Journal of Chromatography B, 919–920 (2013) 62–66

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Short communication

Validation of an efficient LC-microdialysis method for gemifloxacin quantitation in lung, kidney and liver of rats

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ARTICLE INFO

Article history:
Received 29 August 2012
Accepted 8 January 2013
Available online 14 January 2013

Keywords:
Gemifloxacin
Liquid chromatography
Microdialysis
Probe recovery
Tissue penetration
Validation

ABSTRACT

A liquid chromatography method has been established for the reliable determination of unbound gemifloxacin concentrations in kidney, lung and liver microdialysates of rats. Microdialysis probes were inserted into tissues of rats, and then dialysates were collected at regular time intervals after intravenous administration of gemifloxacin (40 mg kg^{-1}). A pilot study was performed to assess gemifloxacin penetration in lung, kidney and liver of rats. Gemifloxacin was separated on a C_{18} column eluted using triethylamine solution (0.5%, v/v), adjusted to pH 3.0 ± 0.1 with 85% phosphoric acid, methanol and acetonitrile (71:15:14, v/v/v) as mobile phase at a flow rate of 1.1 mL min^{-1}. The fluorescence detector was set at excitation and emission wavelengths of 344 nm and 399 nm, respectively. The limit of quantitation was found to be 50 ng mL^{-1}. Linearity was found to be over a concentration range of 50–2000 ng mL^{-1}. The intra-assay and inter-assay precision and accuracy values were determined from the analysis of six quality control samples. The results obtained at three concentration levels showed R.S.D. values lower than 6.06% and 4.10% for repeatability and intermediate precision, respectively. The accuracy (R.E.%) ranged from 90.0 to 106.5%. The chromatographic run time of each sample was performed in 9 min. Drug stability in microdialysates was shown at room temperature for 8 h, after three freeze–thaw cycles, in freezer at −80 °C for 14 days, and in the autosampler after processing for 8 h. The relative recoveries determined by extraction efficiency (EE) and retrodialysis (RD) in vitro employing a flow rate of 1.5 μL min^{-1} were 29.24 ± 3.67% and 23.67 ± 3.31%, respectively. In vivo recoveries determined by RD in Wistar rats' kidney, lung and liver were 27.69 ± 2.09%, 23.12 ± 3.79% and 17.38 ± 0.68%, respectively. The method was successfully applied to investigate tissue penetration of unbound gemifloxacin into the kidney, lung and liver of rats.

1. Introduction

Gemifloxacin is a newer generation fluoroquinolone with suitable pharmacokinetic and pharmacodynamic properties to treat pulmonary diseases caused by *Streptococcus pneumoniae*, as well as atypical pathogens and Gram-negative respiratory ones [1,2]. Many considerations derived from saliva, urine, tissue biopsies and indirect modeling of tissue concentrations from plasma curves act as surrogates for true target site concentrations. The in vivo assessment of drug distribution by microdialysis and target site pharmacokinetics has become to better obtain more realistic information from drugs [3]. Microdialysis can be used to acquire concentration variations of protein-free molecules located in interstitial or extracellular spaces and relies on the passive diffusion of analyte across a dialysis membrane [4].

Fluoroquinolones are well distributed in tissues, as seen in several articles published in the literature. Different techniques to determine gemifloxacin tissue distribution were employed, e.g. blister technique [5], tissue homogenate [6,7], radiochemistry [8] and human-microdialysis [9]. Joukhadar et al. [9] investigated the free gemifloxacin concentrations in the interstitial space fluid of skeletal muscle and subcutaneous adipose tissue in human but no one has investigated the free gemifloxacin in the biophase of interest (lungs) yet. Tissue penetration of quinolones is variable among this class of antimicrobial drugs. For example, the penetration of ciprofloxacin was found to be 0.89 and 1.23 for subcutaneous adipose tissue and skeletal muscle, respectively, considering tissue and plasma levels [10]. Levofloxacin ratios between 0.85 in skeletal muscle and 1.1 in subcutaneous adipose tissue were also observed [11,12]. So, it is important to determine the free levels

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http://dx.doi.org/10.1016/j.jchromb.2013.01.006

in different tissues to better know the antimicrobial penetration. Membrane transport process such as an active influx and/or efflux also may contribute to tissue distribution of antimicrobials. The efflux pump (P-glycoprotein) can corroborate with different levels of antimicrobials into tissues and the investigation of free levels can be explained considering this observation.

