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Optimization and validation of a high performance liquid chromatography method for rapid determination of sinafloxacin, a novel fluoroquinolone in rat plasma using a fused-core C_{18}-silica column

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ABSTRACT

A novel, simple and rapid high performance liquid chromatographic method has been developed and validated for the determination of sinafloxacin, a new fluoroquinolone, in rat plasma using 96-well protein precipitation, fused-core C_{18}-silica column (4.6 mm × 50 mm, 2.7 μm) packed with a new solid support, which is made of 2.7 μm particles that consist of a 1.7 μm solid core covered with a 0.5 μm thick shell of porous silica.The chromatographic separation was achieved with a mobile phase of 20:80 (v/v) of acetonitrile and phosphate buffer (pH = 3.0) at a flow rate of 1 ml min^{-1}. Fluorescence detection was employed with λ_{ex} 295 nm and λ_{em} 505 nm. Lomefloxacin was used as internal standard (IS). The total analysis time was as short as 3 min. The method was sensitive with a limit of detection (LOD) of 2 ng ml^{-1}, with good linearity (R^2 = 0.9996) over the linear range of 5–500 ng ml^{-1}. The intra-day and inter-day precision was less than 5.8% and accuracy ranged from 100.3% to 103.5% for quality control (QC) samples at three concentrations of 10, 50 and 400 ng ml^{-1}.The fused-core C_{18}-silica column method offered high sample throughput, low injection volume and low consumption of organic solvents. The method was successfully employed in the pharmacokinetic study of sinafloxacin formulation product after tail vein injection to healthy rats.

1. Introduction

Sinafloxacin (7-(7-amino-5-azaspiro[2,4]heptan-5-yl)-1-cyclopropyl-6-fluoro-8-methoxy-4-oxo-1,4-dihydroquinoline-3-carboxylic acid compound with methane sulfonic acid (1:1)) (Fig. 1), is a new fourth generation quinolone derivative developed in China and has been applied for a patent in State Intellectual Property Office of the People's Republic of China (patent number: 03150047). The State Food and Drug Administration of China has completed the data verification of the pre-clinical study of sinafloxacin and approved its application in clinical trial. The microbiological, pharmacological and toxicological investigation of sinafloxacin has shown that it has a broad spectrum against gram-positive and gram-negative bacterium in vitro and in vivo, with more potent antibacterial activity but lower cytotoxicity than ciprofloxacin, levofloxacin and gatifloxacin [1–5].

In this paper a rapid, novel, effective HPLC assay suitable for pharmacokinetic and toxicokinetic studies employing 96-well protein precipitation to measure sinafloxacin in biological specimen is reported using special fused-core C_{18}-silica column.

The trademark "Halo" implies a fused-core C_{18}-silica column, packed with a new kind of material, which is made of 2.7 μm particles that consist of a 1.7 μm silica solid core covered with a 0.5 μm thick shell of porous silica [6]. Thus, the ability of this column to generate fast separation comes not only from its small particle size (2.7 μm), but also from its 0.5 μm porous shell fused to a solid core particle. The special particles address the limitation by providing a very small path (0.5 μm) for the diffusion of solute molecules into and out of the stationary phase, thereby reducing the time solutes molecules spend inside the particles thus minimizing a major barrier to fast chromatographic separations [7,8]. What is more, in a recent study, Gritti et al. reported that the fused-core C_{18}-silica column provides a smaller axial diffusion coefficient B and smaller eddy dispersion term A than other columns,

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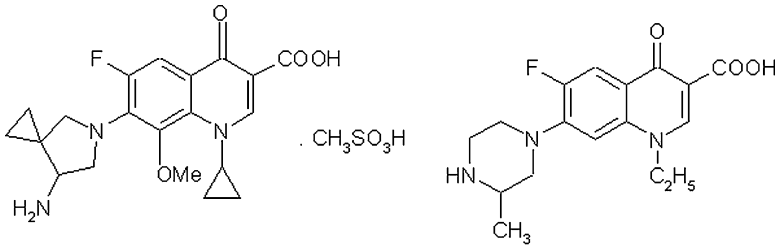

Fig. 1. Structures of sinafloxacin (I) and lomefloxacin (IS).

owing to its lower internal porosity and narrower size distribution of particles [9]. DeStefano et al. also demonstrated that very narrow particle size distribution of the fused-core C_{18}-silica column can be preferred for better packed beds with higher efficiency and stability [10]. In a word, this fast HPLC technology provided by this special column is comparable with ultra-high-pressure liquid chromatography in terms of chromatographic performance, but demands neither expensive ultra-high-pressure instrumentation nor new laboratory protocols. In spite of these advantages, fused-core C_{18}-silica column has not been widely used and some articles so far have focused mainly on the separation testing, physical and chromatographic properties [11–14].

