Journal of Chromatography B, 878 (2010) 3437–3441

Contents lists available at ScienceDirect

Journal of Chromatography B

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

Short communication

High performance liquid chromatography assay with ultraviolet detection for moxifloxacin: Validation and application to a pharmacokinetic study in Chinese volunteers

Yu Hong Xu[a], Dong Li[b], Xin Yu Liu[a], Yu Zhen Li[a], Jian Lu[c,d,*]

[a] Department of Clinical Pharmacy, Futian People's Hospital, Guangdong Medical College, Shenzhen, China
[b] Department of Clinical Pharmacy, Shenzhen People's Hospital, The Second Medical College of Jinan University, Shenzhen, China
[c] National Institute of Drug Clinical Trials, Shenzhen Third People's Hospital, Guangdong Medical College, Shenzhen, China
[d] Department of Medicine and Nosocomial Infection, Shenzhen Third People's Hospital, Guangdong Medical College, Shenzhen, China

ARTICLE INFO

Article history:
Received 31 August 2010
Accepted 25 October 2010
Available online 30 October 2010

Keywords:
Moxifloxacin
HPLC-UV
Validation
Pharmacokinetics

ABSTRACT

A specific, sensitive and widely applicable high performance liquid chromatography with ultraviolet detection (HPLC-UV) method for the determination of moxifloxacin in human plasma was developed and validated in this study. The method involved a single step of liquid–liquid extraction with dichloromethane and the extraction yields more than 80% were achieved. The separation was performed on a common Kromasil C_8 column with an isocratic mobile phase. The total time was within 10 min per run. The calibration curve for moxifloxacin was linear in the concentration range of 0.05–5.0 μg/ml with correlation coefficient of 0.9997. The developed method was validated with excellent specificity, sensitivity, accuracy, precision and stability. Using this developed method, the pharmacokinetics of moxifloxacin in healthy Chinese volunteers was studied.

1. Introduction

Moxifloxacin is a new fourth generation synthetic 8-methoxy fluoroquinolone antibacterial agent with powerful activity against both Gram-positive and Gram-negative bacteria, as well as atypical organisms and anaerobes [1]. With the increasing worldwide use of moxifloxacin, there is an urgent need to develop a simple, rapid, reliable and low-cost analytical method for the pharmacokinetic study and clinical routine monitoring.

The analytical methods used in earlier studies were derived mainly from the HPLC with fluorescence detection method developed by Stass and Dalhoff [2], in which complex gradient elution with changeable flow rate was used and the technique of on-column focusing had to be employed to avoid the peak broadening, interferences and shift of retention times. Recently, some more HPLC methods with expensive fluorescence detector were developed for moxifloxacin determination in biological fluids [3–9]. To solve the tailing problem, special analytic columns and complicated mobile phase with ion-pair reagents as well as gradient elution were employed [3–5,8]. The addition of ion-pair reagents such as tetrabutylammonium salts not only increase the complexity of mobile phase, but also could cause series of chromatography problems [10]. Some methods involved the application of displacing agent and special columns/cartridges in sample processing as well as pre-column derivatization or column switching technique [6–9]. These expensive specific equipments and complex technique would increase analytical cost and the complexity of operation. For clinical routine use, the disadvantages of these methods were obvious. LC/ESI-MS/MS methods have also been reported [11,12], but this expensive apparatus would be not cost-effective for clinical routine determination of moxifloxacin.

Since numerous HPLC systems widely used in healthcare settings, especially in most developing countries, equipped only with UV detector, it is very necessary and useful to develop an HPLC-UV method for moxifloxacin determination. However, up to now, several limited HPLC-UV methods were developed [13–15], in which similar disadvantages mentioned above were involved, and poor liquid–liquid extraction recovery (less than 70%) as well as long run time was observed [15]. Although the existing HPLC-fluorescence methods possessed sufficient sensitivity and could be modified to fit the UV detector, some problems are difficult to be overcome. Firstly, compared to the fluorescence detector, the sensitivity of UV detector is much lower. Secondly, endogenous interferences, which were absent under fluorescence detection, could emerge under UV detection. All these factors probably made previous methods unavailable [16]. Even if they are applicable, the restricted and

* Corresponding author at: National Institute of Drug Clinical Trials, Shenzhen Third People's Hospital, Buxin Road 2019, Shenzhen 518020, China.
Tel.: +86 755 25618139; fax: +86 755 83980683/25618139.
E-mail address: szlujian@yahoo.com (J. Lu).

1570-0232/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2010.10.024

expensive experimental conditions used in many studies were difficult to be satisfied in many healthcare settings due to funding limitation.

In this study, we developed a specific, sensitive, simple, reliable and widely applicable as well as low-cost HPLC-UV method for moxifloxacin determination in human plasma. The separation can be performed under the most common experimental conditions, without adding expensive special equipments. Also, using this developed method, the pharmacokinetics of moxifloxacin in healthy Chinese volunteers was studied.

