Abstract

The simple yet rapid and accurate spectrophotometric method for the determination of fluoroquinolone family member, moxifloxacin in human plasma was developed. The determination was carried out in a citrate – phosphate buffer (pH = 7.2) in the presence of sodium dodecylsulfate (12.0 mmol L\(^{-1}\)). Second order derivative spectra were employed for the quantitation of moxifloxacin by measuring peak – to – peak amplitude in a wavelength range 335 – 345 nm. Linear dynamic range was 0.25 – 10 μg mL\(^{-1}\) with a limit of detection 0.03 μg mL\(^{-1}\). Recovery was between 95 – 102%. Addition of surface active substance improved the sensitivity of the method. The method was successfully applied for the analysis of plasma of healthy volunteers. A HPLC method was also developed as a reference method, to validate and confirm spectrophotometric results.

Keywords: Moxifloxacin; Antibiotic; Plasma; Derivative spectrophotometry; Sodium dodecylsulfate (SDS)

Introduction

Moxifloxacin (1-cyclopropyl-7-(S,S)-2,8-diazabicyclo(4.3.0)-non-8-yl-6-fluoro-8-methoxy-1,4-dihydro-4-oxo-3-quinoline carboxylic acid hydrochloride), Fig. 1, is a new fourth generation 8-methoxy fluoroquinolone developed primarily for the treatment of community acquired pneumonia and upper respiratory tract infections. It is active against Gram negative pathogens, Gram positive cocci, aerobic intracellular bacteria, atypical organisms and anaerobic bacteria [1, 2].

Widespread use of moxifloxacin requires development of number of analytical methods for its determination in various matrices. However, not many methods have been developed for moxifloxacin determination in biological fluids and dosage forms.

Moxifloxacin has been determined in biofluids by HPLC methods [3 – 11], fluorometry [12], capillary electrophoresis [13], differential pulse polarography [14], dif-
Differential pulse voltammetry [15] and spectrophotometry [16]. The HPLC based procedures are methods of choice in the analysis of fluoroquinolones in biological matrices. They offer wide dynamic linear range of determination from about 5 ng/mL up to 3 μg/mL with a limit of detection as low as 2 ng/mL. These techniques however, are time consuming and require expertise. Therefore, alternative methods for routine clinical analysis are desirable. Very suitable may be spectrophotometric methods. So far only two papers dealt with spectrophotometric determination of moxifloxacin [14,16] using zero order UV spectra.

No methods have been reported which use derivative spectrophotometric determination of moxifloxacin in human plasma.

In clinical practice frequent determination of moxifloxacin levels in plasma is often required to follow success in therapy or to evaluate the efficacy of moxifloxacin and possible emergence of resistance. Such determinations are time consuming because of the need to examine a large number of samples. Therefore, rapid analytical methods are required for labor saving. So, the aim of the present investigation was to develop a fast, sensitive, accurate and simple procedure for determination of moxifloxacin in biofluids, without prior, tedious, extraction procedures.

In continuation of our previous determination of fluoroquinolones [17] in the present paper, second-order derivative UV spectrophotometry in a micellar medium was used for the direct assay of moxifloxacin in human plasma.

Experimental

Reagents and standard solution

Moxifloxacin hydrochloride standard (declared purity > 99%), yellow powder, Mr = 437.9, was obtained from BayerPharma AG (Germany). Tablets “Avelox” (400 mg) were products BayerPharma AG. Standard solutions of NaOH and HCl (0.1 mol L⁻¹) were prepared by diluting from concentrated ampoule solution Merck (Darmstadt, Germany). Citrate buffers (pH = 4 – 8) were prepared by mixing appropriate volumes of 0.1 mol L⁻¹ citric acid and 0.2 mol L⁻¹ sodium-monohydrogenphosphate. Borate buffers (pH = 8.5 – 9) were prepared by mixing appropriate volumes of 0.025 mol L⁻¹ sodium borate and 0.1 mol L⁻¹ HCl. All buffer solutions were prepared according to Perrin and Dempsey [18]. Solution of sodium dodecylsulfate, SDS, (0.1 mol L⁻¹) was prepared from purified sodium dodecylsulphate (Merck) by direct weighing and subsequent dissolution in doubly distilled water. Human plasma samples were separated from human pool whole blood obtained from the Department of transfusion of clinical hospital “Dr Dragisa Misovic”, Belgrade.