The primary goal of the present work was to develop and validate a novel, high-throughput, economical method using special fused-core C_{18}-silica column to determine sinafloxacin in biological matrices. Lomefloxacin (Fig. 1), a structure analog of sinafloxacin, was used as the internal standard (IS).

2. Experimental

2.1. Chemicals and reagents

Sinafloxacin injection (Batch no. 20050804) and sinafloxacin (98.5% purity) were supplied by Jiangsu Hengrui Medicine Co. Ltd. (Jiangsu, China). Norfloxacin (97.8% purity) and ofloxacin (98.9% purity) were obtained from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Lomefloxacin (99.6% purity), gatifloxacin (98.3% purity) and ciprofloxacin (99.4% purity) were obtained from Tianjin Biochemical Pharmaceutical Factory (Tianjin, China). Ulifloxacin (NM394, 99.0% purity) was obtained from Shanghai Modern Pharmaceutical Co. Ltd. (Shanghai, China). HPLC-grade reagents such as acetonitrile were purchased from J.T. Baker (Darmstadt, Germany), orthophosphoric acid and triethylamine were purchased from Tedia Company Inc. (Fairfield, CT, USA). Deionized (18 MΩ cm^{-1}) water was generated in-house using a Milli-Q system from Millipore (Bedford, MA, USA).

2.2. Apparatus

The analyses on fused-core C_{18}-silica column were performed using the Shimadzu UFLC chromatographic system (Shimadzu Corporation, Kyoto, Japan), which is equipped with two LC-20AD pumps, a model DGU-$20A_3$ degasser unit, a SIL-20A autosampler, a CTO-20AC thermostatted column compartment and a model RF-10AXL fluorescence detector. Data were processed with Shimadzu LC-Solution version 1.21 SP1 chromatography software.

2.3. Chromatographic conditions on fused-core C_{18}-silica column

Separations were carried out on fused-core C_{18}-silica column (Halo, 2.7 μm, 50 mm × 4.6 mm i.d.) supplied by Advanced Materials Technology Co. Ltd. (Wilmington, DE, USA). The mobile phase consisted of 0.5% triethylamine solution adjusted to pH = 3.0 with orthophosphoric acid:acetonitrile (80:20, v/v) and was pumped at a flow rate of 1.0 ml min^{-1}. The mobile phase was filtered under vacuum through a 0.45 μm membrane filter and ultrasonically degassed before using. Sinafloxacin and IS were monitored at λ_{ex} of 295 nm and λ_{em} of 505 nm. Column temperature was 25 °C and injection volume was 5 μl.

2.4. Preparation of standards and quality control (QC) samples

The stock solution of 100 μg ml^{-1} sinafloxacin was prepared by dissolving in 60% acetonitrile and stored in dark volumetric flasks. Working standard solutions at 0.05, 0.1, 0.2, 0.5, 1, 2, and 5 μg ml^{-1} were prepared from stock solution in high purity water. 1 μg ml^{-1} lomefloxacin solution was prepared by dissolving in 60% acetonitrile and stored in dark volumetric flask. Primary stock solution was kept at 4 °C and remained stable for at least 15 days.

Calibration standards were prepared by spiking 90 μl blank plasma with 10 μl of the standard working solutions of sinafloxacin to give nominal concentrations of 5, 10, 20, 50, 100, 200, and 500 ng ml^{-1}. For each validation and assay run, the calibration curve standards were prepared fresh from the standard working solutions. QC samples, which were used in the validation and during the pharmacokinetic studied, were independently prepared at three-level concentrations of 10, 50, 400 ng ml^{-1}. The QC samples were stored at −20 °C and brought to room temperature before processed together with the SD rat samples.

2.5. Sample preparation

Samples were prepared using protein precipitation in 96-well format plate (1 ml, Varian, Inc., Palo Alto, CA, USA). An eight-channel 50 μl pipetting tool and an eight-channel 300 μl pipetting tool (Finnpipette®, Thermo Electron Corporation, Waltham, MA, USA) were used for liquid transfer steps. Aliquots of 90 μl rat blank plasma were transferred to 96-well plates and 10 μl of standard working solutions were added to make the fresh calibration standards. 100 μl aliquots of plasma samples and three-level QC samples respectively were also spiked into 96-well plates. In order to precipitate the plasma protein, 100 μl acetonitrile solution containing 0.15 μg ml^{-1} of the internal standard was added to each sample (standards, QCs and samples). Plates were capped and mixed by vortex for 3 min and then subjected to centrifuge at 2500 × *g* for 15 min at 4 °C to remove any precipitated material. A 150 μl aliquot of the supernatant was transferred to another 96-well plate and centrifuged at 2500 × *g* for 15 min at 4 °C. Because the 96-well plate is not suitable for the autosampler, the supernatant was transferred to microcentrifuge tube and analyzed by Shimadzu UFLC chromatographic system.

