasma with 10 µl of the appropriate working solution resulting in concentrations of 0.025, 0.050, 0.1, 0.4, 0.6, 0.8, 1.6 and 3.2 µg/ml of MOX per ml of plasma. A plot with the resulting peak area ratios of MOX to IS was obtained against the concentrations. Quality control (QC) samples (low quality control (LQC), 0.025 µg/ml; medium quality control (MQC), 1.6 µg/ml; high quality control (HQC), 3.2 µg/ml; lowest limit of quantification (LLOQ), 0.009 µg/ml were prepared by spiking 0.1 ml aliquot of blank plasma with 10 µl of spiking solution of drug as well as the IS. All solutions were stored in the refrigerator at 4.0 ± 2.0°C. The bulk spiked CC and QC samples were stored at -20°C and brought to room temperature before use.

Preparation of lung Calibration Curve (CC) and Quality Control Samples (QC)

Stock solutions of MOX and gatifloxacin was same for plasma and lung samples. Calibration standards were prepared daily by spiking 0.1 ml of blank lung sample with 10 µl of the appropriate working solution in concentrations of 1.0, 5.0, 7.5, 10, 15,

20, 25 and 30 µg/ml were prepared by appropriate dilution. The resulting standards ranged in concentration from 0.1 to 3.0 µg/g. Quality control (QC) samples were prepared by the same procedure as the calibration sample (low quality control (LQC), 0.2µg/g; medium quality control (MQC), 1.25µg/g; high quality control (HQC), 2.25 µg/g; lowest limit of quantification (LLOQ), 0.1µg/g. Then samples were crushed and centrifuged at 3000 × *g* for 15 min.

Sample Preparation

To a 100 µl of rat plasma or lung (0.5 g crushed in 0.5mL of water), 10 µl of IS and 200 µL of MOX were added and the mixture was incubated at 37 °C for 1 h MOX was then extracted using 100 µl of acetonitrile (liquid–liquid extraction; LLE) followed by vortexing for 2 min. After vortexing, the samples were subjected to centrifuge at 12,000 × g for 15 min using cold centrifuge (Remi Model TC 650 D). Supernatant (100 µl) was mixed with sterile water (400 µl) and transferred to the auto sampler vials; finally, 20 µl of this solution was injected into the chromatographic system. For the lung, as described above, gatifloxacin solution (20 µl) was added to 100 µl of crushed lung. Then, repeating the plasma sample process, 20 µl of the solution was injected into the chromatographic system.

System suitability tests

Before sample analysis, the chromatographic system employed must pass the system suitability parameters in command to assist the precision and accuracy of the developed HPLC method. The capacity factor injection repeatability (n=6), tailing factor, theoretical plate number, and resolution for the two drug peaks were the constraints tested on a combination solution containing 3.2 µg/ml of MOX and 0.2 µg/ml of IS.

Method Validation Study

Selectivity, specificity and linearity Selectivity was verified by analyzing the blank plasma and lung tissue samples from rats to test interference at the analyte retention times. By employing the proposed extraction procedure each blank plasma and lung sample was tested and then comparing with the results of plasma and lung samples spiked with MOX (n=6) in calibration standard to ensure no interference of MOX from plasma. Spiked plasma and lung samples that contained increasing concentrations of MOX from 0.025 to 3.2 µg/ml for plasma and 0.1 to 3.0 µg/g for lung was analyzed according to the procedure described above. The linearity was detected by calculating the correlation coefficient (r) of the curves by means of least-squared linear regression method. All calibration curves of MOX were constructed prior to the experiments with correlation values of at least 0.9998 and 0.9997. The LOD was determined as the plasma and lung tissue concentration giving a signal-to-noise ratio of 3 and LOQ was the least concentration on the standard curve.

Recovery

The LLE efficiency was calculated by comparing the peak areas of extracted plasma and lung tissue standards with areas of reference standards added to blank plasma and lung tissue extract. The reference standard was prepared by extracting rat control plasma and lung tissue and reconstituting the evaporated extracts with stock solutions of MOX, and IS. The concentration of the internal standard was 0.2 µg/ml. Recovery studies for MOX were accomplished at three concentration levels (0.025, 1.6 and 3.2 µg/ml) in plasma and (0.2, 1.25 and 2.25 µg/g) in lung.

Matrix effect

To study the matrix effect, blank plasma and lung tissue samples were processed and spiked afterward to achieve MQC and HQC concentrations. The response (area) was evaluated with directly injected samples at MQC and HQC levels.

Inter-day and intra-day precision and accuracy

Precision of an analytical method articulate how close the data values are to each other for a numeral of measurements acquired from numerous sampling of the identical homogeneous sample, under the same analytical conditions and expressed as relative standard deviation (RSD) (Gao *et al.*, 2007). Accuracy of an analytical method describes the closeness of the test results obtained by the method to the normal value of the analyte. Inter-day and intra-day precision and accuracy were evaluated by spiking known amounts of MOX and IS in plasma and lung samples (n=6). The precisions were expressed as % RSD and % accuracy was expressed by using the formula (2):

$$\frac{\text{Measured Concentration}}{\text{Spiked Concentration}} \times 100 \qquad (2)$$

Three different concentrations 0.025, 1.6 and 3.2 µg/ml for plasma and 0.2, 1.25 and 2.25µg/g for lung tissue were used, and samples were prepared according to the procedure as mentioned above. Intra-day precision and accuracy were assessed within one batch using replicate (n=6) determinations for each concentration of the spiked plasma and lung sample, whereas inter-day precision and accuracy was assessed on three separate occasions using replicates (n=6) for each concentration used.