Apparatus

Spectrophotometric measurements were performed on a Perkin-Elmer (USA), Model Lambda 35, and GBC (Australia) Cintra Model 40, UV-Vis, double-beam spectrophotometers, interfaced to an IBM PC computer. Derivative spectra were obtained with the software supplied by the manufacturer. Quartz cuvettes of 1 cm pathlength were used. Optimized working settings were: slit width 0.5 nm, scan speed 120 nm min⁻¹, time response 0.1 ms and Δλ = 1 or 2 nm [19]. HPLC measurements were performed on an Agilent (Waldbronn, FRG) Series 1100 chromatograph equipped with binary pump and fluorescence detector. The analysis was carried out on Supelco (USA) direct injection shielded hydrobolic phase column, 150 mm × 4.6 mm i.d., 5.0 μm particle size, with a mobile phase consisted of acetonitrile and 0.25 mol dm⁻³ Na₃PO₄ (pH = 6.5, adjusted with phosphoric acid) mixed in a volume ratio 15:85, respectively. Isocratic elution with flow rate 1.0 μL min⁻¹ and fluorescence detection with λex = 290 nm and λem = 500 nm was used. Structural analog of moxifloxacin, ofloxacin, was used as an internal standard. The data were processed with HP ChemStation software.

Optimization procedure

Stock solution of moxifloxacin hydrochloride (50.0 μg mL⁻¹ of moxifloxacin) was prepared by dissolving moxifloxacin standard in doubly distilled water. For the purpose of preliminary optimization of measurement conditions, different volumes, 0.02 – 2.00 mL, of the stock solution were transferred to 5 mL volumetric flasks using Eppendorf pipettes, and diluted with either water, water + SDS, buffer or buffer + SDS to the mark and shaken well. The concentration of SDS in solu-
tions was 12 mmol L\(^{-1}\). In water solutions the pH was adjusted by the addition of either HCl or NaOH and measured potentiometrically (pH from 3.0 to 9.0). The concentration range of moxifloxacin thus covered, was 0.2 – 20.0 µg mL\(^{-1}\). The zero order spectra were recorded in a wavelength interval 200 – 450 nm while second-order derivative spectra were recorded in 320 – 400 nm wavelength range against appropriate reagent blank. For the construction of the calibration graph the standard solutions of moxifloxacin prepared in citrate buffer (pH = 7.2) with the addition of SDS (12 mmol L\(^{-1}\)) were used. Peak to peak amplitude was measured in second order derivative spectra in the wavelength range 335 – 345 nm and plotted against concentration.

**Calibration graph and procedure for plasma samples**

A human pool plasma (1.0 mL) was mixed with different volumes of standard solution of moxifloxacin to give the final drug concentration of 0.25 – 10.0 µg mL\(^{-1}\). According to literature data moxifloxacin in plasma is bound to plasma proteins (43%). To coagulate proteins and prevent moxifloxacin binding to proteins (mainly albumin) sodium-dodecylsulphate was added so that its final concentration was 12 mmol L\(^{-1}\). SDS also creates hydrophobic enviroment which enhances the solubility of moxifloxacin. The plasma samples were then centrifuged at 6000 rpm for 15 min. Supernatant was separated and after filtration through a Minisart plus syringe filter (0.2 µm pore size, Supelco, USA) was transferred into a volumetric flask. The flask was filled with citrate buffer (pH = 7.2) to the mark. Zero and second order UV derivative spectra of the prepared solutions were taken in a wavelength interval 200 – 450 nm and anionic forms. Relative concentrations of these forms strongly depend on pH of the solution. Thus, the strict control of the pH of the solution is necessary for the determination of moxifloxacin by spectrophotometric method.

**Results and Discussion**

Moxifloxacin is a weak heterocyclic amino acid which may exist in solution in cationic, neutral, dipolar and anionic forms. Relative concentrations of these forms strongly depend on pH of the solution. Thus, the strict control of the pH of the solution is necessary for the determination of moxifloxacin by spectrophotometric method.

