Journal of Chromatography B, 879 (2011) 3639–3644

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

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

Short communication

Reversed phase liquid chromatography method with fluorescence detection of gemifloxacin in rat plasma and its application to the pharmacokinetic study

Moacir Kaiser[a], Lauren D. Grünspan[b], Teresa Dalla Costa[a], Leandro Tasso[b,*]

[a] Centro Bioanalítico de Medicamentos, Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul (UFRGS), Avenida Ipiranga 2752, CEP 90610-000, Porto Alegre, RS, Brazil
[b] Curso de Farmácia, Laboratório de Fisiologia e Farmacologia, Universidade de Caxias do Sul (UCS), Rua Francisco Getúlio Vargas 1130, Bairro Petrópolis, CEP 95020-972, Caxias do Sul, RS, Brazil

ARTICLE INFO

Article history:
Received 27 April 2011
Accepted 4 September 2011
Available online 10 September 2011

Keywords:
LC
Gemifloxacin
Fluorescence detection
Rat plasma
Pharmacokinetics

ABSTRACT

A simple, accurate and precise high-performance liquid chromatographic method with fluorescence detection was developed and validated for the determination of gemifloxacin (GEM) in rat plasma using furosemide as internal standard (I.S.). Plasma samples were pretreated by direct deproteinization and all samples and standard solutions were chromatographed at 45 °C using triethylamine solution (0.5%, v/v, pH 3.0 ± 0.1), methanol and acetonitrile (63:30:7, v/v/v) as the mobile phase. Chromatographic resolution was achieved using a RP-C_{18} column (Atlantis, Waters, 150 mm × 4.6 mm, 5 μm) at a flow rate of 1.0 mL min^{-1} and an injection volume of 30 μL. The analytes were measured by fluorescence detection with excitation and emission wavelengths of 344 nm and 399 nm, respectively. The retention times for GEM and I.S. were approximately 7.5 and 12.6 min, respectively. The lower limit of quantitation (LLOQ) was 20 ng mL^{-1} and the calibration curves were linear over a concentration range of 20–5000 ng mL^{-1}. The intra- and inter-day precisions, expressed by relative standard deviation (R.S.D.) were lower than 6.24% and 4.49%, respectively. The accuracy ranged from 91.3% to 112% and from 98.8% to 106% for the lower and upper limit of quantitation of the calibration curve, respectively. Ratio of peak area of analyte to I.S. was used for quantification of plasma samples. No interferences from endogenous substances were found. The recovery of GEM and I.S. from plasma was greater than 90%. Drug stability in plasma was shown at room temperature for 4 h, after three freeze–thaw cycles for 24 h, in freezer at −80 °C for 60 days, and in the autosampler after processing for 12 h. The utility of the assay was confirmed by the successful analysis of plasma samples from GEM pharmacokinetics studies in the rats after intravenous administration.

1. Introduction

Increasing resistance in *Streptococcus pneumoniae* to the more commonly prescribed antimicrobial classes is leading to develop new agents to prescribe according to infection severity. Respiratory fluoroquinolones have been approved to treat community-acquired pneumonia (CAP). CAP is a serious illness and important cause of morbidity and mortality worldwide. *S. pneumoniae* is the most significant bacterial pathogen causative agent in CAP. To treat this infection, fluoroquinolones have been successfully used. GEM (R, S)-7(3-aminomethyl-4-syn-methoxyimino-1-pyrrolidinyl)-1-cyclopropyl-6-fluoro-1,4 dihydro-4-oxo-1,2 naphthyridine-3-carboxylic acid) is a new fluoroquinolone developed as antibacterial agent with a broad spectrum of activity against Gram-positive and Gram-negative organisms (Fig. 1a). Also, this compound has enhanced activity against other pathogens involved in respiratory tract infections, including *Haemophilus influenza* and *Moraxella catarrhalis* [1–4]. It is the only fluoroquinolone in its dual targeting capacity to inhibit both topoisomerase IV and gyrase sites at the therapeutically achievable drug concentrations, whereas older compounds preferentially target topoisomerase IV [5,6].