Sensitivity

The LOD and the LOQ were calculated by measuring the analytical background response, running blanks plasma and lung samples using the maximum sensitivity allowed by the system. The signal-to-noise ratio was used to determine the LOD, and it was estimated as the concentration of MOX in plasma and lung tissue that generated a peak with an area at least three times higher than the baseline noise. LOQ was considered to be 10 times superior to the baseline noise analyzed using the maximum sensitivity allowed by the system.

Preparation of MOX MPs by double emulsification solvent evaporation (w/o/w) method

MOX MPs were prepared by double emulsification solvent evaporation (w/o/w) method using Ultra Turrax IKA T25 digital high shear homogenizer. First, an appropriate amount of drug (MOX) was dissolved in aqueous phase water (2ml) and then this drug solution was added to organic phase dichloromethane (8ml) consisting polymer solution (PLGA 50:50) in a ratio (1:2) with vigorous stirring at 10,000 rpm for 60 seconds to yield a water-in-oil emulsion (w/o). Then an aqueous solution of surfactant 10ml (cetrimide 2%) was added dropwise to water-in-oil (primary emulsion) and further emulsified for 60 seconds. Next, this primary emulsion was added in a thin stream into aqueous phase containing rigidizer, polyvinyl alcohol (PVA) solution (50ml) to form water-in-oil-in-water (w/o/w) emulsion. Stirring was continued for 8 hr using mechanical stirrer to evaporate the organic solvent. The formed microemulsion was centrifuged at 2,000 rpm using cold centrifuge for 15 min. Resultant, MOX MPs were isolated, washed three times with deionised water followed by lyophilization at -50°C for 24 h (Lamprecht *et al.*, 2004).

Pharmacokinetic study

After the method was validated as described above, it was applied to determine the concentrations of MOX in plasma samples following oral administration of free MOX and MOX MPs in the rats. Male adult Wistar rats (250-300 g; n = 18) were used. The animals were housed in polypropylene cages (n = 3/cage) filled with sterile paddy husk. The animals were acclimatized to laboratory conditions over the week before experiments and fed with standard rat diet, under controlled conditions of a 12:12 h light: dark cycle, with a temperature of 22 ± 3 °C and a relative humidity of 50 ± 5 % RH. The experimental protocols were approved by the Institutional Animal Ethical Committee (AACP/IAEC/Feb-2014-01). The animals were fasted overnight before dosing. Handling and sacrificing of rats were in full accordance with the committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines, India, for the care and management of laboratory animals.

Eighteen rats were randomly separated into three groups (six animals in each group). The grouping of animals was as follows:

Group I: control normal rats (received saline solution)

Group II: administered with pure drug (as solution) (10mg/kg/rat)(Raju *et al.,* 2012)

Group III: administered with MOX MPs (as dispersion in 1mL of water) (10mg/kg/rat) and then administered orally using oral gavage needle (No18)

At regular time intervals 0, 0.083, 0.166, 0.25, 0.5, 1, 2, 4, 6, 8, 10, 12, 20 and every 24 h for 3 days, samples of blood were withdrawn (100μl) from the retro orbital plexus by micro capillary technique under light ether anaesthesia into heparinised microcentrifuge tubes (50 units heparin/mL of blood). Plasma was separated by centrifugation at 12,000 ×g for 15 min and analyzed by the following method.

Plasma samples were deproteinated with 1mL of acetonitrile, vortexed for 30 s, and centrifuged at 8,000 ×g for 15 min. The supernatant was decanted into a China dish and evaporated to dryness at room temperature. This was further reconstituted with 100 μL of mobile phase and vortexed for 30 s and 20 μL was injected into an HPLC system. MOX was detected at a wavelength of 290 nm. The proficiency of microparticulate formulations was appraised by administering pure drug orally and measuring the blood levels at 0, 0.083, 0.166, 0.25, 0.5, 1, 2, 4, 6, 8, 10, 12, 20 and 24 h.

In vivo lung biodistribution of MOX-loaded PLGA microparticles

The animals and dosing protocol were the same as in pharmacokinetic study. To study *in vivo* lung biodistribution, 18 rats were randomly divided into two groups (Nine animals in each group). Group I was treated with pure drug, Group II was treated with MOX MPs. At every 24 h for 3 days after dosing, three rats of each group were sacrificed by cervical dislocation under general pentobarbital anaesthesia. Lungs was collected and stored at -16°C until analysis. The extent of *in vivo* biodistribution and retention following oral administration of MPs was analysed for 3 days.

RESULTS AND DISCUSSION

Optimization of Separation

The development of an optimized method requires plenty of experiments that increase exponentially

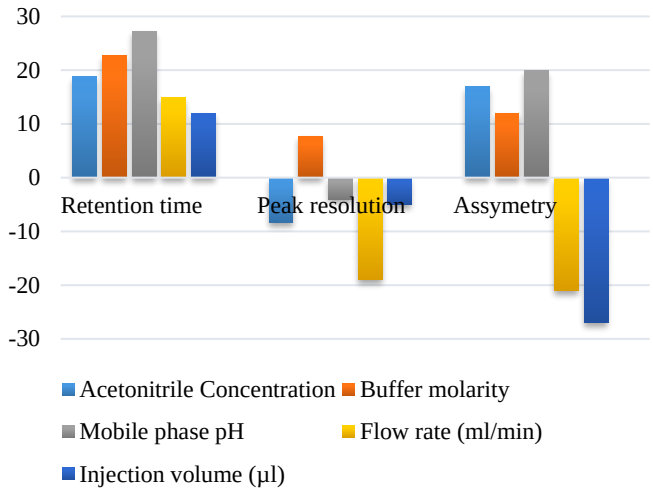


Figure 2: Influence of selected parameters on the response magnitude.

with the number of independent variables. To decrease the number of experiments, a decrease in dimensions of independent variables was considered in a series of preliminary-screening experiments. Optimization of the chromatographic method was achieved in three steps: a series of preliminary experiments followed by two sets of different experiments. The experimental designs were performed to achieve maximum resolution in short analysis time and optimal peak asymmetry.