Examination of the spectra of moxifloxacin in aqueous phase, which was pH adjusted with the addition of strong acid or strong base, were measured potentiometrically, and their comparison with the spectra where the pH was adjusted by buffer shows only negligible influence of buffers on moxifloxacin absorption. Addition of SDS enhances the intensity of absorption peaks for about ca. 5% and affects the shape of absorption bands which become more symmetrical (Fig. 2). SDS also shifts isoelectric point of moxifloxacin from 7.44 to 8.21 [17] thus keeping the solubility at the acceptable level.

The UV spectrum of moxifloxacin consists of two major bands with the maximum at 290 nm for the first band and 340 nm for the second band. The high energy band is mainly due to the \(\pi \rightarrow \pi^*\) transition in the aromatic ring. The low energy band is due to \(n \rightarrow \pi^*\) transition in diazabicyclo substituent at position 7, and consists of two subpeaks. These subpeaks also reflect the participation of non-bonding electron pair on nitrogen at position 1 and are caused by an intermolecular hydrogen bond equilibrium between moxifloxacin and water as well as intramolecular hydrogen bond between 4-keto and 3-carboxylic groups [21,22]. Upon increasing the pH from ca. 4 to 9 higher energy band shows only small changes in position and maximum intensity (hypochromic shift). The lower energy band exhibits however, significant changes in a shape, position and intensity (bathochromic shift). At pH values lower than 7, this band is fairly symmetrical with a shoulder at 350 nm, but at pH values higher than 7, two separated absorption maxima at 335 and 355 nm are obtained. Intensity of the band increases upon increasing the pH up to ca 8 and then decreases. Thus, the optimal pH value for analysis was set to 7.2. The absorption of plasma proteins interferes with the band at 290 nm but becomes negligible at 300 nm. (Fig. 3).

Therefore, the peak centered at 340 nm was chosen for analysis. Since this peak is not well separated from the peak at 355nm the second-order derivative spectrum was used. In this way the background absorption of the plasma was also minimized.

Thus the optimal conditions for the determination

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of moxifloxacin in plasma and in dosage forms were: wavelength at maximum absorption, 340 nm and pH of solution, 7.2. In Fig. 4, the zero (a) and second-order derivative spectra (b), for the different concentration of moxifloxacin in plasma at pH = 7.2 are shown.

The calibration graph for the derivative spectrophotometry was constructed by plotting the peak-to-peak amplitude, in the second derivative spectrum, versus drug concentration. The amplitude was measured in 335 - 345 nm wavelength interval. The equation obtained through regression analysis of the data for standard solution of plasma was:

\[
Y = (2.41 \pm 0.06)10^{-3} X + (3.0 \pm 0.2)10^{-4}
\]

\((n = 7, r = 0.997, S_x = 2.7 \times 10^{-5})\)

where \(Y\) is the peak-to-peak amplitude in second order derivative spectra, in absorbance units, \(X\) is the concentration of moxifloxacin in μg mL\(^{-1}\) and \(S_x\) is the standard error of estimation. The limit of detection, DL, defined as [20]:

\[
DL = 3.3 \times \frac{s_b}{a}
\]

where \(s_b\) is standard deviation of intercept and \(a\) is a slope of the calibration curve was found to be 0.030 μg mL\(^{-1}\).

Table 1 shows the results obtained in the analysis of plasma samples.

The accuracy of measurements, expressed in terms of relative error (R.E.) was about 5% or even less, thus indicating negligible influence of plasma proteins. Clinical investigations have shown that moxifloxacin is rapidly and completely absorbed after oral administration. Peak plasma concentration (approximately 4.5 μg mL\(^{-1}\)) is reached about 1 to 3 hours after administration of single dose of 400 mg. The bioavailability is approximately 90% and together with large AUC area indicate that effect of the first liver pass is negligible. Moxifloxacin has a long plasma half-life (12 h) and is predominantly excreted as either unchanged drug or sulfate and glucuronide metabolites. In this way moxifloxacin metabolizes very slowly in plasma, allowing accurate determination even more than 10 h after administration. Thus, the proposed method for the assay of moxifloxacin in plasma, being simple and rapid, can be applied for the purpose of pharmacokinetic measurements and routine clinical analysis.