2. Materials and methods

2.1. Chemicals and reagents

Moxifloxacin hydrochloride standard (batch No. BXR2C4N, declared purity 94.8%) was friendly obtained from BayerPharma Company (Germany). Gatifloxacin standard (batch No. 30518-200402, declared purity 97.2%), the internal standard (I.S.), was supplied by the National Institute for the Control of Pharmaceutical and Biological Products (NICPBP) of China. Methanol and acetonitrile (HPLC grade) were purchased from Tianjin Shield Fine Chemicals Co. Ltd (Tianjin, China). The other reagents were of analytical grade.

2.2. Chromatographic conditions

Chromatographic analysis was performed on a modular Agilent HP1100 system (Hewlett Packard, Agilent, USA) and signals were processed with HP ChemStation software. The Kromasil C_8 (250 mm × 4.6 mm, 5 μm particle size, EKA chemicals) was used as analytical column. The isocratic mobile phase consisted of acetonitrile, methanol and KH_2PO_4 buffer solution (0.02 M, containing 1% triethylamine, pH 3.0 adjusted with concentrated phosphoric acid) (15:20:65, v/v/v). The flow rate was 1.0 ml/min. The diode array detector was used and detection wavelength was set at 296 nm. The column temperature was maintained at 30 °C.

2.3. Standard stock and working solutions

Standard stock solution of moxifloxacin (1.0 mg/ml) was prepared by direct weighing of standard substance with subsequent dissolution in water. The stock solution was appropriately diluted in KH_2PO_4 buffer solution to obtain working standard solution of moxifloxacin with the final concentrations of 2.5, 5, 10, 20, 40, 80, 160, 250 μg/ml, respectively, used for calibration purposes. Standard stock solution of I.S. (0.2 mg/ml) was prepared by direct weighing of standard substance. The concentration of working standard solution of I.S. was 20 μg/ml, prepared by diluting appropriate stock solution in KH_2PO_4 buffer solution. All prepared solutions were stored at 4 °C until analysis.

2.4. Sample preparation

A simple liquid–liquid extraction was used for the extraction of moxifloxacin in human plasma. To 0.5 ml of human plasma, 15 μl aliquots of working I.S. solution and 40 μl of sodium hydroxide (NaOH, 0.5 M) were added and mixed for 10 s on a vortex agitator (Troody Analytical Instrument, Shanghai, China), followed by extraction with 5 ml of dichloromethane. After vortexed for 5 min, the mixture in a glass screw-capped tube was centrifuged at 10,000 rpm for 5 min (Abbott, USA). The lower organic layer was completely transferred into another conical glass tube and evaporated to dryness at 40 °C under a flow of nitrogen. The final residue was reconstituted in 100 μl aliquots of KH_2PO_4 buffer solution and 20 μl was injected into the sampler for analysis.

2.5. Method validation

2.5.1. Specificity

The specificity of the method was evaluated by analyzing blank drug-free plasma samples obtained from six healthy subjects and actual plasma samples from a volunteer after oral moxifloxacin. The probable interferences from endogenous substances were assessed by observing the chromatograms of blank and spiked plasma samples, as well as samples from the volunteer.

2.5.2. Linearity

The linearity of the standard curves was assessed with the intercept, slope and correlation coefficient (r) and their variations in the range of 0.05–5.0 μg/ml. The calibration samples of moxifloxacin (0.05, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 5.0 μg/ml) were prepared by separately spiking 10 μl of prepared working standard solution of moxifloxacin into 0.5 ml of blank plasma. The standard calibration curve for moxifloxacin was constructed by least square linear regression using peak area ratios of moxifloxacin to I.S. versus the nominal concentrations of the analytes. The deviation of standards from nominal concentration should be within ±15% (±20% for the lower limit of quantification).

2.5.3. Sensitivity

The sensitivity was evaluated by the limit of detection (LOD) and the lower limit of quantification (LLOQ). LOD was defined as the lowest concentration of moxifloxacin in spiked plasma that can be detected by gradual dilution in a signal-to-noise of 3:1. LLOQ was defined as the lowest concentration of moxifloxacin in spiked plasma sample with CV no more than 20% and relative error (R.E.) less than ±20%.

2.5.4. Precision and accuracy

The intra-day and inter-day precision were determined by analyzing six replicates of quality control samples at concentrations of 0.1, 1.6 and 5.0 μg/ml on the same day and six times on six consecutive days, respectively. The precision was evaluated by the coefficient of variation (CV) and the acceptable range of CV was no more than 15% at 1.6, 5.0 μg/ml or 20% at 0.1 μg/ml. The accuracy was assessed by the methodological recovery. The recovery of the method was calculated by comparing the determined concentration of spiked samples to the theoretical concentrations.