Preliminary Studies

The preliminary experiments were executed to decide the essential analytical requirements of the method, such as the type of column, buffer, and pH range. A standard solution containing 2 μg/ml of MOX was used during the initial experiments. In our preliminary study, performance of several kinds of columns (Hypersil C_{18} (200 mm × 4.6 mm, 5 μm), Grace smart C_{18} (150 mm × 4.6 mm, 5 μm), Waters Symmetry C_{18} (200 mm × 4.6 mm, 5 μm), and Phenomenex C_{18} (250 mm × 4.6 mm, 5 μm)) was checked by running dissimilar mobile phases. The best peak asymmetry and peak resolution were obtained with Phenomenex® C_{18}. Therefore, Phenomenex® C_{18} was selected as the analytical column. The resolution of Phenomenex® C_{18} was higher and showed much better peak asymmetry than with other columns. However, the retention times of MOX and IS were longer and the shapes of the peaks were not sharp enough. After careful comparison, a Phenomenex® C_{18} column (250 × 4.6 mm,5 µm)was finally used with a flow rate of 1 ml/min to produce good peak shapes and permit a run time of less than 6 min.

Table 1: Independent variables, dependent variables, and levels of the face centered central composite design.

Factor		**Levels**		
Independent	**Symbol**	**(-1)**	**(0)**	**(1)**
Acetonitrile concentration (%)	A	20	30	40
Mobile phase pH	B	1.5	2.0	2.5
Dependent				
Retention time	Y_1			
Peak Resolution	Y_2			
Peak Asymmetry	Y_3			

Table 2: Experimental conditions according to the central composite design and observed response values.

Exp. no	**Runs**	***A***	***B***	***Y₁***	***Y₂***	***Y₃***
1	7	-1.00	1.00	8.47	2.99	1.6
2	3	0.00	0.00	4.29	7.44	1.0
3	4	0.00	0.00	4.32	7.99	1.1
4	12	0.00	0.00	4.38	7.65	1.0
5	10	0.00	0.00	4.41	8.01	1.0
6	11	1.00	1.00	10.42	4.49	1.9
7	9	0.00	1.00	5.41	8.05	1.0
8	8	-1.00	0.00	5.17	7.61	1.2
9	1	1.00	-1.00	7.44	4.54	1.2
10	2	-1.00	-1.00	9.01	2.32	1.4
11	5	1.00	0.00	12.12	5.42	1.0
12	6	0.00	0.00	4.39	7.98	1.1
13	13	0.00	-1.00	6.15	4.39	1.4

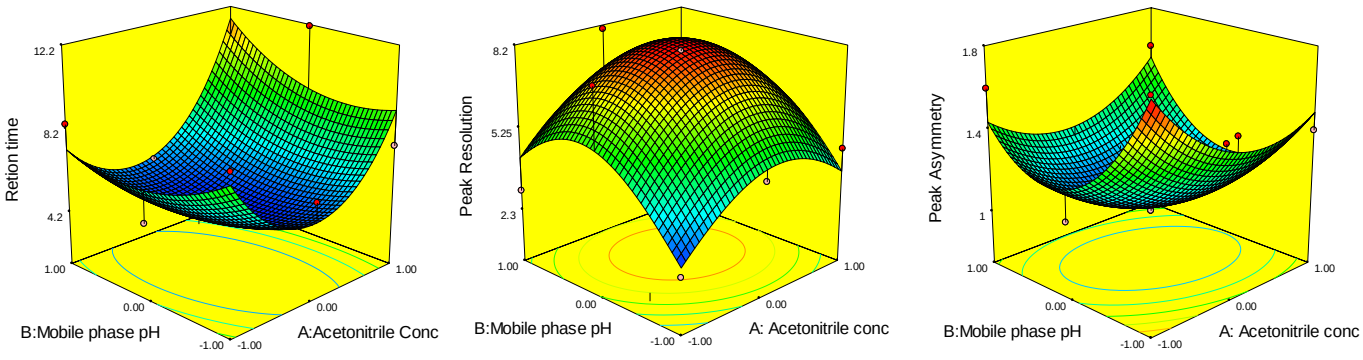
Table 3: System suitability parameters

Parameter	Compound	
	MOX	Gatifloxacin
Retention time (Rt)	4.08	5.84
Tailing factor (T_f)	1.07	1.25
Injection repeatability (RSD)[a]	0.741	0.620
Resolution (Rs)[b]	-	8.57
Capacity factor (K')	4.23	6.31
Theoretical plates (N)	5,822	4,986
Asymmetry	1.55	1.80

[a]RSD of peak areas of six consecutive injections at a concentration of 3.2 and 0.2 µg/ml of MOX and gatifloxacin, respectively; [b]Resolution between (MOX and gatifloxacin)

