An HPLC Method for the Determination of Moxifloxacin in Breast Milk by Fluorimetric Detection with Precolumn Derivatization

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Summary. A new, sensitive, and selective high-performance liquid chromatography (HPLC) method with fluorimetric detection was developed for the determination of moxifloxacin (MOX) in human breast milk. MOX was precolumn derivatized with fluorescamine; the fluorescent derivative was separated on an RP C18 column using a mobile phase composed of acetonitrile–10 mM orthophosphoric acid by isocratic elution with flow rate of 0.5 mL min^{-1}. The method was based on the measurement of the derivative using fluorescence detection at 481 nm with excitation at 351 nm. The calibration curve was linear over the range of 1–40 µg mL^{-1}. Limit of detection (LOD) and limit of quantitation (LOQ) were found to be 0.3 and 1 µg mL^{-1}, respectively. Intra-day and inter-day repeatabilities were less than 3.15%.

Key Words: breast milk, fluorescamine, fluorimetric detection, moxifloxacin, liquid chromatography, precolumn derivatization

Introduction

Moxifloxacin (MOX), 7-[(4aS,7aS)-octahydro-1H-pyrrolo[3,4-b]pyridin-6-yl]-1-cyclopropyl-6-fluoro-8-methoxy-4-oxo-1,4-dihydroquinoline-3-carboxylic acid *(Fig. 1)*, is a fluoroquinolone antibiotic [1]. The drug is available in tablet, parenteral for intravenous infusion and ophtalmic solution forms. MOX can be used to treat infections caused by the following bacteria: aerobic Gram-positive microorganisms: *Corynebacterium* species, *Micrococcus luteus*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus haemolyticus*, *Staphylococcus hominis*, *Staphylococcus warneri*, *Streptococcus pneumoniae*, and *Streptococcus viridans* group; aerobic Gram-negative microorganisms: *Acinetobacter lwoffii*, *Haemophilus influenzae*, and *Haemophilus parainfluenzae*; and other microorganisms: *Chlamydia trachomatis*.
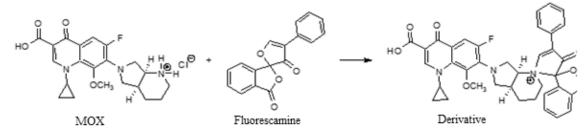
Fig. 1. The reaction between MOX and fluorescamine

MOX is bactericidal, and its mode of action depends on blocking of bacterial DNA replication by binding itself to an enzyme called DNA gyrase, which allows the untwisting required to replicate one DNA double helix into two. Notably, the drug has 100 times higher affinity for bacterial DNA gyrase than for mammalian. MOX is a broad-spectrum antibiotic that is active against both Gram-positive and Gram-negative bacteria. [2].

In literature, it is revealed that MOX has been determined in biological fluids: high-performance liquid chromatography (HPLC) [3–11], voltammetry [12], and capillary electrophoresis with laser-induced fluorescence [13]. None of them is for breast milk analysis. There is no available data for MOX in lactating women. Preclinical studies show that MOX is excreted in milk [14].

The amount of MOX in breast milk in humans is very important. There is a necessity to reveal the effects of MOX in the nursing infants. The proposed analytical procedure will facilitate to conduct researches about MOX.

For this aim, an HPLC method with fluorimetric detection has been developed and validated. The fluorimetric detection is provided by a precolumn derivatization process with a fluorogenic reagent, fluorescamine, which is preffered due to its basic derivatization procedure, fast reactivity, and high sensitivity [15–18]. The derivatization reaction is shown in *Fig. 1*.

Experimental

Chemicals and Reagents

MOX was obtained from Shanghai Yingxuan Pharmaceutical Science and Technology (China), Avelox® tablets containing 400 mg MOX were purchased from local drug store. Acetonitrile, orthophosphoric acide (HPLC grade), and hexane (analytical grade) were supplied from Merck (Darm-

stadt, Germany). Fluorescamine was supplied from Sigma (MO, USA). Water was purified by Human (Japan) ultrawater purification system.

Solutions

A stock solution of MOX (0.1 mg mL^{-1}) was prepared and diluted with water to give standard solutions of from 0.5 to 100 μg mL^{-1}.

Borate buffer was prepared by dissolving 0.620 g boric acid and 0.750 g potassium chloride in 100 mL water. The pH level was adjusted to 8.5 with 0.1 M sodium hydroxide solution, and the volume was made up to 200 mL with water. Fluorescamine solution was freshly prepared in acetone at 1 mg mL^{-1} concentration. The other solutions were stored at 4 °C and were stable at least for 2 weeks.

Instrumentation

Fluorescence spectra and measurements were taken on a Shimadzu spectrofluorimeter Model RF-1501 equipped with xenon lamp and 1-cm quartz cells. Excitation and emission wavelengths were set at 351 nm and 481 nm. pH measurements were conducted with WTW pH 526 digital pH Meter.

