The HPLC method for fast determination of levofloxacin in liquid pharmaceutical formulations

Metoda HPLC do szybkiego oznaczenia lewofloksacyny w płynnych postaciach leku

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Abstract

Background. Levofloxacin is a representative of the third generation of fluoroquinolones. It is an antimicrobial agent with a wide spectrum of the bactericidal activity. It can be found in liquid pharmaceutical formulations such as infusions or eye drops. This paper reports a fast HPLC method for determination of levofloxacin in liquid pharmaceutical formulations. Material and methods. The applied method is RP-HPLC method with UV detection. The sample pre-conditioning involves simple dilution. The calibration curve range obeys 1-6 mg/mL and LOD is 0.019 mg/mL. Results. The validation parameters did not exceed 5% and 2% for interday and intraday assay respectively. The analyte remained stable within the analysis and after the long term of storage. The time of the analysis is very fast – 5 min. Conclusions. The reported method obeys the validation parameters and is suitable for drug analysis. It provides fast and reliable results.

Keywords: levofloxacin, HPLC, infusions, eye-drops, drug analysis

Streszczenie


Słowa kluczowe: lewofloksacyna, HPLC, wlew dożylny, krople do oczu, analiza leku

Introduction

Levofloxacin (LEVO) is an antimicrobial agent which is a representative of the third generation of the fluoroquinolones. It is an L-isomer of ofloxacin. It has a broad bactericidal activity against many Gram-positive, Gram-negative and also atypical bacteria such as Legionella and Mycoplasma. The activity of LEVO is based on the inhibition of the enzymes that are responsible for separation of bacterial DNA i.e. topoisomerase IV and gyrase. It results in the inhibition of cell replication and, in consequence, the cell death [1-4].

The effectiveness of activity of LEVO, as well as the other representatives of fluoroquinolones, strongly depends on its concentration in blood. The bactericidal effect is provided with a repeatable dose in a defined time interval. It prevents the resistance of the bacteria to the treatment. The other factor that may influence the effectiveness of the treatment is the stability of the substance in a pharmaceutical formulation. LEVO is available on the market in liquid pharmaceutical formulations such as infusions and eye-drops. This paper describes the rapid, simple, selective and precise
HPLC method for determination of LEVO in liquid formulations.

**Material and methods**

**HPLC conditions**

Chromatographic analysis was performed at the room temperature. The LiChroCART column (125 × 4 mm, 5 μm, Merck, Germany) with LiChroCART guard column (4 × 4 mm, 5 μm, Merck, Germany) were applied for chromatographic separation. The separation was performed in a chromatograph 1220 Infinity LC (Agilent Technologies). The mobile phase consisted of acetonitrile (HPLC grade, Merck, Germany) and 0.4% aqueous solution of triethylamine (POCH, Gliwice, Poland) (pH 3.0) solutions in the following ratio (24:76 v/v) and it was degassed by ultrasonic degassing. The pH of triethylamine solution was achieved by an addition of concentrated phosphorous acid (Fluka, Germany). The flow rate was 1 mL/min, and the detection wavelength was λ=295 nm. The data were analysed with OpenLab ChemStation ver. A.01.05 software.

**Calibration standards**

The following stock solutions were prepared: LEVO (Sigma-Aldrich, Germany) and moxifloxacin (IS) (Santa Cruz Biotechnology, USA). The concentration of LEVO was 12 mg/mL and for IS was 1 mg/mL. The substances were dissolved in methanol. The standard solutions of LEVO with the following concentrations were prepared: 1 mg/mL, 2 mg/mL, 3 mg/mL, 4 mg/mL, 5 mg/mL and 6 mg/mL. The IS solution concentration was 150 mg/L.

**Sample preparation**

The matrices of a liquid pharmaceutical formulations consisted mainly of isotonic solution of sodium chloride. The concentration of LEVO in the formulation was 5 mg/mL. 10 μL of the investigated infusion was added to 990 μL of methanol. 100 μL of the dilution was mixed with 20 μL of IS solution and filled with methanol to 200 μL. The 20 μL of the final mixture was injected onto HPLC system.

**The validation parameters**

The following validation parameters were assayed: linearity, precision and accuracy.

The linear equation describing the relationship between LEVO concentration and the area under the peak of LEVO and IS ratio. The calibration curve was calculated by the least squares method. Five calibration curves were prepared on five separate days and the validation parameters were calculated.

For the inter- and intra-day assay precision and accuracy for low (1 mg/mL, 2 mg/mL) and high concentrations (5 mg/mL, 6 mg/mL) quality samples were analysed (Table I).

The stability was evaluated during three freeze-thaw cycles and after storage at a room temperature 24 h. The analytes are stable if the deviation from the nominal concentration is within ± 10%.

