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Simultaneous determination of rifampicin and levofloxacin concentrations in catheter segments from a mouse model of a device-related infection by liquid chromatography/electrospray ionization tandem mass spectrometry

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Abstract

The aim of this study was to develop a specific and sensitive liquid chromatography mass spectrometry (LC/MS) method for the determination of rifampicin and levofloxacin concentrations from infected tissues within teflon catheter segments which were subcutaneously implanted in mice. A solid-phase extraction procedure was used to extract analytes from tissue homogenates of the catheter segments and reverse-phase HPLC combined with positive electrospray ionization mass spectrometry was used for analyte separation and quantification. The assay was found to be linear over the concentration range of 0.02–2 μg/g for rifampicin and levofloxacin in tissues and provided good validation data for accuracy and precision. The intra-day accuracy as determined by the relative error was −1.3% for levofloxacin and 6.1% for rifampicin, and precision was evaluated by R.S.D.s with a maximum of 5.1% for levofloxacin and 8.1% for rifampicin. The inter-day accuracy was −3.3% for levofloxacin and −4.6% for rifampicin, and precision was 8.6% for levofloxacin and 7.1% for rifampicin. The assay uses less tissue than previously described methods and can be applied to determine the penetration of rifampicin and the fluoroquinolone in catheter segments from a mouse model of a device-related infection. Finally, the HPLC–MS assay should be applicable to studies of rifamycin + quinolone combination therapies in other animal models of bacterial infection.

Keywords: Levofloxacin; Rifampicin; LC/MS; Device-related infections; Catheter

1. Introduction

Rifampicin is a semi-synthetic rifamycin analog that is active against gram-positive bacteria and some gram-negative bacteria by inhibition of bacterial RNA polymerase [1–4]. It possesses excellent activity against slow-growing bacteria and penetrates into structured bacterial communities on the surfaces of implanted devices known as biofilms [5–8]. Clinically, rifampicin is used mainly in antibiotic combination therapies, because high-level resistance develops rapidly when the agent is administered in monotherapy [9]. Fluoroquinolones have been frequently used in combination with rifampicin for the treatment of device-related infections [10], and efficacy has been demonstrated in some clinical studies [11,12]. Levofloxacin is a second-generation fluoroquinolone that has improved antimicrobial and pharmacokinetic properties. The plasma elimination half-life for levofloxacin ranges from 6 to 8 h, which is complementary to the 3–5 h half-life for rifampicin, making this combination promising for the treatment of infections of indwelling medical devices.

In order to correlate efficacy with the pharmacokinetics of rifampicin and fluoroquinolones in an animal model, a sensitive and selective assay for the determination of drug concentrations is required, preferably within the same assay. Traditional approaches to monitor plasma drug concentrations are less predictive for device-related infections, where the infections are mainly within extravascular tissue sites. Measuring the concentrations of antimicrobial agents at the site(s) of infection is an important parameter in the systematic evaluation of anti-infective agents for the treatment of device-related infections. There are many assay methods for rifampicin and

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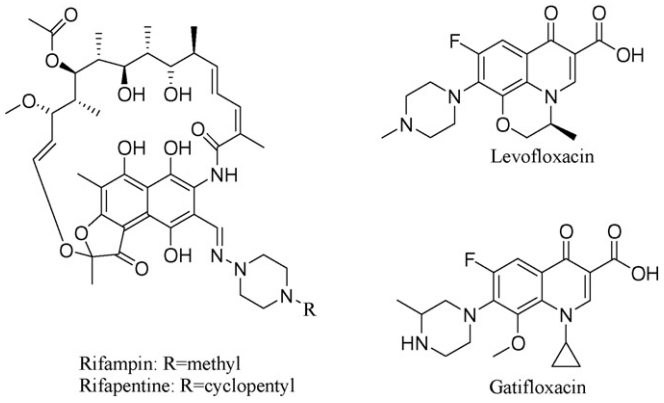

Fig. 1. Chemical structures of levofloxacin, gatifloxacin, rifampicin and rifapentine.

fluoroquinolones, mainly HPLC method with UV–Vis or mass spectrometry detection, described in the literature [13,14], but data on simultaneous quantification of rifampicin and fluoroquinolones is sparse. Salem et al. developed an NMR method for the quantitative determination of levofloxacin and rifampicin in urine samples without chromatographic separation [15], but the quantitation limits were relatively high (1.3 and 1.5 mg/mL for levofloxacin and rifampicin, respectively).

