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First liquid chromatography method for the simultaneous determination of levofloxacin, pazufloxacin, gatifloxacin, moxifloxacin and trovafloxacin in human plasma

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ABSTRACT

For the first time a simple, selective and sensitive liquid chromatography method was developed and validated for the simultaneous determination of levofloxacin (LEV), pazufloxacin (PAZ), gatifloxacin (GAT), moxifloxacin (MOX) and trovafloxacin (TRO) in human plasma. Samples were pre-treated with acetonitrile for precipitation of plasma proteins followed by evaporation and reconstitution steps. Chromatographic separation of the analytes and norfloxacin, used as internal standard (IS), was performed under gradient elution on a LiChroCART® Purospher Star C_{18} column (55 mm × 4 mm, 3 μm). The mobile phase comprised a mixture of 0.1% aqueous formic acid adjusted to pH 3.0 with triethylamine, acetonitrile and methanol pumped at a flow rate of 1.0 mL/min. The detector was set at excitation/emission wavelengths of 260/455 nm. Calibration curves were linear ($r^2 \geq 0.9923$) in the ranges of 0.005–5 μg/mL for GAT, 0.02–5 μg/mL for LEV, PAZ and MOX and 0.04–5 μg/mL for TRO. The intra and interday precision did not exceed 7.32% and the intra and interday accuracy ranged from −11.73 to 8.92%. The limits of quantification were established at 0.005 μg/mL for GAT, 0.02 μg/mL for LEV, PAZ and MOX and 0.04 μg/mL for TRO. No endogenous or tested exogenous compounds were found to interfere at the retention times of the analytes and IS. Since the proposed method proved to be reliable for the quantitative determination of LEV, PAZ, GAT, MOX and TRO it may be a useful tool for routine analysis and to support clinical pharmacokinetic and toxicological studies involving these antibiotics.

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1. Introduction

Fluoroquinolones (FQs) are an important class of synthetic antibiotics widely used in anti-infective chemotherapy due to their remarkably broad spectrum of activity [1,2]. They include a large and continuously expanding group of structurally related compounds that are classified into four generations. Third and fourth generation FQs, e.g. gatifloxacin (GAT), gemifloxacin, levofloxacin (LEV), moxifloxacin (MOX), pazufloxacin (PAZ), sparfloxacin and trovafloxacin (TRO), have several advantages over the earlier ones; they not only exhibit stronger and expanded activity against both gram-negative and gram-positive bacteria and anaerobes but also have improved pharmacokinetic properties. Particularly, these new FQs present high oral bioavailability with plasma drug concentrations comparable to those after intravenous administration, long elimination half-lives and good tissue penetration [3–7].

FQs have a concentration-dependent bactericidal activity and their efficacy can be predicted by measuring two important parameters, the maximum plasma drug concentration (C_{max}) to minimum inhibitory concentration (MIC) ratio and the area under the plasma concentration–time curve from 0 to 24 h (AUC_{0-24}) to MIC ratio. Given the intra- and inter-individual pharmacokinetic variability, particularly significant in hospitalized patients, it is important to monitor plasma drug concentrations to attain the optimal drug dosage regimens and to prevent bacterial resistance [6,8–11]. According to published pharmacokinetic studies, typical values of C_{max} range from approximately 3 to 5/6 μg/mL (depending on the dosage), after a single oral or intravenous dose administration. In the case of pazufloxacin, C_{max} could reach values up to 10 μg/mL after intravenous infusion [7]. This implies the need of simple and adequate analytical methods that can be easily applied in clinical settings for quantification of FQs in human plasma at those expected concentrations.

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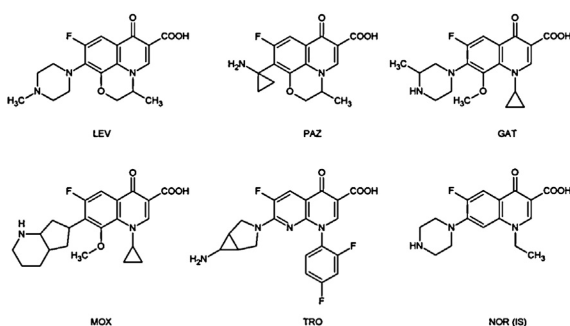

Fig. 1. Chemical structures of levofloxacin (LEV), pazufloxacin (PAZ), gatifloxacin (GAT), moxifloxacin (MOX), trovafloxacin (TRO) and norfloxacin (NOR) used as internal standard (IS).

A large number of high-performance liquid chromatography (HPLC) methods with ultraviolet (UV) or fluorescence (FL) detection have been described for the determination of a single FQ of the third and fourth generations in biological matrices [7,12–18]. However, only a few HPLC methods have been reported in literature for the separation and simultaneous quantification of two or more of these new FQs in human plasma or serum [6,19–23]. Nemutlu et al. [21] and Baietto et al. [23] developed an HPLC-UV method for the measurement of LEV and MOX in human plasma, amongst other drugs and quinolones; whereas Watabe et al. [6] described an HPLC-FL method for the quantification of LEV and PAZ, and the second generation FQ, ciprofloxacin, in the same type of matrix. Three other publications refer to the simultaneous determination of human plasma concentrations of three and four new FQs, namely GAT, LEV and MOX [20]; GAT, MOX and sparfloxacin [22] and GAT, LEV, MOX and TRO [19]. Nguyen et al. [20] developed a fully automated HPLC with a column-switching technique, allowing direct human serum injection into the chromatographic system without any classical pre-treatment steps; however this technique is not feasible in all analytical laboratories. Liang et al. [19] developed an HPLC method with UV and FL detection but it presents the disadvantage of using a complex mobile phase with ion-pair reagent; although four new FQs were separated by optimization and adjustment of chromatographic conditions, the HPLC method was only fully validated for the measurement of LEV in human plasma.