2.6. Pharmacokinetic study in rats

Sprague–Dawley rats of both sexes (obtained from Slac Laboratory animal Co. Ltd, Shanghai, China), weighting 250–300 g, were

randomly divided into three groups (six rats per group). Rats were housed at Animal Care Facility of School of Pharmacy, Second Military Medical University under 12 h light–dark cycles with free access to food and water. Animal welfare and experimental procedures were strictly in accordance with the guide for the care and use of laboratory animals and the related ethical regulations of Second Military Medical University. After an overnight fast each group received sinafloxacin solution via a tail vein injection at doses of 2, 4, and 8 mg kg^{-1}, respectively. Through the catheters which had been implanted into the right external jugular vein of adult SD rats 1 day prior to the experiments, blood samples (0.3 ml) were collected into heparinized tubes before administration and post-dose at 0, 2, 5, 10, 20, 30, 45, 60, 90, 120, 180, 240, 360, 480 and 720 min and physiologic saline (0.3 ml) was administrated to compensate the blood loss after every blood sample. The plasma was separated from heparinized blood by centrifugation and was stored at −20 °C prior to analysis.

3. Results and discussion

3.1. Sample preparation

In order to increase sample throughput, the protein precipitation in 96-well format plates was used. Utilization of 96-well format plates can improve efficiency and robustness of the assay [15,16]. Various protein precipitants, such as 10% perchloric, 0.5% trichloroacetic acid and acetonitrile were tried to achieve good resolution and high recovery of sinafloxacin from spiked biologic matrices. The highest recovery was obtained using acetonitrile as protein precipitation and no interfering peaks were observed at the retention time of sinafloxacin.

3.2. Fused-core C_{18}-silica column method development

Stass and Dalhoff measured moxifloxacin, the structure analog of sinafloxacin, in human body fluids by using fluorescence detection with excitation wavelength 296 nm and emission wavelength 504 nm [17,18]. We have found that sinafloxacin also possess strong fluorescence response under the excitation wavelength 295 nm and emission wavelength 505 nm enabling higher sensitivity to be obtained than with UV detection with decreased interference of endogenous substances in its determination in plasma.

The effect of temperature was investigated at 25, 30, 35 °C. The increase of the column temperature results in a decrease of retention time of sinafloxacin. However, the efficiency of fused-core column decreases [6], and the peak height and peak area were reduced remarkably due to the decrease of fluorescence response. The optimum temperature was selected as 25 °C.

Lomefloxacin was selected as the internal standard for the determination of sinafloxacin because of their similar retention times, fluorescence responses, low endogenous interferences and relatively high extraction recovery. In the course of this search six quinolones were investigated with a mobile phase of 12:88 (v/v) acetonitrile and phosphate buffer (pH = 3.0). The retention times of norfloxacin, ofloxacin, ciprofloxacin, lomefloxacin, NM394 (the metabolite of prulifloxacin) and gatifloxacin respectively were 2.20, 2.21, 2.52, 2.89, 4.26, and 5.13 min. Thus, we can infer that the fused-core C_{18}-silica column could be successfully used to separate different quinolones without applying gradient HPLC method [19], adding extra ion-pairing reagents [20] or using ternary solvent system [21].

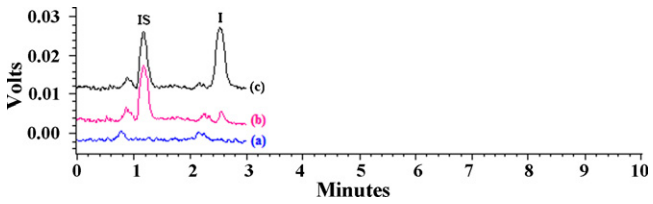

Fig. 2. Fused-core C_{18} column chromatogram for lomefloxacin (IS) and sinafloxacin (I) from: (a) a extracted blank plasma; (b) blank plasma spiked with of sinafloxacin at a LLOQ level (5 ng ml^{-1}) and IS; (c) plasma spiked with IS at 3 h after the dose of 2 mg kg^{-1}.