In the present study, we employed microdialysis to investigate the potential of gemifloxacin to penetrate lung, kidney and liver tissues in healthy rats. To achieve this goal, an efficient HPLC method is described here. To our knowledge, there have been no reports of liquid chromatography method to determine free gemifloxacin in lung, liver and kidney of rats sampled by microdialysis.

2. Materials and methods

2.1. Chemicals and reagents

Gemifloxacin mesylate (purity > 99.5%) was a donation from Aché Pharmaceutical Laboratory (São Paulo, Brazil). LC grade methanol, acetonitrile and triethylamine were purchased from Merck (Darmstadt, Germany). Other chemicals used in the experiment were of analytical reagent grade and purchased from commercial sources. Urethane was purchased from Sigma (St. Louis, USA). Distilled water was prepared using a Milli-Q water purification system from Millipore. Ringer's solution consisted of 149 mM NaCl, 2.46 mM $CaCl_2$, and 4.02 mM KCl.

2.2. Preparation of standard solutions, calibration standards and quality controls

Stock standard solution of gemifloxacin (500 $\mu g\,mL^{-1}$) was prepared in amber volumetric flask by dissolving the appropriate amount of this substance in Ringer's solution and stored at 4 °C. A series of working solutions of gemifloxacin were prepared by subsequent dilutions of the above stock solution with Ringer's solution to reach a concentration range of 50–2000 $ng\,mL^{-1}$, including six calibration standards of 50, 150, 500, 1000, 1500 and 2000 $ng\,mL^{-1}$ and quality control (QC) samples with low (100 $ng\,mL^{-1}$), medium (925 $ng\,mL^{-1}$) and high (1750 $ng\,mL^{-1}$) concentrations. All solutions were kept protected from light.

2.3. Instrumentation and conditions

The chromatographic analysis was carried out on a Shimadzu system equipped with an isocratic LC-10AD VP pump, SIL-10AD VP auto injector, SCL-10A VP system controller and DGU-14A degasser. Chromatographic separation was achieved on a reversed phase C_{18} (Shimadzu Shim-Pack, 250 mm × 4.6 mm i.d.; particle size 5 μm). The flow rate of 1.1 $mL\,min^{-1}$ and the injection volume of 30 μL were performed. All samples and standard solutions were chromatographed at 40 °C using triethylamine solution (0.5%, v/v), adjusted to pH 3.0 ± 0.1 with 85% phosphoric acid, methanol and acetonitrile (71:15:14, v/v/v) as mobile phase. The fluorescence detector was set at excitation and emission wavelengths of 344 nm and 399 nm, respectively. The peak area of gemifloxacin was used for the quantitation of samples. The chromatographic run time of each sample was performed in 9 min. Data were processed by Shimadzu CLASS-VP software (version 6.12).

2.4. Method validation

This method was validated in compliance with the FDA guidelines for biological method validation [13]. The analytical performance parameters evaluated included specificity test, linearity, lower limit of quantitation, precision (intra and inter-assay), accuracy and stability of gemifloxacin under various test conditions.

The specificity test was assessed by comparing the chromatograms of blank dialysates from six different rats with the chromatograms for the corresponding spiked dialysate samples to test for endogenous interference.

To evaluate the linearity, the calibration curves were constructed using six standards ranging from 50 to 2000 $ng\,mL^{-1}$. Calibration standards were freshly prepared every day during ongoing analysis. Calibration equation obtained from regression analysis was used to calculate the corresponding predicted responses. The concentration of gemifloxacin in dialysate samples was determined using the linear regression line (unweight) of the concentration standard versus peak area. The lower limit of quantitation (LLOQ) was estimated in the process of calibration curve construction and was defined as the lowest concentration for which precision (R.S.D.) was better than 20%.