Few analytical methods are reported for quantification of GEM in pharmaceutical forms by spectrophotometry [7,8], spectrofluorimetry [9] and microbiological assay [10], as well as in human biological fluids by LC–MS [11–13], LC–MS/MS [14,15], LC–UV [16] and LC–FL [9,17]. However, a pre-clinical LC–FL methodology involving a simple one step extraction (protein precipitation) that reduces the amount of solvent used to determine GEM in rat plasma with a short run and excellent accuracy has not been reported yet in the literature.

The aim of this study was to develop and validate a simple, accurate, specific and reproducible LC–FL method for the quantification of GEM in rat plasma. The analytical method is successfully applied

* Corresponding author. Tel.: +55 54 3218 2123; fax: +55 54 3218 2190.
E-mail address: ltasso@ucs.br (L. Tasso).

1570-0232/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2011.09.011

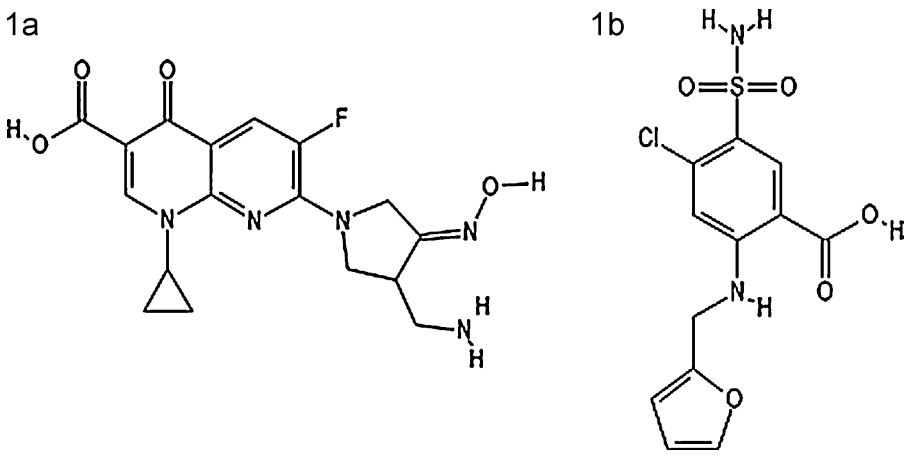

Fig. 1. (a) Chemical structure of gemifloxacin (GEM). (b) Chemical structure of furosemide. (I.S.)

to determine GEM in rat plasma without any interference from matrix, and it may be applied to support pharmacokinetic studies, metabolites characterization/quantification and microdialysis studies, allowing the analysis of low concentration samples.

2. Materials and methods

2.1. Chemicals and reagents

GEM mesylate (99.6%) was a gift from Aché Pharmaceutical Laboratory (São Paulo, Brazil). Furosemide (99.4%), the internal standard (I.S.) (Fig. 1b), was purchased from Galena (São Paulo, Brazil). Acetonitrile and methanol (LC grade) and triethylamine were obtained from Merck (Darmstadt, Germany). Other chemicals were of analytical grade. Water was purified by a Milli-Q system (Millipore, MA, USA).

2.2. Apparatus

LC apparatus of a Shimadzu system equipped with LC-10AD VP pump with a low-pressure gradient flow control valve FCV-10AL VP, SCL-10A VP system controller, SIL-10AD VP autoinjector, CTO-10A VP column oven, RF-10AXL detector and a DGU-14A degasser. Shimadzu CLASS-VP software (Version 6.12) was used for data acquisition and mathematical calculations. The chromatography was performed on a RP-C_{18} Atlantis (Waters, 150 mm × 4.6 mm i.d.; particle size 5 μm) column preceded by a guard column (C_{18} Phenomenex, 3.9 mm × 20 mm). The mobile phase consisted of triethylamine solution (0.5%, v/v), adjusted to pH 3.0 ± 0.1 with 85% phosphoric acid, methanol and acetonitrile (63:30:7, v/v/v). Before delivering the mobile phase into the system, it was degassed for 10 min by sonication and filtered through 0.45 μm filter (Sartorius, Germany) using vacuum. The flow rate of 1 mL min^{-1} and the injection volume of 30 μL were performed. The LC system was operated isocratically. Separation was performed at a controlled temperature of 45 °C. The detection wavelengths were set at 344 nm and 399 nm for excitation and emission, respectively. The ratio of peak area of GEM to I.S. was used for the quantitation of samples.