Screening Based on a Taguchi Orthogonal Array Design

If the number of factors is high, an absolute response surface would be a complex multidimensional structure needing much more experimental research in order to be fully determined. Thus a screening study was applied to choose momentous parameters on separation. Taguchi orthogonal array design permitted evaluation of whether variables have a considerable influence on the chosen response or not. The parameters considered in the Taguchi orthogonal array design was the acetonitrile percentage, mobile phase ratio and pH, injection volume, and flow rate. The experimental ranges of the variables were elected on the basis of preliminary experiments. The distinct responses of the variables were the retention times, peak resolution, and peak asymmetry. As can be seen in Figure 2, acetonitrile concentration, buffer molarity, flow rate, and injection volume had a negative effect on peak resolution and peak asymmetry while all the above parameters had a positive effect on retention time. Acetonitrile concentration and mobile phase pH had significant effect on retention time and peak asymmetry and were selected for further optimization. The flow rate and injection volume were fixed to their optimum levels, which were 1 ml/min and 20 μL, respectively, to decrease analysis time and maximize resolution.



(a) **(b)** **(c)**

Figure 3: Three-dimensional graph showing (a) the effect of mobile phase and acetonitrile concentration on retention time. (b) Three dimensional graph showing the effect of mobile phase and acetonitrile concentration on peak resolution. (c)Three-dimensional graph showing the effect of mobile phase and acetonitrile concentration on peak asymmetry

Response Surfacing Based on 3^2 Central Composite Design

Response surface mapping was an efficient way to find the optimum condition. In this case, the 2-factor-3-level CCD was employed to draw response surface graphs to determine the optimal conditions and to investigate parabolic interactions between parameters (acetonitrile concentration and mobile phase pH). The variables with their relative experimental values are reported in Table 1. This design permitted the response surface to be modeled by fitting a second-order polynomial with the number of experiments equal to $2k$ +$2k$ +1, where k is the number of variables, which composed a total of 13 experiments to be executed as per CCD design Table 2. Experiments were executed according to the design listed in Table 2 and responses measured are given in the same table. Three-dimensional surface plots are presented in Figure 3 and are extremely valuable for studying the interaction effects of the factors on the responses. The retention time for MOX decreases as the acetonitrile (v/v)%, augmented from lower to intermediate level (Figure 3(a)), when the buffer pH of mobile phase was at intermediate level. The effect of mobile phase pH on the retention time of MOX was therefore investigated in a pH range from 1.7 to 2.4. Retention time was considered a more critical parameter in terms of analytical run time and sampling throughout analysis.

A classical second-degree model with a 3D experimental domain was hypothesized. The coefficients for the second order polynomial model were estimated by least squares regression. The equation for the Y_1 (retention time) factor is shown in (3). The regression coefficients calculated from CCD are given as follows:

$$Y_1 = 2.21 + 0.21x_1 + 0.043x_2 + 0.15x_1x_2 + 0.68x_1^2 + 0.21x_2^2 \quad (3)$$

The optimized chromatographic conditions were then used for all future analytical studies. To establish peak asymmetry, a line was drawn through the peaks generated following analysis of samples. In general, peak symmetry was improved at intermediate level of mobile phase pH and acetonitrile concentration as shown in Figure 3(b). Peak resolution was extensively affected when the mobile phase pH and acetonitrile concentration decreased to low level as depicted in Figure 3(c). This result designates that both acetonitrile concentration and mobile phase pH were one of the most important parameters that can be manipulated to optimize the separation and analysis of MOX. In the mobile phase pH range investigated, the resolution of MOX was improved as the pH was increased from lower to intermediate level. The effect of mobile phase pH and acetonitrile concentration on peak resolution is depicted in Figure 3(c). When using an acetonitrile concentration, mobile phase pH at intermediate level resulted in improved peak resolution. As shown in Figure 3(c), a decrease in resolution was observed as mobile phase of lower pH was used. When the acetonitrile concentration was at intermediate level peak resolution was improved. The mathematical relationship in the form of polynomial equations for the measured responses Y_2 and Y_3 is given as follows (4) and (5):

$$Y_2 = 2.84 + 0.94x_1 + 0.16x_2 - 0.054x_1x_2 - 0.42x_1^2 - 0.49x_2^2 \quad (4)$$

$$Y_3 = 1.02 - 0.019x_1 - 0.028x_2 + 0.040x_1x_2 + 0.083x_1^2 + 0.15x_2^2 \quad (5)$$

The model was authenticated by analysis of variance (ANOVA) employing Design Expert software that had been used to develop the