The intra- and inter-day precisions were evaluated by assessing six replicate QC samples in Ringer's solution at three different concentrations (100, 925 and 1750 $ng\,mL^{-1}$) on two consecutive days. The assay accuracy was calculated as relative error (R.E.%). The precision was given as the relative standard deviation (R.S.D.%). Accuracy and precision values within ±15% covering the actual range of experimental concentrations were considered acceptable.

Drug stability in microdialysates was assessed in the autosampler at room temperature for 8 h, after three freeze–thaw cycles, on storage at −80 °C for 14 days, and in the autosampler after processing for 8 h.

2.5. Sampling by microdialysis

To measure the unbound fraction of gemifloxacin in vitro and in the interstitial space fluid, microdialysis probes (CMA 20; CMA, Stockholm, Sweden) with a molecular cutoff of 20 kDa were used. The Bioanalytical (Indiana, USA) microinfusion pump employed consisted of a 1020 Bee Hive controller and a 1001 Baby Bee Syringe Drive. We employed in vitro (extraction efficiency – EE and retrodialysis – RD) and in vivo (RD) methods to assess the relative recovery of the microdialysis probes. The EE is defined as the ratio between the loss/gain of analyte during its passage through the probe ($C_{in} - C_{out}$) and the difference in concentration between perfusate and sampling site ($C_{in} - C_{sample}$). The recovery for RD can be computed as the ratio of drug lost during passage ($C_{in} - C_{out}$) and drug entering the microdialysis probe (C_{in}). Both methods were carried out at 37 ± 1 °C.

The in vitro recoveries induced by EE and RD of gemifloxacin were evaluated considering a constant flow rate of 1.5 $\mu L\,min^{-1}$. Three different gemifloxacin concentrations were tested in this experiment: 500, 1000 and 2000 $ng\,mL^{-1}$. Dialysate samples were collected every 30 min after equilibration time (1.5 h).

The protocol used for determination of gemifloxacin by in vivo microdialysis was previously approved by the Ethics in Research Committee of University of Caxias do Sul – UCS. Animal experiments were performed according to the National Institutes of Health Guide for Care and Use of Laboratory Animals. Male Wistar rats (250–300 g), purchased from Technology and Science Foundation (Santa Maria RS, Brazil) were used in the study.

The assessment of in vivo microdialysis was performed considering RD method. The animals were initially anesthetized with ethyl carbamate (1.25 $g\,kg^{-1}$, i.p.) and immobilized in a supine position. For probes calibration in liver, lung and kidney an incision was made in the skin and a guide cannula was inserted through the organ. The probes ($n = 3$) were continuously perfused with gemifloxacin in Ringer's solution at a flow rate of 1.5 $\mu L\,min^{-1}$. After 1 h equilibration three samples were collected with