The aim of this study was to develop and validate a sensitive liquid chromatography tandem mass spectrometry method that simultaneously determines levofloxacin and rifampicin concentrations from tissues recovered from catheter segments subcutaneously implanted in mice in a previously described model of a device-related infection [11]. The method is simple, requires a small amount of tissue sample, and can therefore be used in our mouse model for a rapid evaluation of compounds to treat device-related infections.

2. Experimental

2.1. Chemicals

Levofloxacin and gatifloxacin (Fig. 1) were obtained from LKT Laboratories (St. Paul, MN). Rifampicin and rifapentine were obtained form Sigma–Aldrich and Sequoia Research Products, respectively. Acetonitrile and water used for the mobile phase were HPLC grade. All buffer solutions were prepared with ACS reagent grade chemicals.

2.2. Instrumentation

The LC/MS system was comprised of a Surveyor pump, a Surveyor autosampler and a LCQ-Deca XP ion-trap mass spectrometer equipped with an electrospray ionization (ESI) probe operated in positive mode (Thermo Finnigan, San Jose, CA). The system was operated using Xcalibur software (version 1.4, Thermo Finnigan). The LCQ capillary temperature was 275 °C, sheath gas flow was 30 (arbitrary units). The automatic gain control was turned on. The electrospray voltage was set to 4.5 kV, the normalized collision energy was 30%, with wideband activation turned off. Selected reaction monitoring (SRM) data were obtained by monitoring four transitions: levofloxacin m/z 362 → 318, gatifloxacin (internal standard for levofloxacin) m/z 376 → 332, rifampicin m/z 823 → 791 and rifapentine (internal standard for rifampicin) m/z 877 → 845 for analyte and internal standard quantitation. The spectrometer was tuned with levofloxacin and rifampicin infused through a syringe pump.

2.3. Chromatography

Chromatographic separation was performed on a Symmetry C18 column (2.1 mm × 100 mm, 3.5 μm; Waters, Milford, MA). Mobile phase A consisted of acetonitrile–water–formic acid (3:97:0.2, v/v/v) and mobile phase B consisted of acetonitrile–formic acid (100:0.2, v/v). Gradient elution was performed with 95:5 A–B for 1 min, 95:5 A–B to 45:55 A–B for 7 min, 1 min hold at 55% B, return to 95:5 A–B for 3 min, 12 min run time. The flow-rate was 0.4 mL/min and the column was used at ambient temperature.

2.4. Sample extraction procedure

To 0.4 mL of tissue homogenate, 10 μL of an internal standard working solution was added and vortexed for 1 min followed by centrifugation at 3000 × *g* for 20 min. The precipitate was re-suspended in 40 μL of methanol and centrifuged at 3000 × *g* for 20 min. The supernatants were combined and applied to a 30 mg/mL StrataX cartridge (Phenomenex, Torrance, CA) that was preconditioned with 1 mL of methanol and 1 mL of phosphate buffered saline pH 7.2 (1× PBS). The cartridge was then washed with 1 mL of methanol–water (5:95, v/v), followed by elution with 1 mL of releasing solution (methanol with 20 μg/mL ascorbic acid–formic acid, 100:0.1 (v/v). Ascorbic acid was added to prevent oxidation of rifampicin and rifapentine). The eluate was evaporated to dryness under reduced pressure. The residue was reconstituted in 0.1 mL of methanol–0.3 mg/mL ascorbic acid–formic acid (50:50:0.1, v/v/v) and 3 μL of this solution was injected into the chromatographic system.

2.5. Preparation of standards and quality controls

Stock solutions of levofloxacin and gatifloxacin were prepared by dissolving the appropriate amount of drug, accurately weighed, in deionized water to yield a final concentration of 2 mg/mL. Stock solutions of rifampicin and rifapentine were prepared in DMSO at a final concentration of 2 mg/mL. Working stock solutions of 8, 0.8 and 0.08 μg/mL levofloxacin and rifampicin were prepared by appropriate dilution in methanol. Working stock solutions were further diluted into blank tissue homogenate to obtain calibration standards for levofloxacin and rifampicin at concentrations of 0.02, 0.04, 0.1, 0.2, 0.4, 1 and 2 μg/g. Quality controls representing 0.06, 0.2 and 2 μg/g of levofloxacin–rifampicin were prepared by the same procedure as the calibration standards. The working internal standard solution containing 1 μg/mL rifapentine, 3 μg/mL gatifloxacin and 15 mg/mL ascorbic acid was prepared by appropriate dilution in methanol.