3.3. Method validation

3.3.1. Selectivity

The selectivity of the method was tested by comparing the chromatograms of five different lots of blank plasma and the spiked plasma. Under the above conditions, the retention time of sinafloxacin was 2.56 min and lomefloxacin was 1.21 min (Fig. 2). All plasma lots were found to be free of interferences with the compounds of interest.

3.3.2. Linearity

A calibration curve ranging from 5 to 500 ng ml^{-1} of sinafloxacin was used in each run by plotting the peak area ratios of the analyte to IS against the nominal standard curve concentrations. The calibration model was selected based on the analysis of the data by linear regression with/without intercepts and weighing factors ($1/x$, $1/x^2$ and none). The best linear fit and least-squares residuals for the calibration curve were achieved with a $1/x$ weighing factor. Finally, the mean calibration equation was $y = 0.2848$(R.S.D. = 9.70%, $n = 5$) + 0.02473(R.S.D. = 4.95%, $n = 5$)x, where y represents the peak area ratios of analytes to that of IS, and x represents the plasma concentration of analytes in ng ml^{-1}. Calibration curves showed excellent linearity in the range 5–500 ng ml^{-1} with the concentration coefficient of 0.9996.

3.3.3. Lower limit of quantification (LLOQ) and limit of detection (LOD)

The lower limit of quantitation (LLOQ) of the assay, defined as the lowest concentration on the standard curve that can be quantitated with accuracy within 15% of nominal and precision not exceeding 15% R.S.D., was 5 ng ml^{-1}. The LLOQ samples of five different plasma independent from the calibration curves were analyzed and the accuracy was 106.0%, intra- and inter-day precision was 7.8% and 10.7%, respectively (Table 1). Typical chromatograms of LLOQ samples are shown in Fig. 2(b). The LLOQ was sufficient for pharmacokinetic studies of sinafloxacin formulation products in rat plasma. LOD (S/N > 3) was 2 ng ml^{-1}.

Table 1
Intra- and inter-day precision and accuracy for sinafloxacin in rat plasma ($n = 5$).

QC sample	Concentration (ng ml^{-1})	Intra-day			Inter-day		
		Mean ± S.D. (ng ml^{-1})	Accuracy (%)	R.S.D. (%)	Mean ± S.D. (ng ml^{-1})	Accuracy (%)	R.S.D. (%)
HQC	400	401.2 ± 16.4	100.3 ± 4.1	4.1	414.1 ± 20.3	103.5 ± 5.1	4.9
MQC	50	51.0 ± 2.7	102.0 ± 4.5	4.4	51.5 ± 2.9	102.9 ± 5.8	5.6
LQC	10	10.2 ± 0.4	102.3 ± 4.1	4.0	10.2 ± 0.6	101.5 ± 5.8	5.8
LLOQ	5	5.0 ± 0.4	100.9 ± 7.9	7.8	5.3 ± 0.6	106.0 ± 11.3	10.7

3.3.4. Accuracy and precision

The intra- and inter-day precision and accuracy were estimated by analyzing five replicate samples at each QC concentration on the same day and on five consecutive days. Accuracy was determined by calculating the ratios of the predicted concentrations to the spiked values and with the precision expressed as R.S.D.

Table 1 summarizes intra- and inter-day precision and accuracy of QC samples. The results of intra-day and inter-day analysis indicated that the present method was accurate, reliable and reproducible.

3.3.5. Extraction efficiency

To investigate extraction recovery, a set of samples ($n = 5$ at each concentration in unique lots of plasma) were prepared by spiking sinafloxacin into plasma at 10, 50, 400 ng ml^{-1} and IS at 75 ng ml^{-1}. The samples were subsequently processed using the procedure described previously. A second set of plasma samples were processed and spiked post-extraction with the same concentrations of sinafloxacin and IS. Extraction recovery for each analyte was determined by calculating the ratios of the raw peak areas of the pre-extractions spiked samples to that of the samples spiked after extraction. The mean extraction recovery of sinafloxacin at the three concentrations was 74.4% (LQC), 78.8% (MQC) and 75.5% (HQC), respectively and the extraction recovery of lomefloxacin was 71.5%.

3.3.6. Stability

The stability tests of sinafloxacin in rat plasma were studied under various conditions: short-term temperature stability, long-term stability, autosampler stability and freeze–thaw cycles stability, which were evaluated by analyzing three QC levels in quintuple. The mean values and standard deviations of the ratios between the concentration found and initial concentration were used for stability evaluation. Sinafloxacin had an acceptable stability at room temperature for 2 h, at −20 °C for 1 month, in the autosampler at room temperature for 8 h after protein precipitation and after three freeze–thaw cycles with the values 97.4–101.7%, 94.2–101.4%, 96.5–99.1%, and 94.7–98.2%, respectively.