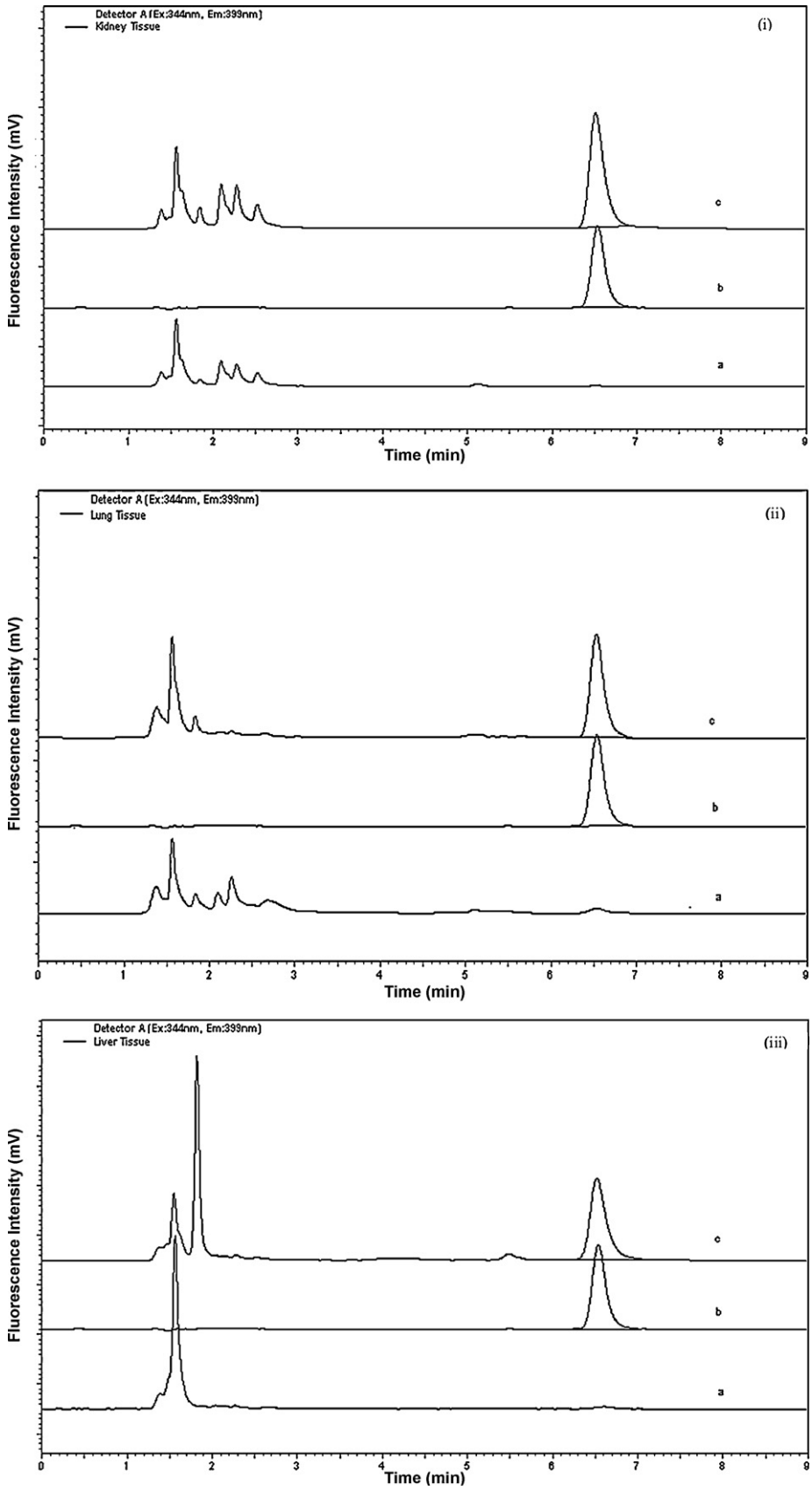

Fig. 1. Representative chromatograms of blank Ringer's solution (a), dialysate spiked with gemifloxacin 500 ng mL^{-1} (b) and 0.5 h dialysate sample (c) from kidney (i), lung (ii) and (iii) liver.

30 min intervals. The in vivo recovery was determined by measuring the relative loss of the analyte diffusing from the perfusate into the extracellular fluid according to the following equation: $RD = (C_{in} - C_{out})/C_{in}$, where C_{in} and C_{out} correspond to the gemifloxacin concentrations in the perfusate and in the dialysates collected, respectively.

For probes calibration in lung, after anesthesia, the animals were intubated by tracheotomy and artificially ventilated with room air using a rodent respirator (Harvard Apparatus, model 683) with a frequency of 62–66 min^{-1} and an air volume of 2.5 mL. The right lung was exposed through an open space cut between two ribs. A microdialysis probe was inserted into the intermediate lobe through a small incision made in the pleura. The probe was held in place with ties around the probe shaft and the lung. The lobe was carefully put back in place and the chest was superficially closed. After surgery, the procedure was the same described for probes calibration in kidney and liver.