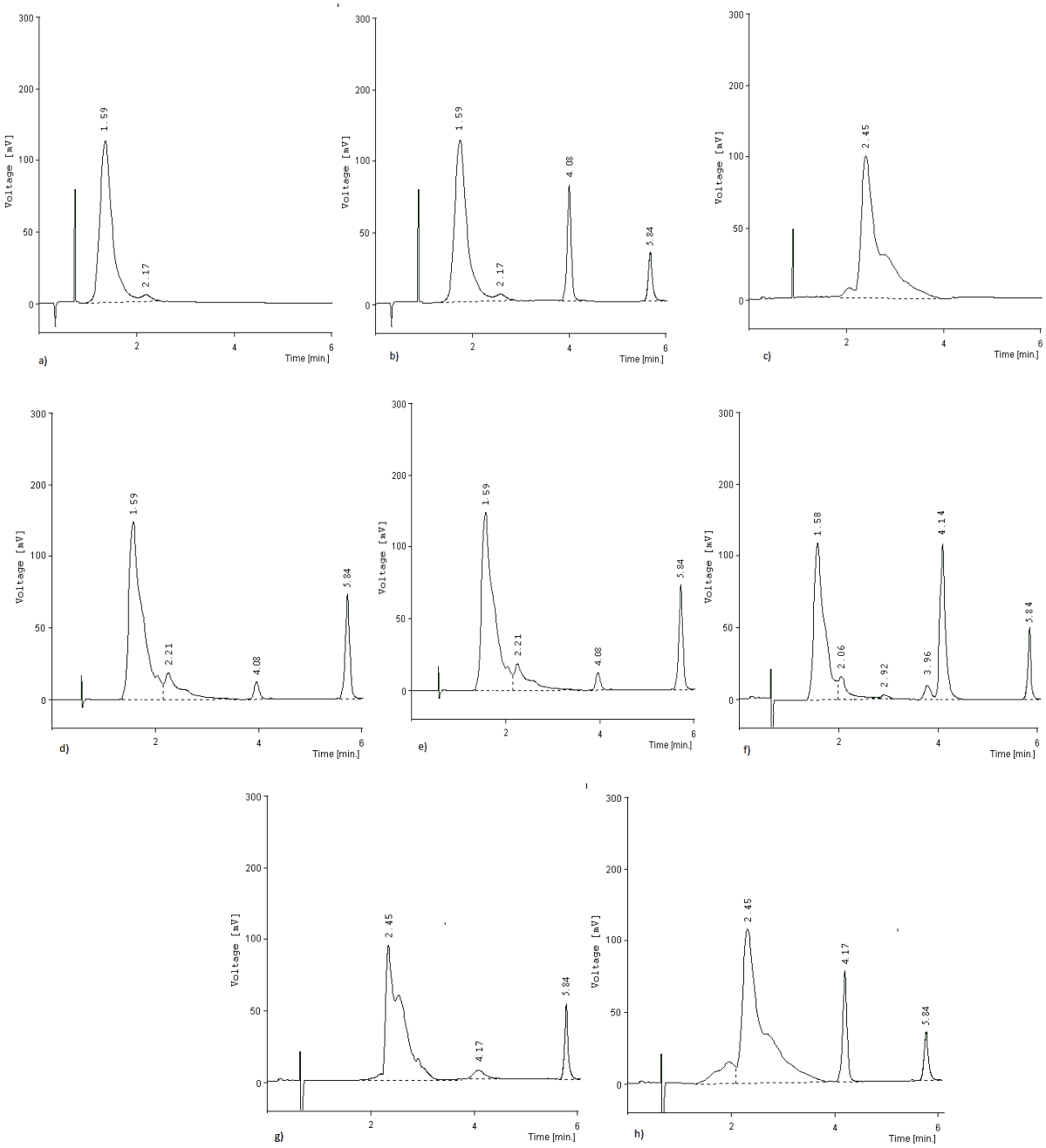


Figure 4 a. blank plasma; Figure 4 b. blank plasma spiked with 1.6 µg/ml MOX and 0.2 µg/ml of IS; Figure 4 c. Blank lungs tissue sample; Figure 4 d. blank lung tissue spiked with 0.009 µg/ml MOX and 0.2 µg/ml of IS; Figure 4 e. Plasma sample collected from a rat 30 min after receiving a 10 mg/kg oral administration of free MOX; Figure 4 f. Plasma sample collected from a rat 12 h after receiving oral administration of (equivalent to 10 mg/kg of MOX) MOX MPs; Figure 4 g. Lung sample collected from a rat 30 min after receiving a 10 mg/kg oral administration of free MOX; Figure 4 h. Lung sample collected from a rat 12 h after receiving oral administration of (equivalent to 10 mg/kg of MOX) MOX MPs

experimental plan for RSM. The ANOVA tests demonstrated that the models materialized to be adequate, with significant lack of fit ($P < 0.0001$) and with a satisfactory coefficient of correlation (r). It should be noted that the peak asymmetry achieved with the optimized chromatographic conditions was 1.1 and was considered suitable for this method. The final optimum conditions for chromatographic separation were 0.01% Triethanolamine in distilled water, pH 2, and Acetonitrile concentration 30%. The optimized mobile phase consisted of a mixture of (0.01% Triethanolamine in distilled water): Acetonitrile in the ratio 70:30 v/v pH 2 adjusted with ortho-phosphoric acid, at a flow rate of 1 ml/min.

Sample pre-treatment

A pre-treatment procedure is regularly needed to eradicate protein and potential interferences prior to HPLC analysis because of the complex nature of plasma and lung tissue. The instrumentation is not the limiting factor in high-through put analysis, but the sample preparation. Unlike liquid-liquid extraction and solid-phase extraction, protein precipitation is frequently used for rapid sample clean-up and distracting protein-drug binding. In order to increase sample throughput, the protein precipitation was used, which resulted in shorter sample preparation time. To assure the feasibility and adequacy of the proposed method for estimation of MOX in routine pharmaceutical application and verify the resolution, column efficiency, and chromatographic repeatability system suitability tests were performed Table 3. The capacity factor (k') was between 1 and 10, indicating good resolution with respect to the void volume. The RSD of peak areas of six consecutive injections was found to be less than 2%, thus showing good injection repeatability, and excellent chromatographic and environmental conditions. The tailing factor (T_f) for the MOX was found to be close to 1, reflecting good peak symmetry. The resolution (R_s) between the peaks was found to be > 2, indicating good separation of the MOX. The values for theoretical plate number (N) demonstrated good column efficiency. Resolution between MOX and gatifloxacin was 8.57.