2.3. Preparation of standard solutions

GEM was dissolved in methanol to prepare a primary stock solution at a final concentration of 100 μg mL^{-1}. Working standard solutions were prepared daily by serial dilution of primary stock solution with methanol to obtain analyte concentration from 200 ng mL^{-1} to 50,000 ng mL^{-1}. The I.S. working solutions were prepared in acetonitrile in a similar manner, providing finally a concentration of 50,000 ng mL^{-1}. The intermediate stock solutions were prepared weekly, while working stock solutions used for the calibration curves were prepared daily. All solutions were kept protected from light.

Calibration standards in plasma (from 20 ng mL^{-1} to 5000 ng mL^{-1}) samples were prepared by spiking blank rat plasma with 20 μL and 10 μL of working stock solutions of GEM and I.S., respectively. The final concentration of I.S. was 2500 ng mL^{-1}. The quality control (QC) plasma samples of GEM (60 ng mL^{-1}, 2500 ng mL^{-1} and 4000 ng mL^{-1}) were similarly prepared. The QC samples were prepared from a stock solution that was different from the one used to generate standard curve samples. These QC samples were used to investigate intra- and inter-run variations.

2.4. Sample preparation

The plasma samples were stored in freezer at −80 °C until use and allowed to thaw at room temperature before processing. To 180 μL of plasma samples, 150 μL ice-cold acetonitrile containing 0.5% formic acid was added, resulting in protein precipitation. After rigorous vortex mixing for 3 min in an automatic mixer, the samples were centrifuged at 6800 × *g* for 10 min at 4 °C. The supernatant was loaded in the autosampler tray and 30 μL was injected into the LC system for analysis.

2.5. Bioanalytical method validation

The validation was carried out according to Food and Drug Administration (FDA, 2001) [18]. The described method was validated considering linearity, lower limit of quantitation (LLOQ), specificity, recovery, precision, accuracy and stability. The QC sample results in all runs were used to calculate the precision and accuracy of the analytical method.

2.5.1. Linearity and LLOQ

In order to determine the linearity, six calibration curves were performed on two consecutive days. Seven point calibration curves were constructed in plasma over the selected concentration range and tested for linearity. Calibration standards were freshly prepared every day during ongoing analysis. The fitting of the calibration curves was performed by using the non-linear regression software Scientist® v. 2.01 (Micromath®, Salt Lake City, USA). The data was weighted by 1/concentration. Linear regression analysis of the data from calibration curves gave slope (*a*), intercept (*b*) and correlation coefficients, which were used to determine the concentration of analyte in the QC samples. The lowest concentration level

giving a chromatographic response with acceptable coefficient of variation was defined as the LLOQ.

2.5.2. Specificity

Specificity of the method was determined by analyzing eight different batches of blank plasma obtained from healthy rats.

2.5.3. Precision and accuracy

The reproducibility of the analytical procedure was determined by calculating intra- and inter-day relative standard deviation (R.S.D.%) and accuracy (%bias) for each calibration level. Precision and accuracy were performed by six replicate analysis of spiked plasma QC samples at three concentrations (60 ng mL^{-1}, 2500 ng mL^{-1} and 4000 ng mL^{-1}) followed by their comparison with the calibration curves prepared on the same day and on two consecutive days.

2.5.4. Recovery

The absolute recovery was performed by calculating the mean of the response of each concentration (60 ng mL^{-1}, 2500 ng mL^{-1} and 4000 ng mL^{-1} for GEM and 2500 ng mL^{-1} for I.S.) and dividing the extracted sample mean by the unextracted (spiked blank plasma extract) sample mean at the corresponding concentration. Comparison with unextracted samples, spiked in plasma residues, was performed to eliminate matrix effects, giving a true recovery. The relative recovery was obtained using the equation: relative recovery = (response peaks of extract/unextracted) × 100.