The dialysate samples were measured by liquid chromatography on the day of sampling. The unbound gemifloxacin concentrations in kidney, liver and lung of rats were calculated from the measured dialysate concentrations and the relative recovery determined by RD in vivo.

To determine gemifloxacin penetration into different tissues (lung, kidney and liver) of rats the RD method was employed. The animals ($n = 3$/group) were initially anesthetized with ethyl carbamate (1.25 g kg^{-1}, i.p.) and immobilized in a supine position. The same procedure as described for in vivo recoveries was performed here. Gemifloxacin, at a dose of 40 mg kg^{-1} was administered intravenously by the lateral tail vein.

3. Results and discussion

3.1. Method validation

Representative chromatograms of blank Ringer's solution (a), rat dialysate spiked with gemifloxacin 500 ng mL^{-1} (b) and 0.5 h dialysate sample (c) from kidney (i), lung (ii) and (iii) liver of rat are presented in Fig. 1. The results indicated that there were no significant endogenous interferents at the retention time of gemifloxacin peak (around 6.6 min), showing the specificity of the method.

The linearity of the standard curves was checked in six different runs after calculating individual slopes and intercepts of each individual curve. The slopes of the calibration curves obtained did not vary considerably, and the intercepts obtained were near to theoretical zero value, demonstrating good constancy of the measuring system. The calibration curves were validated over the concentration range of 50–2000 ng mL^{-1}. The goodness of fit (r^2) was found to be greater than 0.997. No deviation from linearity was found ($p > 0.05$) and the regression was highly significant ($p \leq 0.01$) by means of ANOVA. All analytical results were not more than 15% coefficient of variation for precision and not more than 15% deviation from the nominal value for accuracy. The LLOQ for gemifloxacin with acceptable precision and accuracy was 50 ng mL^{-1}.

The intra-assay and inter-assay precision and accuracy values were determined from the analysis of six QC samples at low, medium and high concentrations of gemifloxacin. The results are reported in Table 1. The results obtained at three concentration levels showed R.S.D. values lower than 6.06% and 4.10% for repeatability and intermediate precision, respectively. The accuracy (R.E.%) ranged from 90.0 to 106.5% (Table 2).

The results of stability are summarized in Table 3. A maximum deviation of 4.62% in microdialysates was observed. The results showed that gemifloxacin was stable under all conditions employed in this study.

Table 1
Intra-assay and inter-assay precision of quality control samples.

Spiked conc. QC (ng mL^{-1})	Day	Mean concentration found	S.D. (ng mL^{-1})	R.S.D. (%)
Intra-assay				
1750	1	1852	53.5	2.89
	2	1821	33.7	1.85
925	1	918	18.1	1.97
	2	972	26.9	2.77
100	1	94.4	5.4	5.73
	2	95.7	5.8	6.06
Inter-assay				
1750		1837	22.0	1.20
925		945	38.7	4.10
100		95.1	1.0	1.05

S.D., standard deviation; R.S.D., relative standard deviation.

Table 2
Accuracy for the analysis of quality control samples.

Quality control (ng mL^{-1})	Range (ng mL^{-1})	Accuracy (R.E.%)
1750	1715–1864	98.0–106.5
925	940.9–1032	96.5–105.8
100	90.0–102.2	90.0–102.2

3.2. Microdialysis in vitro and in vivo recoveries

The relative recoveries determined by EE and RD in vitro employing a flow rate of 1.5 μL min^{-1} were 29.24 ± 3.67% and 23.67 ± 3.31%, respectively. In vivo recoveries determined by RD in Wistar rats' kidney, lung and liver were 27.69 ± 2.09%, 23.12 ± 3.79% and 17.38 ± 0.68%, respectively. The recoveries were dependent on the tissue investigated. The recoveries were not concentration dependent (500, 1000 and 2000 ng mL^{-1}). These values were used to correct gemifloxacin concentrations determined in kidney, lung and liver of rats.