The HPLC analyses were conducted on a Shimadzu (Japan) LC 20 liquid chromatograph which includes an LC-20AT pump, SIL AT-HT autosampler part, an SPD-20A HT fluorimetric detector, which was set at 351 nm for excitation and 481 nm for emission, and CTO 10 AC column oven. Chromatographic separation was performed by isocratic elution at room temperature on a GL Sciences (Japan) C18 (ODS) column with acetonitrile–10 mM orthophosphoric acid (pH 2.4) at a flow rate of 0.5 mL min^{-1}.

Optimization Studies for Derivatization Procedure

The different experimental parameters affecting the development of the reaction product were tested, and optimal conditions were determined. Some parameters were changed individually while others were kept constant. These were pH, reagent concentration, temperature, heating period, and acetone–water ratio in the reaction medium.

Sample Preparation and General Procedure

Breast milk samples were collected from a 35-year-old volunteer mom (informed consent form was obtained according to ethical commitee approval) into polyethylene storage packs. Different amounts of MOX were spiked to milk samples. The milk samples were stored at −20 °C. To extract the drug from the milk samples, 2 mL milk was alkalinized with 250 µL 0.1 M NaOH, and the solution was then extracted into 5 mL of hexane. The contents were mixed with vortex mixer at moderate speed for 5 min and centrifuged at 4500 × *g* for 3 min. The aqueous layer was discarded. The organic layer was evaporated to dryness under a stream of nitrogen at room temperature. The residue was dissolved in 100 µL water, and 200 µL pH 8 borate buffer and 100 µL 1 mg mL^{-1} fluorescamine solution were added. The solution was vigorously mixed with a vortex mixer for 30 s, and 20 µL derivatized sample was injected into the HPLC system.

Results and Discussion

Derivatization

The optimum reaction time, temperature, pH, buffer type, proportions of acetone–water, and mole ratio of fluorescamine–MOX were determined.

Effect of pH

The reaction of fluorescamine with primary amines is pH dependent [15]. It was proved that the fluorescence emission was developed only in alkaline medium by using borate buffer [15–18], and therefore, different pHs were trialed within the range of 7–11 using borate buffer. At pH 8, maximum absorbance was observed.

Effect of Time and Temperature

In order to determine the optimum temperature and time required for the reaction, the derivatization reaction was carried out at different temperatures and durations. It was found that the fluorophore was formed immediately at room temperature.

Effect of Fluorescamine Concentration

The effect of fluorescamine concentration on the derivatization reaction was studied. It was found that 0.25×10^{-3} mmol (0.1 mL of 1% (*w/v*)) fluorescamine solution was sufficient to obtain maximum intensity.
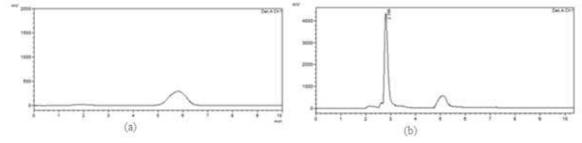
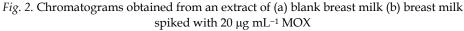## Effect of Acetone to Water Ratio in Derivatization Medium

Different volumes of acetone and water were tested where the concentrations of drug, buffer, and fluorescamine solutions were kept constant. The maximum peak area was observed by using a ratio of acetone to water as 1:3.

Stoichiometry of the Reaction

The molar ratio of fluorescamine to MOX in the reaction mixture was studied according to Job's method of continuous variation [19]. Utilizing equimolar solutions of MOX and fluorescamine, the reaction stoichiometry was found to approximate to a 1:1 ratio (reagent–drug). According to fluorescence intensity measurements, it is correct to say that all of the reagent is consumed, and there is no shortfall or excess of the reagent in this stoichiometric ratio.

For the optimization studies, fluorescence intensities of the derivatives were measured by spectrofluorimeter. Derivatives, prepared under the above mentioned conditions, remained stable for at least 24 h.

Fig. 2. Chromatograms obtained from an extract of (a) blank breast milk (b) breast milk spiked with 20 µg mL⁻¹ MOX

A good separation of the derivatives and endogenous compounds of breast milk was obtained using an isocratic elution system and reversed phase (RP)-HPLC as described above. Representative chromatograms of the

blank milk and milk samples spiked with MOX (20 µg mL⁻¹) are shown in *Fig. 2a* and *b*, respectively. No interference was detected with the milk constituents. The retention time of MOX is about 3 min.

Validation of the Method

Validation of the method was carried out according to the following guidelines given by the International Conference on Harmonization (ICH) [20]:

- Calibration and sensitivity: The linearity of the method was evaluated by a calibration curve in the range of 1.0–40.0 µg mL⁻¹ of the drug (*n* = 6). Calibration curves were prepared by the analysis of 2 mL breast milk samples spiked with various volumes of each working standard MOX solution. The samples were then submitted to the process of extraction, derivatization, chromatographic separation, and fluorometric detection described above. Calibration curves were obtained using linear least-squares regression analysis by plotting of peak areas of the derivative, versus the corresponding MOX concentrations. The equation of the calibration curve (*n* = 6) obtained from five points was: *y* = 25,008*x* + 85,305 (correlation coefficient = 0.9994), where *y* represents the peak area of MOX–fluorescamine derivative and *x* represents the concentration of MOX.