The method was applied to the analysis of moxifloxacin in plasma of two healthy volunteers to whom an “Avelox” tablet (400 mg) was administered after overnight fasting. The blood samples (1 mL) were taken by cubital vein puncture at 1 h after administration. The blood was allowed to clot at room temperature and after-
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Fig. 3. The UV spectra of blank plasma, plasma with the addition of moxifloxacin and plasma with the addition of moxifloxacin and sodium dodecylsulfate.

Table 1. Results obtained in determination of moxifloxacin in plasma samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>Added (µg mL⁻¹)</th>
<th>Found (µg mL⁻¹)</th>
<th>R.E. (%)</th>
<th>Recovery (%) ± R.S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.250</td>
<td>0.238 ± 0.01</td>
<td>4.8</td>
<td>95.2 ± 4.3</td>
</tr>
<tr>
<td>2</td>
<td>0.575</td>
<td>0.547 ± 0.02</td>
<td>4.9</td>
<td>95.1 ± 3.7</td>
</tr>
<tr>
<td>3</td>
<td>2.070</td>
<td>2.001 ± 0.03</td>
<td>3.3</td>
<td>96.7 ± 1.5</td>
</tr>
<tr>
<td>4</td>
<td>4.025</td>
<td>4.109 ± 0.07</td>
<td>2.1</td>
<td>102.1 ± 1.7</td>
</tr>
<tr>
<td>5</td>
<td>5.010</td>
<td>4.987 ± 0.02</td>
<td>0.5</td>
<td>99.5 ± 0.4</td>
</tr>
</tbody>
</table>

*Mean, standard deviation, relative error (R.E.) and relative standard deviation (R.S.D.) in five determination
Fig. 4a. Zeroth spectra for moxifloxacin determination in plasma. Concentrations of moxifloxacin are in μg/mL.

Fig. 4b. Second order derivative spectra for moxifloxacin determination in plasma.
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wards centrifuged at 1500 rpm for 30 min. The superna-
tant (0.5 mL) was subjected to the same procedure as for plasma calibration graph. The zero order UV spectrum of one patient plasma specimen is shown in Fig. 5.

The results of analysis after five repeated measure-
ments were: for sample 1; 3.56 ± 0.15 μg mL⁻¹ and for sample 2; 3.89 ± 0.10 μg mL⁻¹. These results are in good agreement with the literature data [23].

An HPLC method was developed to compare the validity of the spectrophotometric method. In the development of the method we aimed to achieve the pH values near to neutral as this proved to be optimal for spectrophotometry. However, the column used does not allow higher pH values than 7 so we varied experimental parameters in such a way that pH was set to 6.5 while at the same time the percentage of acetonitrile was raised in comparison with that used in our previous work [17] so that chromatographic parameters were acceptable. Spiked plasma samples were directly injected onto the column. The calibration line was constructed by plotting the peak areas ratio of moxifloxacin to ofloxacin versus moxifloxacin concentration. The concentration of ofloxacin was 0.5 μg mL⁻¹. The method was fully validated and validation parameters were: linearity range 50 – 1500 ng mL⁻¹, correlation coefficient, 0.9983, mean recovery, 98.5%, limit of quantification, 12.0 ng mL⁻¹ and limit of detection, 5.0 ng mL⁻¹. The chromatogram of patient plasma sample is shown in Fig. 6.

The concentration of moxifloxacin in patient plasma read from the calibration curve was 4.05 ± 0.08 μg mL⁻¹ for sample 2. Relative error related to spectrophotometric result is 4.1%, which means that the accuracy and precis-

Fig. 5. UV spectra of patient plasma specimen in the presence and without SDS.

Fig. 6. Chromatogram of patient plasma specimen taken 1 hr after administration of an Avelox tablet (400 mg).
sion of the proposed method is satisfactory.

Conclusions

The proposed method is fast and accurate with minimal sample pre-treatment. Plasma proteins do not interfere and addition of sodium dodecylsulfate enhances sensitivity of determination. The method could be reliably used in the analysis of the large number of samples in a short time e.g. in clinical and pharmacological analysis.

Acknowledgements

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References