**Limit of detection (LOD)**

The limit of detection was calculated from the following equation:

\[
LOD = \frac{3.3\times s}{b}
\]

s-standard deviation of the interception, b- slope of the equation curve

**Results and discussion**

The fast method for the determination of the drug is useful in drug analysis. The mobile phase consisted of acetonitrile and triethylamine (24:76, v/v) solution which was an ion pair reagent. This proportion provides...
the total resolution of the LEVO and IS. The chromatographic profile is shown in Fig. 1. The retention time for levofloxacin is 2 min. and IS is 3.7 min. This method characterizes a short time of analysis – 5 min.

The addition of ion pair reagent causes a better interaction of LEVO with the stationary phase and it reduces the tailing of the peaks when combined with the proper value of pH. The separation mechanism is an interaction of LEVO and with the free silanol groups of the stationary phase. Triethylamine reduces the availability of these groups for LEVO and it resulted in reducing of the tailing effect. The silanol groups are ionized at the pH above 3.5 and they interact with 1° and 2° amines. The pH of the mobile phase was 3.0. The increase of pH resulted in the peak tailing. According to the literature data, the concentration of ion pair reagent up to 1% combined with a slightly acidic pH provides a good resolution of the analytes [5,6]. In our study, 0.4% triethylamine combined with a pH value 3.0 resulted in a total resolution of the analytes. Too high concentration of the ion pair reagent results in a long time of the column equilibration. The other factor that was considered for improving the shape of the peak was the phosphate buffer, however it did not improve the quality of the resolution of analytes. The applied organic solvent was acetonitrile. The use of methanol resulted in longer time of analysis. The peaks were

Figure 1. The chromatogram of the sample of commercially available infusion. The concentration of levofloxacin 5 mg/mL (levofloxacin (1) tr – 2 min and IS (2) tr – 3.7 min)

Rycina 1. Chromatogram próbki pobranej z komercyjnie dostępnego preparatu do wlewu dożylnego. Stężenie levofloksacyyny 5 mg/mL (levofloksacyna (1) tr – 2 min and IS (2) tr – 3.7 min)

Figure 2. The chromatogram of the: A) calibration curve sample (LEVO concentration 5mg/mL), B) blind sample, C) zero sample, where 1- levofloxacin, 2- IS

Rycina 2. Chromatogram: A) próbki z krzywej wzorcowej (stężenie LEVO 5mg/mL), B) próbki ślepej, C) próby zerowej, gdzie 1-levofloksacyyna, 2- IS
broad and the pressure on the column was high, the resolution was poor. The mixtures of acetonitrile and methanol in different proportions were also tested but it resulted in poor resolution. The chromatogram of the sample of the calibration curve, the zero and blind sample was showed in Fig 2.

Many separation methods were reported for LEVO determination in pharmaceutical formulation. They involved capillary electrophoresis [7], HPLC with ultraviolet detection [8, 9]. The developed method was a modification of the method developed by the Santoro et al. [9], however in our study the internal standard was used. The method was tested on the commercially available infusion of levofloxacin at the concentration 5 mg/mL and the separation provided the total resolution of the analyte and IS (Fig.1). The temperature was ambient. The higher temperature does not influence the chromatographic separation significantly [10].

The validation parameters obey the ICH recommendations for bioanalytical methods. The inter-day precision and accuracy do not exceed the value of 4.21% and 3.01 respectively. The intra-day precision and accuracy do not exceed the value 1.74% and 1.61% respectively (Table I).

The sample pre-conditioning involved a simple dilution with methanol. The matrix was not complex and it was not necessary to apply more laborious techniques of sample preparation such as liquid-liquid extraction or solid-phase extraction. The dilution of the liquid formulation was also applied by Gupta et al. [11], Kalarya et al. [5] and Razzaq et al. [12].

The analyte remained stable in stability tests. After storage for 24 hours at the room temperature and after storage at the temperature -20°C for three months the accuracy (denoted as RE) did not exceed 2.0%. After three freeze-thaw-cycles the accuracy did not exceed 2.1% (Table II).

The calibration curve was linear within the range 1-6 mg/mL. The regression coefficient was higher than 0.999. The test value in Mandell’s test was 6.35 vs. the critical value 34.11 and it also confirmed the linearity.

The lowest concentration from the calibration curve is 1 mg/mL. The LOD is 0.019 mg/mL and it is satisfactory for the determination of LEVO in infusions.

Conclusions
In this study, the fast and cheap method for determination of LEVO was developed. The method fulfills the validation parameters for the analytical methods and can be applied in drug analysis.

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Conflict of interest
None

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Table II. The stability of levofloxacin
Tabela II. Stabilność lewofloksacyny

<table>
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<tr>
<th>Experimental conditions</th>
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