2.5.5. Stability

The stability of GEM in rat plasma was performed using low (60 ng mL^{-1}) and high (4000 ng mL^{-1}) QC samples under different conditions: three freeze–thaw cycles, short-term, long-term and after processed. For the short-term stability, the frozen plasma samples (−80 °C) were kept at room temperature for 4 h before sample preparation. Also, stability of GEM was assessed after storage at −80 °C for 60 days and after three freeze–thaw cycles at the same temperature. Post-preparation (extracted) storage stability was tested after keeping the samples in an autosampler at 4 °C for 12 h. Stability was evaluated by comparing measured concentration before and after storage. The samples were analyzed and the results were compared with those obtained for the freshly prepared samples. Three repeated determinations were made in each case and at each stipulated time period.

2.6. Pharmacokinetic study

Application of this LC method in a pilot study by determining plasma concentration of GEM in male Wistar rats is presented in this article. Animal experiments were performed according to the National Institutes of Health Guide for Care and Use of Laboratory Animals and institutional guidelines. The protocol used to quantify GEM in rat plasma was previously approved by the Ethics in Research Committee of University of Caxias do Sul – UCS. Three rats (250–350 g) (purchased from Technology and Science Foundation (Santa Maria, Brazil)) were used in the study. The animals were housed under standard conditions and kept on a 12-h light:12-h dark cycle. Food and water were available *ad libitum*. On the day of the experiment, GEM at a dose of 40 mg kg^{-1} was administered intravenously by the lateral tail vein. Blood samples were serially collected prior to dosing (as the time zero) and 0.08, 0.5, 1.5, 3.0, 6.0 and 8.0 h after intravenous administration. Plasma was separated by centrifugation at 6800 × *g* for 10 min at 21 °C and stored at −80 °C until analysis by the LC developed and validated method. High plasma levels were diluted in blank rat plasma to give concentrations into the calibration curve and the concentrations were back-calculated.

3. Results and discussion

3.1. I.S. selection

Regarding the I.S. selection, other quinolone drugs such as, norfloxacin, ofloxacin, ciprofloxacin and levofloxacin were tested, due to structural similarity and fluorescence capacity. The octanol/water partition coefficient (Log *P*) may have contributed to the short retention time observed to all of these compounds when compared to GEM. Therefore, furosemide, which presented suitable characteristics including extraction reproducibility, higher relative retention time compared to GEM, and no plasmatic matrix interference, was chosen as I.S.

3.2. Extraction optimization

During the bioanalytical method development, one of the most important steps is to extract the drug from the plasmatic matrix. Preferably, the selection of simple and efficient extraction procedures, as protein precipitation, are recommended due to ease, quickness and high reproducibility. In this context, different organic solvents, mainly methanol and acetonitrile, were tested. The results showed that the GEM recovery was higher using acetonitrile when compared to methanol. Besides, an increase of the drug peak tailing factor was observed. Also, to increase the protein precipitation and GEM recovery, the influence of different co-precipitation agents, such as acetic acid, formic acid, trichloroacetic acid and trifluoroacetic acid, was investigated. So, a mixture of acetonitrile and formic acid (0.5%, v/v) was chosen as final solvent for GEM extraction from plasma samples. This procedure offers advantages regarding the methods described in the literature. Some of the methods described the use of liquid–liquid extraction followed by evaporation [11,15,16]. Liquid–liquid extraction, widely employed, is a tedious, solvent and time-consuming sample preparation. The literature also presents the use of an ultrafiltration tube to remove protein of the matrix [17] that can be more expensive than a single protein precipitation step employing organic solvent, as well as the use of fluorogenic reagents [9] to quantify the gemifloxacin molecule. This approach is time-consuming and more expensive and the concentration range analyzed was limited (40–200 ng mL^{-1} and 100–1200 ng mL^{-1}) depending on the fluorogenic reagent used.