Method validation

The extraction methods were validated according to the United States Pharmacopoeia (United States Pharmacopeial Convention 2008) and by the US FDA Guidance for Bioanalytical Method Validation (US DHHS FDA CDER 2001; Martins *et al.*, 2012). Validation of the method included an evaluation of the following characteristics viz., assay accuracy and precision, calibration curve, analyte stability, and assay specificity/selectivity. These validation experiments followed the blank plasma and lung tissue was obtained from rats that were not administered with MOX.

Specificity

Specificity is expressed as the capability of a method to distinguish the analyte from all potentially intrusive substance. The specificity of the method was scrutinized by blank plasma and lung tissue detection, peak purity and spiking blank plasma and lung tissue with pure standard compounds. Both the blank rat plasma and lung tissue had no interference, when MOX and the IS eluted (Figure. 4a and Figure. 4c). Each chromatographic run was completed within 6 min. Under the optimum chromatographic conditions, the retention times obtained for MOX and IS, were

Table 4: Intra-day precision and accuracy of MOX in rat plasma and lung samples (n =6).

Concentration (µgmL−1)	**Observed concentration(µgmL−1)**	Precision% (%RSD)	Accuracy% (Relative error, %)
Plasma			
3.2	3.19 ± 0.12	3.76	-0.31
1.6	1.58 ± 0.09	5.69	-1.25
0.025	0.0249 ± 0.003	12.05	-0.40
Lung[a]			
2.25	2.19 ± 0.03	1.37	-2.60
1.25	1.33 ±0.13	9.77	6.40
0.2	0.21 ±0.01	4.76	5.00

RSD relative standard deviation, [a]Theoretical concentration and concentration found in µg/g for lung samples.

Table 5: Inter-day precision and accuracy of MOX in rat plasma and lung samples (n =6).

Concentration (µgmL−1)	**Observed concentration(µgmL−1)**	Precision% (%RSD)	Accuracy% (Relative error, %)
Plasma			
3.2	3.21 ± 0.16	4.98	0.31
1.6	1.572 ± 0.11	6.99	-1.75
0.025	0.0251 ± 0.002	7.96	0.40
Lung[a]			
2.25	2.17 ± 0.05	2.30	-3.50
1.25	1.30 ±0.11	8.46	4.00
0.2	0.196 ± 0.006	3.06	-2.00

RSD relative standard deviation, [a]Theoretical concentration and concentration found in µg/g for lung samples

Table 6: Recovery of MOX and gatifloxacin in rat plasma and lung samples (n = 6)

Spiked concentration of MOX	**Mean recovery (%)**	**Spiked concentration of gatifloxacin (µg/ml)**	**Mean recovery (%)**
Plasma (µg/ml)			
3.2	93.72 ± 2.1		83.42 ± 3.1
1.6	89.56 ± 0.5	0.2	92.27 ± 4.8
0.025	95.37 ± 2.0		87.46 ± 1.9
Lung (µg/g)			
2.25	95.4 ± 3.2		85.42 ± 1.1
1.25	90.0 ± 2.5	0.2	91.15 ± 3.6
0.2	98.4 ± 2.3		82.36 ± 1.8

♣Exposed at ambient temperature (25 ˚C) for 4 h. ►After three freeze-thaw cycles.♦Stored at -16 ˚C.

.4.08 (±0.012) and 5.84 (±0.026) min for plasma, and 4.17 (±0.016) and 5.84 (±0.022) for lung tissue respectively.

Selectivity and linearity

The LLOQ of the assay, defined as the lowest concentration on the standard curve that was quantitated with accuracy within 10 % of nominal and precision not exceeding 15 % RSD, was 0.009µg/ml for plasma and 0.1µg/g for lung tissue. The reproducibility of LOQ was determined by examining four LOQ samples independent of the standard curve. A typical chromatogram of a LOQsample is shown in Figure. 4d. The LOD and

LOQ were 0.006 and 0.009 µg/ml for plasma and 0.05 and 0.1 µg/g for lung tissue respectively. The reported LOQ of HPLC-UV method for MOX determination in lung tissue was 0.4µg/g (Lemoine T et al. 2000).

Using relative cheaper and commonly available instruments such as HPLC the developed method was sensitive enough for the determination of MOX from our samples with the LOQ of 0.009 µg/ml for plasma and 0.1 µg/g for lung tissue. This method is sensitive enough to characterize the pharmacokinetic profiles of free MOX and MOX MPs in rats. The linearity of an analytical method is its capability to acquire test results which are directly proportional to the concentration of analyte in the sample. The linearity of the assay for the test compounds was evaluated with a total of nine calibration standards. Calibration curves consisted of eight concentrations of MOX spiked in rat plasma: 0.025, 0.050, 0.1, 0.4, 0.6, 0.8, 1.6 and 3.2 µg/ml. Similarly Calibration curves consisted of eight concentrations of MOX spiked in lung tissue: 0.1, 0.5, 0.75, 1.0, 1.5, 2.0, 2.5, and 3.0 µg/g. The extraction procedure and HPLC analysis described above were performed on the calibration samples. Calibration curves were constructed by plotting the peak area ratios (MOX/IS) of plasma and lung tissue standards versus nominal concentration. The analysis of MOX in plasma and lung tissue exhibited excellent linearity through the coefficient of correlation r^2 = 0.9998 for plasma and r^2 = 0.9997 for lung tissue respectively.