Daily calibration curves were constructed using peak ratios of levofloxacin/gatifloxacin or rifampicin/rifapentine. Unknown concentrations were computed from the weighted ($1/x^2$) linear regression equation of the peak area ratio against concentration.

2.6. Accuracy, precision and recovery

The accuracy and precision of the method were estimated by the intra- and inter-day relative standard deviation (R.S.D.) and relative errors (deviation between the concentrations spiked and found) from the back-calculated quality controls at three concentrations. Means, standard deviations, relative errors and R.S.D. were calculated by standard methods. Absolute recoveries of both analytes and internal standards were determined as the response of each compound in spiked tissue homogenate divided by the response for the compound in reconstitution solvent at the same concentration level.

2.7. Specificity and selectivity

The specificity of the assay in the presence of endogenous components was evaluated using catheter tissue homogenate obtained from mice dosed with a drug-free vehicle.

2.8. Limit of detection and limit of quantitation

The limit of detection (LOD) was defined by the concentration with a signal-to-noise ratio of 3. The limit of quantitation (LOQ) is defined as lowest concentration that gives both intra-day and inter-day R.S.D.s and relative errors were less than 20%.

2.9. Method application

The analytical method was subsequently used to analyze samples from a mouse model of a device-related infection [11]. Briefly, teflon intravenous catheter segments 1 cm in length, infected with *Staphylococcus aureus*, were subcutaneously implanted into each flank of Balb/c mice. After a 7-day stabilization period, mice were given 25 mg/kg rifampicin, 30 mg/kg levofloxacin or a combination of the two drugs by intraperitoneal (i.p.) administration, twice daily for 14 days. Twelve hours after the last administration, catheter segments were excised into 1 mL of 1× PBS. Tissue blocks inside catheter segments (approximately 0.05 g in each segment) were mechanically disrupted by 5 min vortexing at high speed, 0.4 mL of the homogenate was used for efficacy study, and the rest was snap frozen and stored at −80 °C until analysis.

Comparisons between drug concentrations obtained from monotherapy and combination therapy were performed with a Student's *t*-test. A *P*-value <0.05 was considered to indicate a statistically significant difference.

3. Results and discussion

A selective and reliable method was developed to simultaneously determine levofloxacin and rifampicin concentrations in host tissues, bacteria and biofilm matrix materials recovered from the luminal content of catheter segments in a mouse model of device-related infection. During the sample preparation, homogenization of the tissue contained inside catheters was not necessary because of the relatively soft matrix. Five minutes of vortexing was sufficient to give a homogeneous tissue suspension. The difference in hydrophobicity between fluoroquinolones and rifamycins was the major challenge for extraction and chromatographic separation. A second extraction with methanol was utilized to ensure good recovery of rifampicin, which is known to be relatively highly protein bound. Gatifloxacin and rifapentine were used as the internal standards for levofloxacin and rifampicin, respectively. A good LC separation on a Symmetry C18 column was obtained with solvent gradient consisting of water and acetonitrile (Fig. 2). A total run time of 12 min was allowed between successive injections to avoid endogenous interfering peaks. Levofloxacin and rifampicin are analyzed in the same run, reducing analysis time and variability of results. Since gradient chromatographic separation is used, it should be possible to apply this method with minor modification for the determination of other fluoroquinolones or rifamycin analogs.