3.3. Optimization of chromatographic conditions

Initially, the system performance was tested by means of the same proportion of methanol and water (50:50, v/v) as mobile phase. However, a short retention time of GEM was observed. Thus, the aqueous proportion of mobile phase was changed to 70% (v/v), leading to an increase of the retention time and split of GEM peak. To correct this situation, 0.5% (v/v) of triethylamine in aqueous solution (pH adjusted to 3.0 ± 0.1 with orthophosphoric acid) was used, and typical chromatographic peaks were obtained at a flow of 1 mL min^{-1}. In order to decrease the chromatographic run, 7% (v/v) of acetonitrile was added to the mobile phase composition, and the oven temperature was adjusted to 45 °C. This modification was satisfactory, keeping the good resolution, but increasing the peak symmetry and decreasing the tailing factor for both chromatographic peaks. So, the ratio of triethylamine solution (0.5%, v/v), methanol and acetonitrile (63:30:7, v/v/v) was selected as final mobile phase composition. For validation purposes, the maximal excitation and emission wavelengths (344 nm and 399 nm, respectively) were used to increase the method sensibility, allowing the measurement of low GEM concentrations in rat plasma.

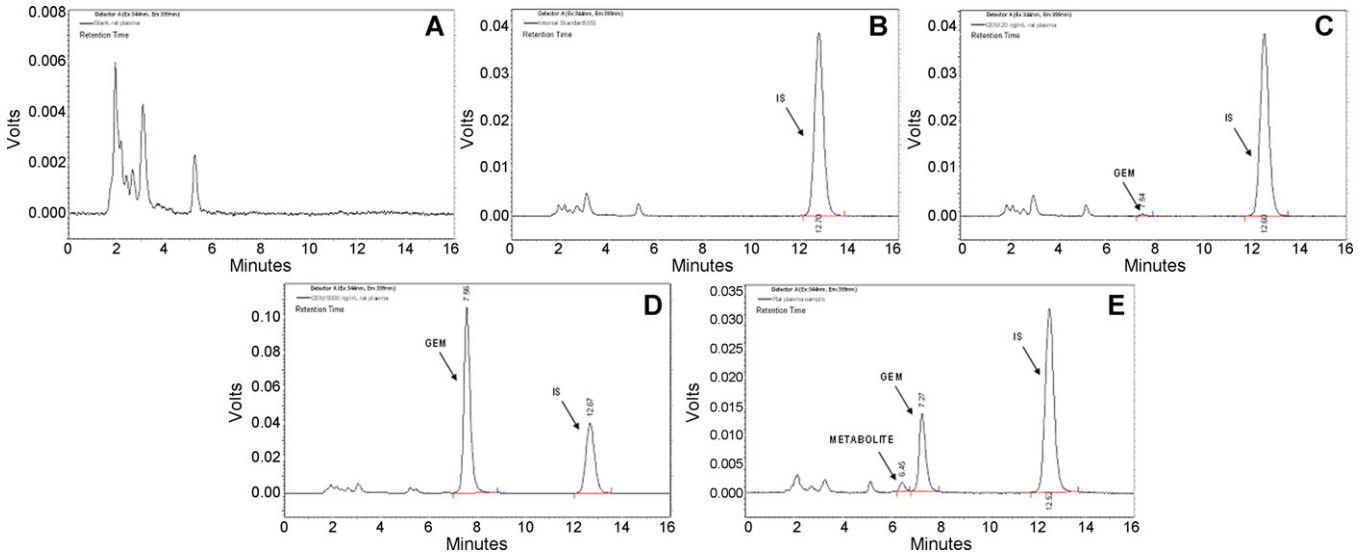

Fig. 2. Representative chromatograms of blank rat plasma (A), rat plasma with furosemide (I.S.) at 2500 ng mL^{-1} (B), rat plasma with GEM at 20 ng mL^{-1} (C), rat plasma with GEM at 5000 ng mL^{-1} (D), and rat plasma sample at 3.0 h with GEM and I.S. (E.)