  The limit of detection (LOD) and limit of quantitation (LOQ) were determined using the formula: LOD or LOQ = *k*SDa/*b*, where *k* = 3 for LOD and 10 for LOQ, SDa is the standard deviation of the intercept and *b* is the slope. The parameters for the analytical performance of the proposed method are summarized in *Table I*.

Table I. Analytical parameters of the method

Parameters	Method
Concentration range[a] (µg mL⁻¹)	1–40
Regression equation[b]	
Intercept ± SD	85,305 ± 37.92
Slope ± SD	25,008 ± 41.02
Correlation coefficient (r^2)	0.9994
LOD (µg mL⁻¹)	0.3
LOQ (µg mL⁻¹)	1

[a]Average of six determinations.
[b]*y* = *x*C + b where *C* is the concentration in µg mL⁻¹ and *y* is the peak area.

- Accuracy, precision, and recovery: Accuracy and precision were assessed by determination of the QC samples at three concentration levels. QC samples at three different concentrations (1, 10.0, and 40.0 mg mL^{-1}) that can be classified as low, medium, and high concentrations (n = 5) in milk and aqueous sample were prepared. The accuracy was expressed by recovery values and relative mean error (RME), and the precision, by relative standard deviation (RSD). The absolute recovery of MOX from milk was examined by extraction and derivatization of spiked milk samples and comparison with peak areas obtained after derivatization of the same amounts of aqueous unextracted MOX solutions. The mean absolute recovery of MOX was of 89.43%. The relative recovery was calculated as 98.26% by the comparison of the amounts that are added on to spiked and measured by the calibration curve.

  Five replicates of samples at each concentration were assayed on the same day for intra-day and on five different days for inter-day precision and accuracy. The RSD values of both intra-day and inter-day assays were all less than 3.15%. According to all these results summarized in *Table II*, good precision and accuracy were observed.

Table II. Accuracy and precision of the method

Added concentration (µg mL^{-1})	Foundconcentration (µg mL^{-1}) (Mean ± SD^1)	Recovery (%)	RSD of recovery	RSD of intra-day variation	RSD of inter-day variation
1.00	1.64 ± 0.04	100.64	0.56	2.26	3.15
10.00	97.68 ± 0.82	97.68	2.28	2.75	2.86
40.00	38.58 ± 1.15	99.10	1.45	1.67	2.92
Mean relative recovery = 98.26					

For each concentration, n = 3.

- Robustness: Robustness was assessed by determination of the QC samples at three concentration levels as described at validation section above (n = 3). The parameters, that are changed to measure the robustness of the method, are flow-rate, column oven temperature, acetonitrile, and aqueous phase contents of the mobile phase. The column temperature was changed from room temperature to 30 °C. The mobile phase proportions were changed from 35:65 (acetoni-

trile–acidic solution) to 30:70, and the flow rate was changed from 0.5 to 0.3, 0.6, and 1 mL min^{-1}. These changes had no significant effect on peak area and resolution. According to standard conditions, resolution of derivative's peak is 4.3 ± 0.6; in the trials for robustness, resolution was observed between 3.8 to 4.6.

- Stability: The effects of freezing and thawing on MOX concentrations were studied using spiked MOX milk standards at 1, 20, and 40 μg mL^{-1}, which were subjected to four freeze–thaw cycles before analysis. The stability of derivatives in spiked milk stored at room temperature for 24 h and −20 °C for 2 weeks was evaluated, as well. Stock solutions of MOX were stable at least for 30 days when stored at −20 °C. After 20 days, no decrease was observed in the concentration of MOX in milk. Derivatized solutions were found to be stable for 4 days if the samples were kept at 4 °C using a sample cooler, and 1 day if kept at room temperature.

    The main advantage of the proposed method is the ability to determine MOX in human breast milk and supply the deficiency in the literature. The cited methods are for plasma assays (3–11, 13, 14), urine assay (12), and saliva and interstitial space fluid assay (13). The other advantages are related to simplicity of the derivatization process and extraction procedure from milk with high recovery values. Even though fluorogenic derivatization procedures require heat and longer time, in this study, no heating procedure or long reaction time is needed. Moreover, the RSD, RME, and recovery values indicate that the proposed method is more precise and accurate than some previously reported methods (4–9). Most of the reported methods also depend on RP-HPLC techniques (3–8), but differently, the presented method has the shortest retention time.

Conclusion

The presented HPLC method is sensitive, cost-effective, and reproducible. The retention time of the drug substance is about 3 min, which shows that the duration of the analysis is very short. Highly fluorescent derivatives of MOX with fluorescamine allow fluorometric detection of the drug substance with simple HPLC, which can be reliably used by almost every drug laboratory. The presented method can certainly be used for the researches about the effect of the drug in infants and analysis of the drug in breast milk.

Acknowlegments

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