3.3.7. Sample dilution

To demonstrate the ability to dilute and analyze samples containing sinafloxacin at concentrations above the assay upper limit of quantitation, a set of plasma samples were prepared containing sinafloxacin at a concentration of 45,000 ng/ml and placed in a −20 °C freezer overnight prior to analysis. A 10 μl aliquot was withdrawn for analysis ($n = 5$), diluted with 990 μl of blank plasma, and processed as described in Section 2.5. The results are shown in Table 2.

Table 2
Sample dilution accuracy and precision.

Assayed concentration (ng ml^{-1})	Reported concentration (ng ml^{-1})
447.2	45,720
454.4	45,440
453.3	45,330
460.8	46,080
451.5	45,150
Mean	45,344
R.S.D. (%)	1.0
Accuracy (%)	100.7

Nominal concentration: 45,000 ng ml^{-1}; dilution factor: 100.

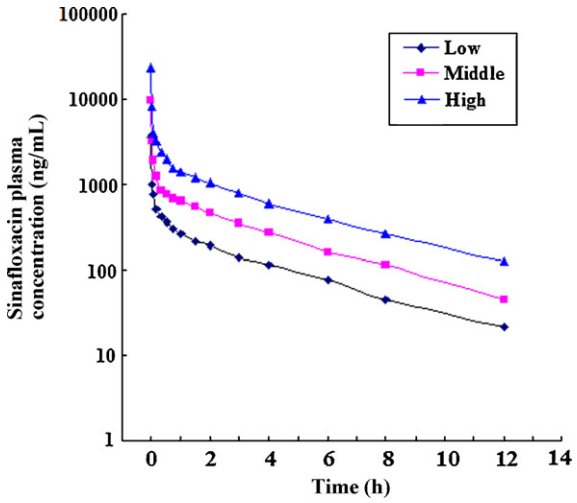

Fig. 3. Mean plasma concentration–time curve (semilogarithm) of sinafloxacin in rats after a single i.v. dose of 2, 4, 8 mg kg^{-1}.

Table 3
Pharmacokinetic parameters of sinafloxacin in rats (mean ± S.D., $n = 6$) after intravenous administration of doses of 2, 4 and 8 mg kg^{-1}.

Parameter	2 (mg kg^{-1})	4 (mg kg^{-1})	8 (mg kg^{-1})
C_{max} (μg ml^{-1})	6.8 ± 1.3	13.2 ± 8.3	27.5 ± 15.0
$T_{1/2}$ (h)	2.7 ± 0.8	2.6 ± 1.0	2.3 ± 0.9
MRT (h)	2.7 ± 0.9	2.6 ± 0.4	2.4 ± 0.7
AUC_{0-12} (μg h ml^{-1})	1.7 ± 0.6	4.1 ± 1.6	8.2 ± 4.0
$AUC_{0-\infty}$ (μg h ml^{-1})	1.8 ± 0.7	4.3 ± 1.8	8.7 ± 4.4
CL (L h kg^{-1})	1.3 ± 0.6	1.1 ± 0.4	1.2 ± 0.7

3.4. Application

This method was successfully applied to study the pharmacokinetics of sinafloxacin injection in SD rats. The plasma concentration–time curves of sinafloxacin in rats following intravenous injection of 2, 4, 8 mg kg^{-1} body weight are shown in Fig. 3. The plasma concentration of sinafloxacin was detected up to 12 h. The concentration–time data were analyzed by non-compartmental method and the pharmacokinetic parameters are summarized in Table 3. The C_{max} values versus dose are linear over the administered dose range ($r = 0.9996$) and the AUC_{0-12} ($r = 0.9993$), $AUC_{0-\infty}$ ($r = 0.9994$) values also verses doses. Sinafloxacin exhibited linear pharmacokinetic characteristics in rats after intravenous administration of 2, 4, and 8 mg kg^{-1} body weight.

4. Conclusion

A novel, fast and simple HPLC method using fused-core C_{18}-silica column has been developed and validated for determination of sinafloxacin in rat plasma using protein precipitation in 96-well format for sample preparation. The special fused-core C_{18}-silica column method provided adequate selectivity, sensitivity, precision and accuracy, significantly decreased the injection volume and analysis time which resulted in low consumption of solvents and high sample throughput. This rapid and simple HPLC assay meets the requirements of a high-throughput bioanalysis of sinafloxacin in clinical studies at relatively low cost.

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