3.4. Specificity

The developed and validated LC method with fluorescence detection was used to quantify GEM in rat plasma for pharmacokinetic studies.

Representative chromatograms of blank rat plasma (A), rat plasma with furosemide (I.S.) at 2500 ng mL^{-1} (B), rat plasma with GEM at 20 ng mL^{-1} (C), rat plasma with GEM at 5000 ng mL^{-1} (D) and rat plasma sample with GEM and IS at 3.0 h (E) are presented in Fig. 2. A different peak was observed in rat plasma and could be a metabolite. As it can be seen, GEM and I.S. were sufficiently separated from each other and no interfering peak was observed in the chromatograms from these samples, showing the specificity of the method. Moreover, the mean retention times for GEM and I.S. were at ~7.5 min and ~12.6 min, respectively, indicative of good resolution (upper to 2.0) between both compounds in relation to the endogenous substances.

The method developed and validated was able to quantify the metabolite and original drug in the same run without interference. This point was not presented in the literature before and contributes to the novelty of the method. Pre-clinical pharmacokinetic investigations require reliable analytical performance for drug measuring in different biological matrix. In this context, it is very important to develop a chromatographic system able to identify and characterize metabolites in the same analytical run. Therefore, this work reports the first bioanalytical method by LC–FL for quantification of GEM in rat plasma with the presence of a metabolite. The characterization of such a substance was not the objective of this work but this methodology could be employed to quantify it in plasma sample as well as the original drug.

3.5. Linearity and LLOQ

The linearity of the method was observed in the expected concentration range (20–5000 ng mL^{-1}) demonstrating its suitability for analysis. The goodness-of-fit (r^2) was found to be greater than or equal to 0.9955 for all curves indicating functional linear relationship between the concentration of analyte and area under the peak. The mean linear regression equation of calibration curve for GEM was $y = 0.00037x - 0.00062$ ($r^2 = 0.9996$). No deviation from linearity was found ($p > 0.05$) and the regression was highly significant ($p \leq 0.01$) by means of ANOVA. All back-calculated values of the individual calibration standards were lower than 7.10% of the spiked value as well as the accuracy range. All analytical results are not more than 15% coefficient of variation for precision and not more than 15% deviation from the nominal value for accuracy (bioanalytical methods). The LLOQ measured with acceptable precision and accuracy under the stated experimental conditions for this method in rat plasma was 20 ng mL^{-1} for which the R.S.D. was lower than 20%.

Table 1
Intra and inter-day precision for the GEM quality control in rat plasma.

Concentration spiked QC (ng mL^{-1})	Day	Measured concentrations		
		Mean concentration found (ng mL^{-1})	S.D. (ng mL^{-1})	R.S.D. (%)
Intra-day				
4000	1	4060	47.2	1.16
	2	3954	50.5	1.28
2500	1	2487	25.8	1.04
	2	2493	91.2	3.66
60	1	61.1	0.55	0.90
	2	58.7	3.67	6.24
Inter-day				
4000		4007	72.9	1.82
2500		2489	60.0	2.41
60		60.0	2.69	4.49

3.6. Precision and accuracy

A summary of the precision and accuracy results at low, medium and high concentrations of GEM in rat plasma are reported in Tables 1 and 2, respectively. The precision of the assay was determined by repeatability (intra-day precision) and intermediate precision (inter-day precision) expressed as R.S.D. The results obtained at three concentration levels showed R.S.D. values lower

Table 2
Accuracy for the analysis of GEM in rat plasma.

Quality control (ng mL^{-1})	Range (ng mL^{-1})	Accuracy (%)
4000	3900–4109	97.5–102.7
2500	2394–2511	95.8–100.4
60	56.0–62.9	93.3–104.8

Table 3
Relative recovery and corresponding relative standard deviation (R.S.D.) of GEM and I.S. in rat plasma ($n = 6$).