To the best of our knowledge, no bioanalytical method has been reported for the simultaneous determination of five of the new FQs in human plasma or serum. The aim of this research work was to develop and validate a simple and reliable reversed-phase HPLC method coupled with FL detection to quantify LEV, PAZ, GAT, MOX and TRO (Fig. 1) in human plasma. The proposed method was validated in a wide concentration range for each compound and therefore can be applied to therapeutic monitoring of these FQs in clinical practice and to support other clinical pharmacokinetic- and toxicokinetic-based studies.

2. Material and methods

2.1. Chemicals, materials and reagents

LEV (lot no. 1395156, ≥98.00% purity), TRO (lot no. 020M47081, ≥98.00% purity) and norfloxacin (NOR) (lot no. 028K1480, ≥98.00% purity), used as internal standard (IS), were purchased from Sigma–Aldrich (St. Louis, MO, USA). GAT (lot no. M11254/07-10, 99.97% purity) and PAZ (lot no. M11529/12-10, 99.80% purity) were obtained from Biokemix (New Mills, Derbyshire, UK) while MOX (lot no. 51587, 99.63% purity) was acquired from Molekula (Shaftesbury, Dorset, UK). Methanol and acetonitrile (both HPLC gradient grade) were purchased from Fisher Scientific (Leicestershire, UK). Ultrapure water (HPLC grade, >15 MΩ) was prepared by means of a Milli-Q water apparatus from Millipore (Milford, MA, USA). Reagents such as fuming hydrochloric acid (37%), formic acid (98–100%) and triethylamine (TEA) were acquired from Merck KGaA (Darmstadt, Germany).

Blank human plasma samples from healthy donors were kindly provided by the Portuguese Blood Institute after written consent of each subject.

Table 1
Gradient elution programme for HPLC analysis. Solvents A, B and C correspond to 0.1% aqueous formic acid (pH 3.0, triethylamine), acetonitrile and methanol, respectively.

Time (min)	Solvent A (%)	Solvent B (%)	Solvent C (%)
0	80	0	20
2	80	0	20
3	82	2	16
5	82	2	16
7	42	2	56
11	42	2	56
13	80	0	20
18	80	0	20

2.2. Apparatus and chromatographic conditions

The chromatographic analysis was carried out on a Shimadzu HPLC system (Shimadzu Corporation, Kyoto, Japan) equipped with a solvent delivery model (LC-20A), a degasser (DGU-20A5), an autosampler (SIL-20AHT), a column oven (CTO-10ASVP) and fluorescence detector (RF-20AXS). The HPLC apparatus and data acquisition were controlled by LCsolution software (Shimadzu Corporation, Kyoto, Japan).

The chromatographic separation of the five analytes (LEV, PAZ, GAT, MOX and TRO) and IS was performed under gradient elution using a reversed-phase LiChroCART® Purospher Star C_{18} column (55 mm × 4 mm, 3 μm particle size; Merck KGaA, Darmstadt, Germany) thermostated at 25 °C. The mobile phase consisted of a mixture of 0.1% aqueous formic acid adjusted to pH 3.0 with TEA (solvent A), acetonitrile (solvent B) and methanol (solvent C) and was pumped at a flow rate of 1.0 mL/min, applying the time gradient elution programme described in Table 1. After restoring the initial composition of the mobile phase, the column was re-equilibrated for 5 min, leading to a total run time analysis of 18 min. The FL excitation and emission wavelengths were set at 260 and 455 nm, respectively, and the sample injection volume was 20 μL.

2.3. Stock solutions, calibration standards and quality control samples

Stock solutions were individually prepared for LEV, PAZ, GAT, MOX, TRO and IS at the concentration of 1 mg/mL. Stock solutions of PAZ and IS were prepared by dissolving appropriate amounts of each compound in a mixture of methanol and 37% hydrochloric acid (99.5:0.5, v/v), while stock solutions of LEV, GAT, MOX and TRO were prepared in pure methanol. Appropriate dilutions of stock solutions were made with methanol to obtain two intermediate solutions per analyte at the concentrations of 250 and 20 μg/mL. Each of the stock or diluted solutions were combined and diluted in methanol to prepare six spiking solutions, containing all five analytes with final concentrations of 0.1, 0.2, 1.0, 5.0, 30.0, 100.0 μg/mL for GAT; 0.4, 0.8, 2.8, 10.0, 40.0, 100.0 μg/mL for LEV, PAZ and MOX and 0.8, 1.6, 4.0, 12.0, 40.0, 100.0 μg/mL for TRO. The IS stock solution was also diluted in methanol to a concentration of 250 μg/mL; from this intermediate solution, an aqueous IS working solution of 10 μg/mL was prepared daily by dilution with deionised water. All stock solutions were stored at −30 °C for one month and

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