Precision and accuracy

The precision and accuracy data for the analytical procedures are shown in Table 4 and 5. Intra-day and inter-day precision (%RSD) of the methods were lower than 13% and were within the acceptable limits to meet the guidelines for bioanalytical method validation which is considered to be ≤ 15%. The accuracy of the both methods was also good with the deviation between the nominal concentration and calculated concentration for MOX well below the limits of 15 %. Precision and accuracy data indicated that the method to extract MOX from plasma and lung tissue are highly reproducible and robust.

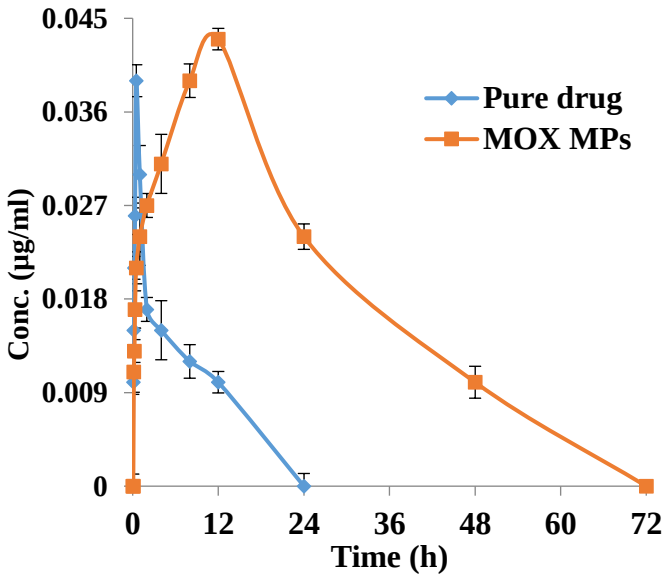


Figure 5: Plasma concentration-time curve of free MOX and MOX MPs after being orally administered in male Wistar rats (n = 6, mean ± S.D). The inset shows the Plasma concentration-time curve in 24h.

Recovery

The absolute recovery of MOX and IS was calculated for replicate spiked QC samples (LQC, MQC and HQC) Table 6. Extraction recovery is calculated by comparing the peak area ratios of MOX and IS respectively, in plasma and lung samples with the peak area of MOX and IS added to blank plasma and lung tissue at concentrations ranging 0.025, 1.6 and 3.2 µg/ml for plasma and 0.2, 1.25 and 2.25µg/g for lung. Results shows an overall mean percent recovery of 92.88 % for

MOX and 87.71 % for IS added to blank plasma extract, and 90.11% for MOX and 85.23 % for IS added to blank lung tissue extract. It is confirmed that recovery of the analyte need not be 100 %, but the amount of recovery of an analyte and the internal standard should be reliable, precise, and reproducible. Hence, the developed method reported showed a recovery of 92.88 % in plasma and 90.32% in lung tissue (for MOX) may be considered suitable taking into account the consistency and reproducibility of the results obtained upon repetitive evaluation.

Stability

Bench-top stability was investigated to ensure that MOX was not degraded in plasma and lung samples at room temperature for a time period to cover the sample preparation. It was measured by divulging the QC samples to ambient laboratory conditions for 10 h. Freeze–thaw stability was measured over three cycles. Because of the need for occasional delayed injection of extraction samples, the stability of reconstituted samples was assessed at ambient temperature for 24 h. The freezer storage stability of MOX in rat plasma and lung tissue at -20°C was evaluated by assaying QC samples at beginning and 1 week later. All stability QC samples were analyzed in six replicates. The results indicated that MOX had an acceptable stability under those conditions Table 7 and 8.

Sample dilution

To investigate the ability to dilute and analyze samples containing MOX at concentrations above the assay upper limit of quantitation, a set of plasma and lung samples was prepared containing MOX at a concentration of 5.0 µg/ml, and placed in a -20°C freezer overnight prior to analysis. After thawing, a 20 µl aliquot was withdrawn for analysis (n = 6), diluted with 100 µl of control rat plasma and lung sample, vortex for 30s, then treated as described above.

Table 7: Stability of MOX in plasma samples (n= 6).

Sample condition	Spiked concentration (µg/mL)	Mean determined concentration (µg/mL)	Accuracy (%)
Bench-top stability♣	3.2	3.11	97.18
	1.6	1.586	99.12
	0.025	0.0246	98.40
Freeze-thaw stability►	3.2	3.17	99.06
	1.6	1.61	100.62
	0.025	0.0244	97.60
One-week stability♦	3.2	3.08	96.25
	1.6	1.51	94.37
	0.025	0.0243	97.20

♣Exposed at ambient temperature (25◦C) for 4 h. ►After three freeze-thaw cycles.♦Stored at -16◦C

Table 8: Stability of MOX in lung samples (n = 6).

Sample condition	Spiked concentration (µg/g)	Mean determined concentration (µg/g)	Accuracy (%)
Bench-top stability♣	2.25	2.18	96.80
	1.25	1.22	97.60
	0.2	0.193	96.50
Freeze-thaw stability►	2.25	2.20	97.77
	1.25	1.24	99.20
	0.2	0.191	95.50
One-week stability♦	2.25	2.13	94.66
	1.25	1.19	95.20
	0.2	0.189	94.50

♣Exposed at ambient temperature (25◦C) for 4 h. ►After three freeze-thaw cycles.♦Stored at -16◦C.