GEM (QCs)	60 (ng mL^{-1})		2500 (ng mL^{-1})		4000 (ng mL^{-1})	
	Conc. found	Recovery (%)	Conc. found	Recovery (%)	Conc. found	Recovery (%)
Mean	55.3	92.2	2327	93.1	3618	90.4
S.D.	3.44	5.73	88.0	3.52	44.0	1.76
R.S.D. (%)	6.22	6.21	3.78	3.78	1.22	1.94

I.S.	60 (ng mL^{-1}) Recovery (%)	2500 (ng mL^{-1}) Recovery (%)	4000 (ng mL^{-1}) Recovery (%)
Mean	92.1	89.4	90.6
S.D.	2.03	2.83	2.94
R.S.D. (%)	2.20	3.16	3.25

than 6.24% and 4.49% for repeatability and intermediate precision, respectively. All results regarding precision and accuracy were within the ranges acceptable for bioanalytical purposes.

3.7. Recovery

Recovery experiments (extraction efficiency) were performed by comparing the analytical results for extracted samples ($n = 6$) with unextracted (spiked blank plasma extract) standards that represent 100% recovery. Comparison among the unextracted samples, spiked and plasma residues was done in order to eliminate matrix effect, giving a true recovery. Data are shown in Table 3. The plasma recovery of GEM and I.S. was greater than 90%.

3.8. Stability

The results of short-term stability, freeze/thaw stability, autosampler stability, and long-term stability are shown in Table 4. A maximum deviation of 4.04% in plasma was observed. The results were found to be within the assay limits during the entire process, independently of the concentration level considered.

3.9. Application of assay

The present LC method was successfully used to investigate the pharmacokinetic of GEM in Wistar rat plasma after intravenous administration at 40 mg kg^{-1} dose. The calibration curve and QC samples were used for quantitative analysis of rat plasma samples. The mean plasma concentration–time profile of GEM is shown in Fig. 3. The pharmacokinetic profile shows a rapid distribution phase of GEM from rat plasma. The corresponding pharmacokinetic parameters obtained after model independent analysis were: $k_e = 0.34\ h^{-1}$, $t_{1/2}\beta = 2.04$ h, $AUC_{0-t} = 43615.85$ ng h mL^{-1} and $AUC_{0-\infty} = 46420.40$ ng h mL^{-1}. The terminal phase of GEM could be well characterized proving that the analytical methodology has adequate sensitivity to detect very low concentration until 20 ng mL^{-1} after dosing. The pharmacokinetic results of GEM will be used to determine the tissue to plasma ratio distribution in another study.

Table 4
Stability of GEM in rat plasma at two quality control levels ($n = 3$).

Stability	Accuracy (%)			
	60 (ng mL^{-1})		4000 (ng mL^{-1})	
	Mean ± S.D.	R.S.D (%)	Mean ± S.D.	R.S.D. (%)
Short-term stability[a]	98.2 ± 1.33	1.35	96.3 ± 3.89	4.04
Freeze–thaw stability[b]	97.1 ± 1.25	1.29	99.2 ± 2.24	2.26
Long-term stability[c]	96.1 ± 3.24	3.37	95.6 ± 2.24	2.89
Processed sample stability[d]	97.4 ± 2.73	2.80	98.2 ± 3.23	3.29

[a] At room temperature for 4 h.
[b] Three freeze–thaw cycles within 3 days for 24 h and thawed at room temperature.
[c] At −80 °C for 60 days.
[d] In an autosampler at 4 °C for 12 h.

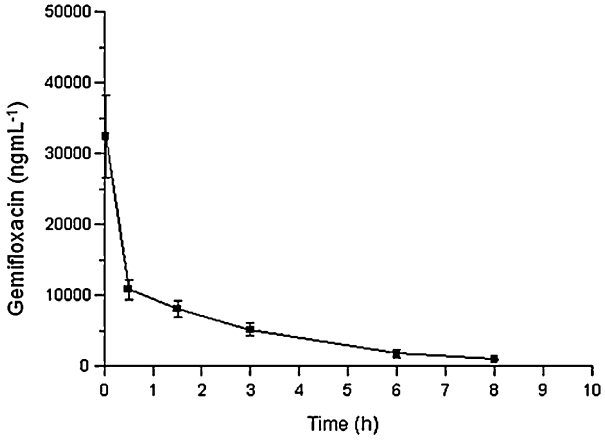

Fig. 3. Mean plasma concentration-time profile after intravenous administration of GEM at 40 mg kg^{-1} ($n = 3$). The data points are means and errors bars are standard deviations.