3.3. Application of the method

The validated method was successfully used to quantify gemifloxacin free concentrations in different tissues of rats employing microdialysis technique. This approach gives the possibility to know the real concentrations of drugs in different parts of the body and possibly optimize the therapy. The pharmacokinetic profiles of unbound gemifloxacin concentration can be seen in Fig. 2. These observations (higher free concentration in kidney > liver > lung) are in agreement with previously results obtained by our group

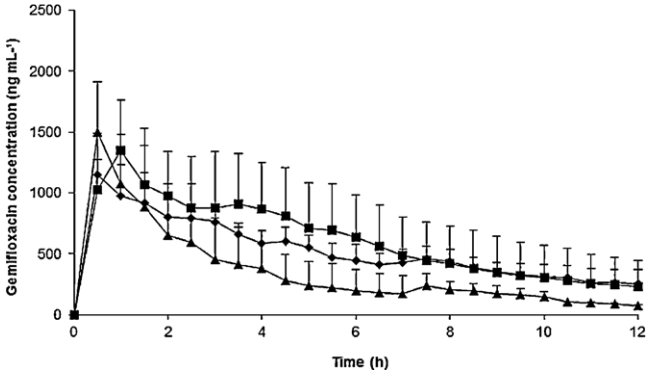

Fig. 2. Concentration versus time profiles of free gemifloxacin levels in kidney (■), liver (♦) and lung (▲) after intravenous administration of 40 mg kg^{-1} to Wistar rats ($n = 3$/group). Data are depicted as means ± standard deviation.

Table 3
Determination of the stability of gemifloxacin (n = 3).

Spiked conc. (ng mL^{-1})	Conditions	Mean measured conc. ± S.D. (ng mL^{-1})	Bias (%)	R.S.D. (%)
50	8 h at room temperature	52.87 ± 1.41	5.73	2.68
	3 F/T	50.02 ± 0.24	0.03	0.48
	14 days at −80 °C	49.94 ± 0.65	−0.12	1.29
	8 h at 4 °C	52.32 ± 1.04	4.64	1.98
2000	8 h at room temperature	2042 ± 79.64	2.14	3.90
	3 F/T	1961 ± 43.41	−1.93	2.21
	14 days at −80 °C	1956 ± 39.82	−2.20	2.04
	8 h at 4 °C	2019 ± 93.23	0.98	4.62

S.D., standard deviation; R.S.D., relative standard deviation; F/T, freeze–thaw cycles.

when tissue homogenate was employed [7]. We have previously investigated the distribution of gemifloxacin under normobaric and hyperbaric exposure employing tissue homogenate technique. The results showed that concentrations of gemifloxacin were also higher in the kidney than those in the liver and lung. Other authors showed similar results when multiple oral dosing (200 mg kg^{-1}) was administered for 7 days [14]. However, calculation of the true concentration in tissues may be imprecise employing tissue homogenate, leading to overestimation of actual active concentrations in the extracellular fluid, once tissue homogenate gives the total amount (bound and unbound) of drug in the organ. The investigation of antimicrobials levels can be performed employing microdialysis technique. The microdialysis can determine only the unbound fraction present in the tissue and this information is more appropriate to know the concentrations to be exposed to microorganisms considering the antibiotic investigated.

The efflux pump (P-glycoprotein) can corroborate with different levels of antimicrobials into tissues. Chang-Koo et al. [15] have investigated the oral bioavailability of gemifloxacin in rats and its possible association with efflux transporters. It was observed that efflux transporters appeared to significantly limit the bioavailability of gemifloxacin in rats, suggesting their possible contribution to the bioavailability of the drug in the human.

4. Conclusion

An efficient, stable, precise, accurate and selective method with fluorescence detection for quantitation of unbound gemifloxacin concentrations was validated. The in vitro and in vivo performance of the microdialysis technique was established for the study of gemifloxacin. The method was successfully applied to investigate this anti-infective drug into the kidney, lung and liver of rats.

Acknowledgments

The authors would like to thank FAPERGS (Brazil) and University of Caxias do Sul for financial support. The authors also acknowledge Laboratory Aché (Brazil) for the donation of gemifloxacin.

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