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Validated liquid chromatographic-fluorescence method for the quantitation of gemifloxacin in human plasma

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ABSTRACT

A highly selective, sensitive and rapid high performance liquid chromatographic method has been developed and validated to quantify gemifloxacin in human plasma. The gemifloxacin and internal standard (ciprofloxacin) were extracted by ultrafiltration technique followed by injection into chromatographic system. Chromatographic separation was achieved on a reversed phase C_{18} column with a mobile phase of acetonitrile:0.1% trifluoroacetic acid (20:80, v/v) using isocratic elution (at flow rate 1 mL min^{-1}). The analytes were detected at 269 and 393 nm for excitation and emission, respectively. The assay exhibited a linear range of 25–5000 ng mL^{-1} for gemifloxacin in human plasma. The lower limit of detection was 10 ng mL^{-1}. The method was statistically validated for linearity, accuracy, precision and selectivity following FDA guidelines. The intra- and inter-assay coefficients of variation did not exceed 7.6% deviation of the nominal concentration. The recovery of gemifloxacin from plasma was greater than 97.0%. Stability of gemifloxacin in plasma was excellent with no evidence of degradation during sample processing (auto-sampler) and at least 3 months storage in a freezer at −70 °C. This validation method is applied for clinical study of the gemifloxacin in human volunteers.

1. Introduction

Gemifloxacin [(R, S)-7-(3-aminomethyl-4-syn-methoxyimino-1-pyrrolidinyl)-1-cyclopropyl-6-fluoro-1, 4-dihydro-4-oxo-1, 8-naphthyridine-3-carboxylic acid methanesulfonate] (CAS number 175463-14-6), is a recently developed fluoroquinolone antibacterial compound with a broad spectrum of activity (Fig. 1) [1–3]. It has shown potent antibacterial activity against clinical isolates and reference strains in both in vitro studies and experimental models of infection in animals [4,5]. It has particularly enhanced activity against gram-positive organisms, and displays fourfold higher activity than that of moxifloxacin against *Streptococcus pneumoniae* (minimum inhibitory concentration to inhibit 90% of isolates [MIC_{90}] is 0.03 μg mL^{-1}) in vitro [5]. Gemifloxacin has also shown potent activity against other major pathogens involved in respiratory tract infections, including *Haemophilus influenzae* and *Moraxella catarrhalis* and the atypical organisms, *Legionella pneumophila*, *Chlamydia* spp., and *Mycoplasma* spp. [6]. Furthermore, the compound has shown potent activity against many organisms that cause urinary tract infections. The adverse reaction profile is similar to that of older members of this class [7]. The pharmacokinetic properties of fluoroquinolone antibacterial agents have been well described [8]. Gemifloxacin is rapidly absorbed with a time to maximum plasma concentration (T_{max}) of 0.5–2 h in healthy subjects and displays linear pharmacokinetics over the dosage range studied (20–800 mg). The long terminal phase half-life ($t_{1/2}$) is 8 h after single or repeated administration. Approximately 20–30% of the administered dose is excreted unchanged in the urine and plasma protein binding of gemifloxacin is about 70% [9,10].

A few analytical methods have been published for quantification of gemifloxacin in human plasma using liquid chromatography–mass (LC/MS) [9–11] and –mass/mass (LC/MS/MS) [12]. However the LC/MS machine is quite expensive and not readily available in the most clinical, bioanalytical, educational research laboratories.

A method was described by Rote and Pingle [13] for the determination of gemifloxacin in spiked human plasma using liquid chromatography with UV detection. The calibration range was 30–600 ng mL^{-1} using liquid/liquid extraction.

Liquid chromatographic methods with fluorescence detection (HPLC-FL) were developed for the determination of gemifloxacin in human serum and urine using a reversed phase, and liquid–liquid extraction (unpublished data) [9,10].

To the best of our knowledge, the following validation parameters: linearity, precision, accuracy, recovery and stability have not been reported combined in a single gemifloxacin HPLC-FL method

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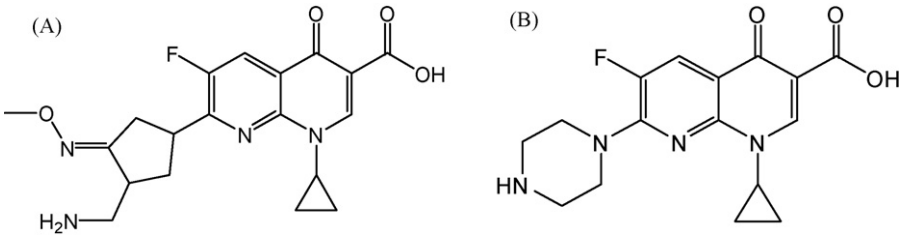

Fig. 1. The chemical structure of: (A) gemifloxacin and (B) ciprofloxacin (IS).

with its clinical application. So we developed a simple, rapid, sensitive and selective HPLC-FL method for the quantification of gemifloxacin in human plasma. The plasma sample containing the drug and IS was ultrafiltrated and the supernatant was injected into analytical column without any further clean up. The drug and internal standard were detected at 269 and 393 nm for excitation and emission, respectively. The lower limit of quantification is 25 ng mL^{-1} using 500 μL of human plasma, with lower limit of detection of 10 ng mL^{-1}. The total run time was ~8 min. The method proved very robust and was successfully applied for the analysis of clinical samples from male volunteers dosed with gemifloxacin.