4. Conclusion

A LC-FL method to quantify GEM in rat plasma for application to pharmacokinetic studies is described. This validated method demonstrates good reproducibility and accuracy and has been successfully applied to the analysis of rat plasma samples. The novel method has a better liquid chromatography separation and sample preparation when compared with other methods published. The chromatographs provide clear separation with no interfering peaks and stability has been demonstrated in different conditions. Also, the validated method can be used to support other pharmacokinetic investigation which will be necessary to quantify the analyte at very low concentration in plasma. The present method can facilitate the development and validation of other method to analyze GEM in tissue homogenate.

Acknowledgements

The authors acknowledge FAPERGS (Brazil) and University of Caxias do Sul for financial support.

References

[1] P. Ball, L. Mandell, G. Patou, W. Dankner, G. Tillotson, Int. J. Antimicrob. Agents 23 (2004) 421.
[2] M.G. Cormican, R.N. Jones, Antimicrob. Agents Chemother. 41 (1997) 204.
[3] J.H. Elliott, N.M. Anstey, S.P. Jacups, et al., Int. J. Infect. Dis. 9 (2005) 15.

[4] B. Roy, A. Bose, U. Bhaumik, A. Das, N. Chatterjee, A. Ghosh, S. Darbar, A.K. Sarkar, P. Sengupta, T.K. Pal, J. Pharm. Sci. 99 (2010) 1535.
[5] E.P. Trallero, C.G. Rey, A.M.M. Sanchez, L. Aguilar, J.G. Lomas, J. Ruiz, Antimicrob. Agents Chemother. 46 (2002) 2665.
[6] V.J. Heaton, J. Ambler, L.M. Fisher, Antimicrob. Agents Chemother. 44 (2000) 3112.
[7] M.V. Krishna, D.G. Sankar, E-J. Chem. 5 (2008) 493.
[8] M.V. Krishna, D.G. Sankar, E-J. Chem. 5 (2008) 515.
[9] S.E.K. Tekkeli, A. Önal, J. Fluoresc. 21 (2011) 1001.
[10] C.S. Paim, F. Führ, A.B. Barth, C.E.I. Gonçalves, N. Nardi, M. Steppe, E.E.S. Schapoval, Talanta 83 (2011) 1774.
[11] A. Allen, E. Bygate, S. Oliver, M. Johnson, C. Ward, C. Cheon, Y.S. Choo, I.C. Kim, Antimicrob. Agents Chemother. 44 (2000) 1604.
[12] A. Allen, E. Bygate, M. Vousden, S. Oliver, M. Johnson, C. Ward, A.J. Cheon, Y.S. Choo, I.C. Kim, Antimicrob. Agents Chemother. 45 (2001) 540.
[13] E. Doyle, S.E. Fowles, D.F. McDonnel, R. McCarthy, S.A. White, J. Chromatogr. B 746 (2000) 191.
[14] V.R. Robledo, W.F. Smyth, Anal. Chim. Acta 623 (2008) 221.
[15] R. Bikash, D. Ayan, B. Uttam, K.S. Amlan, B. Anirbandeep, M. Jayanti, S.C. Uday, K.D. Anjan, T.K. Pal, J. Pharm. Biomed. Anal. 52 (2010) 216.
[16] A.R. Rote, S.P. Pingle, J. Chromatogr. B 877 (2009) 3719.
[17] B.M.H. Al-Hadiya, A.A. Khady, G.A.E. Mostafa, Talanta 83 (2010) 110.
[18] FDA Guidance for Industry, Bioanalytical Method Validation, May 2001.