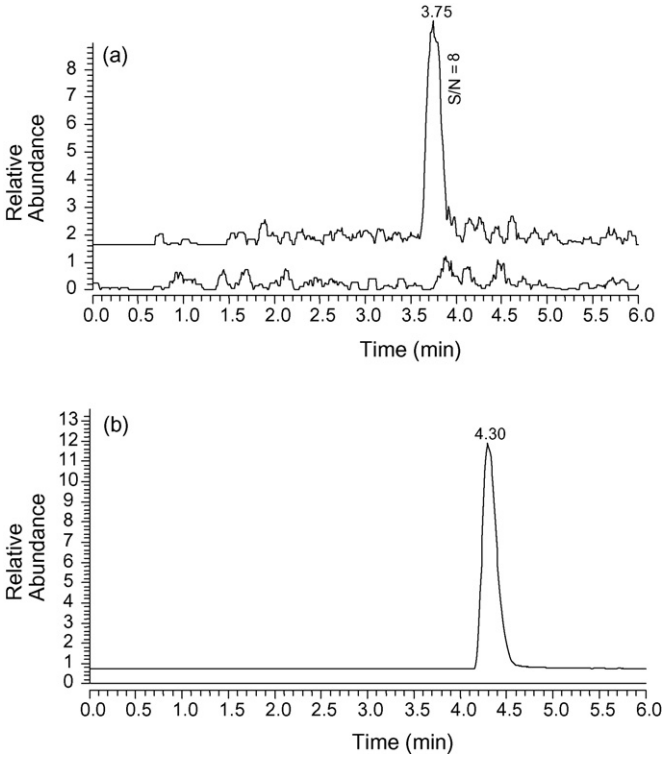

Fig. 2. Chromatograms of (a) tissue homogenate spiked with 0.02 μg/g of levofloxacin and blank tissue homogenate, (b) tissue homogenate spiked with 0.25 μg/g gatifloxacin (IS) and blank tissue homogenate.

The specificity tested the ability of the method to detect and quantitate the analyte in the presence of endogenous components. The chromatographic separation of tissue blank and spiked homogenates at the LOQ of levofloxacin and rifampicin are presented in Figs. 2 and 3. No significant interfering peaks were observed at the retention times of the analytes and inter-

Table 1
Recovery of rifampicin, levofloxacin and internal standards from mouse tissue homogenates within infected catheter segments

Nominal concentration (μg/g)	Mean recovery (%)			
	Levofloxacin	Gatifloxacin	Rifampicin	Rifapentine
0.06	83.8		84.6	
0.2	80.0		83.2	
2	87.2		90.6	
0.25		86.2		
0.75				78.9

nal standards. At 0.02 μg/g, the R.S.D. was 18.3% ($n = 6$) and 16.2% ($n = 6$) for levofloxacin and rifampicin, respectively.

The analysis of levofloxacin and rifampicin exhibited good linearity with the coefficient of correlation $r^2 > 0.99$ in all cases. Standard curves were linear over a range of 0.02–2 μg/g for both drugs with a weighting factor of $1/x^2$ applied during linear regression. Daily calibration curves were used for calibration and calculation purposes.

The recovery efficiency for both analytes in the tissue homogenate was evaluated at three concentrations (Table 1). The mean recovery values were 80–87.2% and 83.2–90.6% for levofloxacin and rifampicin, respectively. The mean recovery values for internal standards were 86.2 and 78.9% for gatifloxacin and rifapentine, respectively. No significant difference in recovery was observed among the four compounds

The intra-day accuracy and precision were calculated from five replicated QC samples at three concentrations for each compound. The results are summarized in Table 2. The accuracy as determined by the relative error was comparable with a maximum of −1.3% for levofloxacin and 6.1% for rifampicin. The precision was evaluated by R.S.D.s with a maximum of 5.1% for levofloxacin and 8.1% for rifampicin, both at the low concentration level.

The inter-day accuracy and precision were calculated from replicated QC samples at three concentrations for each compound on two independent assays (Table 2). The accuracy as determined by the relative error was less than 5% for each compound at three concentrations tested. The R.S.D.s were less than 9% for each compound.