2. Experimental

2.1. Chemicals and reagents

Gemifloxacin mesylate (purity ≥ 99.0%) and ciprofloxacin hydrochloride (purity ≥ 99.0%) were purchased from Sigma chemical (St. Louis, MO, USA). Deionized water was purified using cartridge system (Picotech water system, RTP, NC USA). Acetonitrile and ethanol were of HPLC grade (BDH, England). Trifluoro acetic acid was of analytical grade (Riedel-deHaen, Germany).

2.2. Apparatus

The LC system consisted of a water binary pump, model 1525 (Milford, MA, USA), equipped with a fluorescence detector model 2487, an autosampler model 717. Waters solvent delivery system was used to operate the isocratic flow. The data was collected with Millennium software (version 4.0) for data acquisition analysis. The chromatographic separations were performed using a symmetry LC-18 stainless steel column (150 mm × 3.9 mm, 5 μm) coupled with a symmetry C_{18}-sentry guard column (20 mm). Micron® (ultracell centrifugal filter paper Millipore cooperation MAO 1730, USA).

2.3. Chromatographic conditions

The mobile phase was a mixture of 0.1% trifluoroacetic acid: acetonitrile (80:20, v/v). The mobile phase was freshly prepared, then filtered through a Millipore filter (pore size 0.45 μm) and degassed continuously by an on-line degasser in the HPLC. Separation was performed at room temperature using a 1.0 mL min^{-1} flow-rate and 8 min run time. The injection volume was 75 μL and the detection wavelengths were set at 269 and 393 nm for excitation and emission respectively.

2.4. Standard solution

Standard solutions preparation was conducted at room temperature under subdued light (protected form direct light). The solution were protected from light with aluminum foil wrapping and stored at −70 °C. Gemifloxacin standard stock solution was prepared in 50% ethanol in water to produce a final concentration of 1 mg mL^{-1}. The working standard solution was prepared by diluting 1.0 mL of stock solution into 10 milliliters measuring flask with 50% ethanol in water to give a 100 μg mL^{-1} concentration. The internal standard (IS), ciprofloxacin stock solution was prepared in deionized water to produce a concentration of 1.0 mg mL^{-1}. Ten mL of this stock solution was diluted to 100 mL with sodium dihydrogen phosphate buffer to produce a working solution of 100 μg mL^{-1}.

2.5. Sample processing

Fifty microliters of ciprofloxacin (IS, 100 μg mL^{-1}), were added to 500 μL plasma sample in a 1.5 mL micro centrifuge tube (Eppendorf) thoroughly vortex-mixed for 30 s, then 450 μL sample was transferred to an ultrafiltration tube and centrifuged at 15,000 r.p.m. for 10 min. Aliquot of the ultrafiltrate were loaded in the autosampler tray and 75 μL of this sample was injected onto the analytical column.

2.6. Bioanalytical method validation

The described method was validated in terms of linearity, limit of detection (LOD), limit of quantification (LOQ), recovery, specificity, stability, precision and accuracy according to international guidelines regarding bioanalytical method validation [14–16]. Limit of detection and LOQ were calculated from the residual standard deviation of the regression line (δ) of the calibration curve and its slope (S) in accordance to the following equations: LOD = 3.3 (δ/S) and LOQ = 10 (δ/S).

2.6.1. Calibration and control samples

Appropriate volumes of gemifloxacin working standard solution (100 μg mL^{-1}) were added to drug-free human plasma (20 mL) to prepare eight non-zero standard drug concentration (25, 50, 150, 300, 600, 1500, 3000 and 5000 ng mL^{-1}), and five quality control concentrations (25, 75, 750, 2500 and 4000 ng mL^{-1}). Standard drug concentrations used for the preparation of the calibration curves were different from those employed in the quality control studies. A calibration curve was constructed from blank plasma sample, a zero sample (a plasma spiked with IS) and eight non-zero samples covering the total range (25–5000 ng mL^{-1}), including lower limit of quantification (LLOQ).

Each validation run consisted of system suitability sample, blank sample, a zero sample (a plasma processed with IS) calibration curve consisting eight non-zero samples covering the total range (25–5000 ng mL^{-1}) and quality control samples at five concentration (n = 6, at each concentration). Such validation samples were generated on six consecutive days. Calibration samples were analyzed from low to high concentration at the beginning of each validation run and the other sample were distributed randomly through the run. The calibration curve had a correlation coefficient (r) of 0.9988.

2.6.2. Human plasma applications

Five healthy volunteers participated in this study to support the applicability of the developed method to quantify gemifloxacin for