Method applicability

The validated method was successfully applied to quantify free MOX and MOX MPs to rats, after oral administration. Developed MOX MPs had a particle size of 1.26 µm with an entrapment of 78.31 % and total drug content of 96.24 %. The areas under the concentration versus time curves were 0.375 and 1.454 µg/ml h for free MOX and

MOX MPs respectively, indicating a 1.1 fold increase in bioavailability (BA) of MOX after oral administration of MOX MPs to rats. Oral administration of free MOX in the present study resulted in a sharp Cmax of 0.039µg/ml within 30 min after which the plasma concentration declined rapidly, indicating a rapid metabolism of MOX, whereas, a relatively slow increase and sustained plasma concentration of MOX was observed for a longer time (72 h) after the administration of a single dose of MOX MPs. Significantly ($p < 0.05$) high Cmax of 0.043 µg/ml at 12 h with MOX still detectable at 72 h, confirms the sustained effect of poly(lactic-co-glycolic acid) MPs. Values beyond the limits of detection of the developed calibration curve were determined by extrapolation. The representative chromatogram of a plasma and lung sample, which was collected from a Wistar rats 30 min following oral administration of free MOX (Figure. 4e and Figure. 4g) and 12 h after receiving MOX MPs (Figure. 4f and Figure. 4h). The mean plasma concentration–time profiles after an oral administration of free MOX and MOX MPs as portrays in Figure. 5. Despite the numerous studies have been reported to measure the plasmatic levels of MOX, no one has attempted the pharmacokinetic of MPs of this compound. In support of our work, the pharmacokinetic study after the oral administration of 10 mg/kg of MOX to rats was reported (B. Raju 2012). The lung biodistribution data revealed initial rapid uptake and showed significantly higher concentrations of MOX and MOX MPs, which were 0.022±0.021 µg/gm and 0.028±0.013 µg/gm. At the end of 24 h, faster elimination of pure drug was observed. In comparison, these values were higher considering the MOX MPs on second day (0.049± 0.011 µg/gm). At third day, miniscule concentrations were found (0.021±0.021 µg/gm). After third day, concentrations of MOX MPs were not detectable and reached below detectable limits.

Table 9: Pharmacokinetic parameters of free MOX and MOX MPs at a dose of 10mg/kg/rat.

Pharmacokinetic parameters	MOX	MOX MPs
C_{max} (µgmL^{-1})	0.039±0.011µg/mL	0.043±0.011µg/mL
t_{max} (h)	30 min	12 h
$t_{1/2}$ (h)	13.67±1.08 h	18.933±1.08 h
$AUC_{0-\infty}$ (μgmL^{-1} * h)	0.375 ± 0.095	1.454± 0.131
Ke (1/h)	0.0506± 0.004	0.0366± 0.006
MRT	18.969 ± 0.570	29.87± 1.450

Data presented as mean ± standard deviation (n = 6)

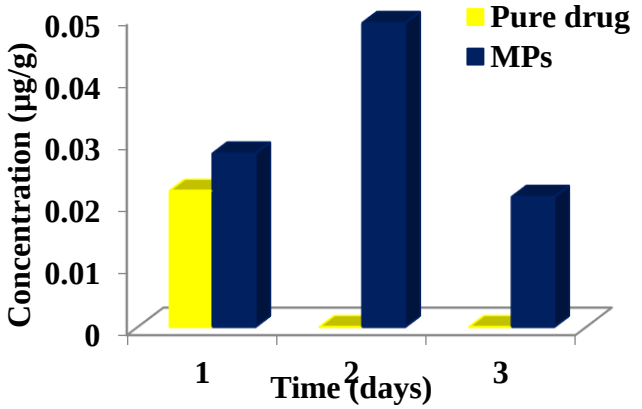


Figure 6: Concentration of pure drug and *MOX* loaded poly (lactic glycolic acid) microparticles (MOX MPs) to rats (y axis) was determined in lungs every 24 h for 3 days after oral administration.

It may thus, be concluded that the developed method employed in the current study is novel more accurate and sensitive. The results substantiate the suitability of the developed method for determining MOX concentration in plasma and lung tissue upon peroral administration of MOX MPs. The pharmacokinetic data of free MOX and MOX MPs after oral administration in rats is shown in Table 9. The lung biodistribution data after oral administration in rats for three days is shown in Figure. 6.

CONCLUSION

The novelty of the current work is the development of microparticulate drug delivery system solely through the judicious selection of apt blend of poly(lactic-co-glycolic acid)and emulsifier and validate by systematically optimized HPLC method using Formulation by Design (FbD). Experimental designs have been employed during the development of the method to minimize retention time and maximize peak resolution and optimal peak asymmetry. The predicted values from the model equation were found to be in good agreement with observed values and to gain a better understanding of the two variables. Finally, the method was applied to investigate the content of MOX in free form and as MPs in rat plasma and lung tissue. Conclusively, the studies can be judiciously explored to develop suitable platform technology (ies) for development of effectual and cost-effectual optimized HPLC method to investigate the pharmacokinetic performance of microparticulate drug delivery system of other anti-TB drugs

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests regarding the publication of this paper.

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ABBREVIATIONS

Moxifloxacin (MOX); poly (DL-lactide-co-glycolide) (PLGA); microspheres (MPs); tuberculosis (TB); Fluoroquinolones (FQs); multi-drug resistance tuberculosis (MDR-TB); extensively drug resistance tuberculosis (XDR-TB)

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