The sensitivity of the method as expressed by the limit of quantification was better to a previously published NMR method [15]. The LOQ was 0.02 μg/g for both compounds, well below

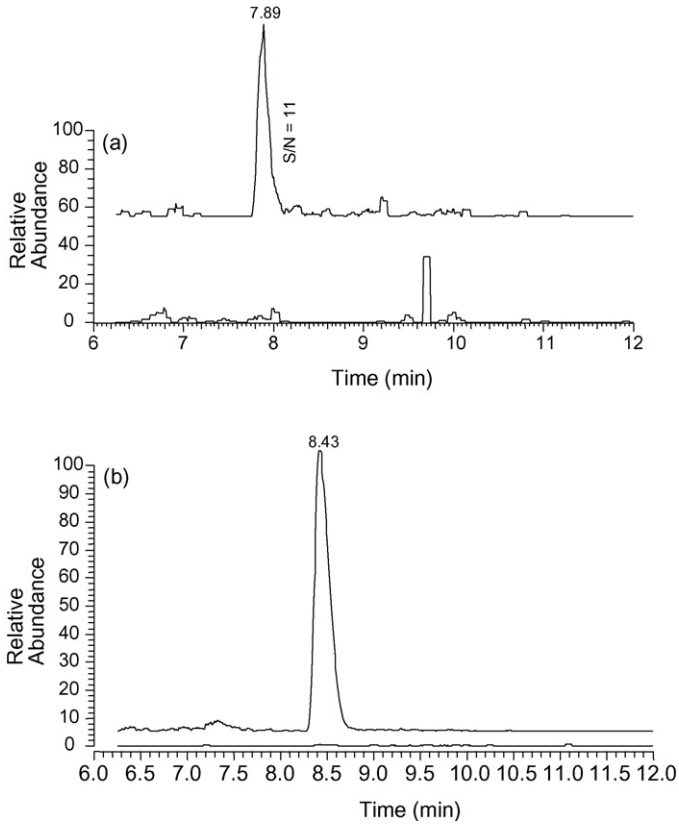

Fig. 3. Chromatograms of (a) tissue homogenate spiked with 0.02 μg/g of rifampicin and blank tissue homogenate, (b) tissue homogenate spiked with 0.75 μg/g rifapentine (IS) and blank tissue homogenate.

Table 2
Precision and accuracy of intra- and inter-day assay of levofloxacin and rifampicin in tissue homogenates from mice

Nominal concentration (μg/g)	Intra-day ($n = 5$)			Inter-day ($n = 10$)		
	Mean	R.S.D. (%)	Relative error (%)	Mean	R.S.D. (%)	Relative error (%)
Levofloxacin						
0.06	0.0592	5.1	−1.3	0.0580	8.6	−3.3
0.2	0.202	5.0	1.1	0.198	5.9	−0.9
2	2.02	2.5	0.9	1.94	5.8	−2.7
Rifampicin						
0.06	0.0637	8.1	6.1	0.0625	7.0	4.1
0.2	0.201	4.6	0.5	0.198	6.8	−0.9
2	1.99	6.1	−0.4	1.91	7.1	−4.6

Table 3
Mean tissue drug concentrations at 12 h after the last i.p. administration of 30 mg/kg levofloxacin, 25 mg/kg rifampicin or combination of levofloxacin and rifampicin for 14 days, twice daily

Dose	*n*	Drug level (μg/g) (mean ± S.D.)	
		Levofloxacin	Rifampicin
Levofloxacin, 30 mg/kg	5	0.09 ± 0.12	N/A
Rifampicin, 25 mg/kg	5	N/A	0.69 ± 0.4
Levofloxacin, 30 mg/kg + rifampicin, 25 mg/kg	4	0.17 ± 0.11	0.72 ± 0.43

the minimal inhibitory concentration (MIC) of 0.5 μg/mL, for levofloxacin against *S. aureus*. Rifampicin has a much lower MIC value, 0.008 μg/mL, but is still comparable with the 0.01 μg/g limit of detection for the method. A better sensitivity for rifampicin could be achieved by increasing the injection volume or by increasing the amount of sample used for the extraction.

The present method was validated with a catheter tissue homogenate. It uses small quantities of biological material (0.05 g of tissue), therefore is applicable to preclinical studies with rodent species. The method was subsequently used to analyze samples from a mouse model of a device-related staphylococcal biofilm infection. Levofloxacin and rifampicin concentrations in mouse tissue after i.p. administration of levofloxacin and rifampicin, alone or in combination, are shown in Table 3. There were no significant differences ($P > 0.05$) in drug concentrations between monotherapy and combination therapy, although rifampicin concentrations were much higher than levofloxacin concentrations 12 h after the last dose. Additional studies will be needed to correlate drug concentrations in catheter segments and *in vivo* efficacy and development of resistance to rifampicin.

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