2.5.5. Extraction recovery

The extraction recovery of moxifloxacin from human plasma was evaluated by comparing the responses (peak area ratios of moxifloxacin to I.S.) of analytes spiked before extraction into plasma with those spiked after extraction into the separated organic layer solution. The extraction recovery was investigated at three different concentration levels of 0.1, 1.6 and 5.0 μg/ml by triplicate analysis.

2.5.6. Stability

The stability of plasma samples under different conditions was evaluated respectively by spiked quality control samples at three concentration levels. Freeze–thaw stability was determined by following three freeze–thaw cycles from −20 °C to room temperature for every 24 h. The long-term and short-term stability of plasma samples were assessed by determining the concentration of the samples kept at −20 °C for 1, 2, 4, 12 weeks and at room temperature for 2, 4, 8, 12 h, respectively. The stability of extracted plasma samples was evaluated by keeping samples at room temperature for 2, 4, 6, 12 h. The stabilities under different conditions were obtained from comparing the measured concentration with the concentration of sample at 0 h.

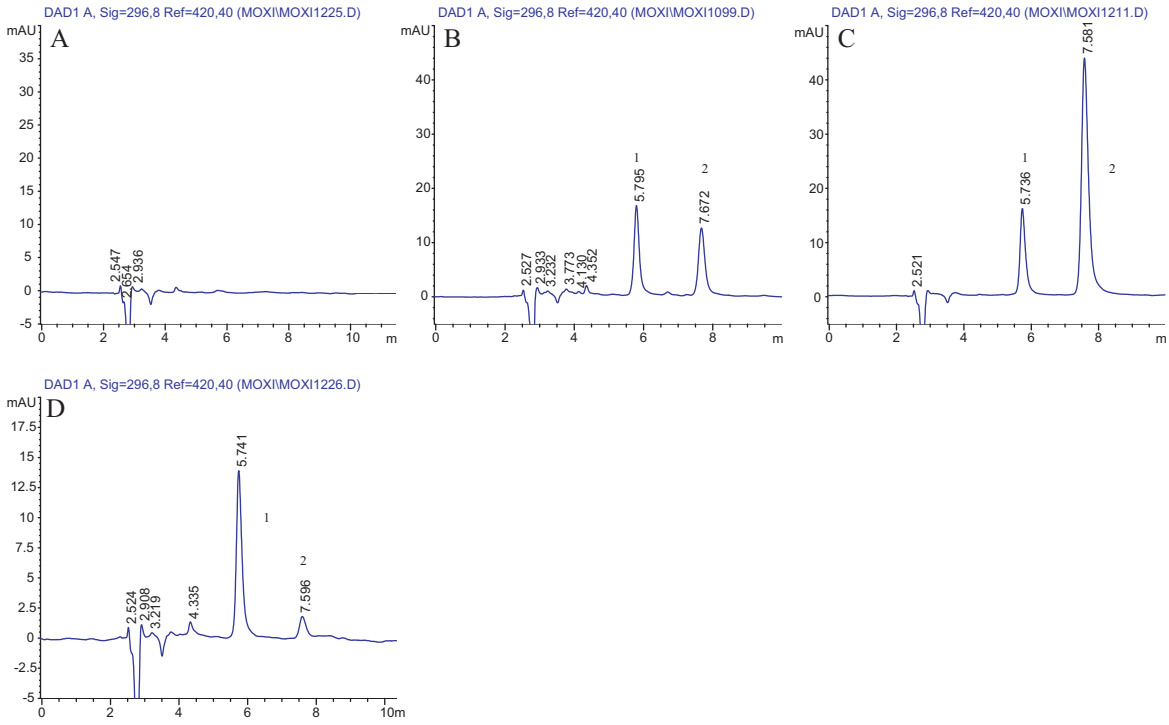

Fig. 1. HPLC chromatograms of moxifloxacin and gatifloxacin in human plasma. (A) blank plasma; (B) blank plasma spiked with moxifloxacin (0.2 μg/ml) and gatifloxacin; (C) plasma sample after administration; (D) blank plasma spiked with lowest moxifloxacin and gatifloxacin; 1. gatifloxacin; 2. moxifloxacin.

2.6. Pharmacokinetic study in Chinese subjects

The study was performed according to the requirements of the guideline for the conduct of pharmacokinetic study of chemical medicines issued by the State Food and Drug Administration of China (Guideline of SFDAC). Eight males and four females of eligible Chinese volunteers (aged between 22 and 42 years, bodyweight ranged from 50 to 81 kg, respectively) were recruited. Written informed consents were obtained from all subjects before the study. 4 ml of venous blood sample was collected into heparinized vacuum blood-collecting tubes before dosing (0 h) and at 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 10, 12, 24, 48 h after oral administration. The blood samples were centrifuged at 3000 rmp for 10 min within 1 h. The separated plasma samples were immediately frozen at −20 °C until analysis.

The plasma concentration–time data were analyzed by standard non-compartmental model with the help of the Drug and Statistics software (DAS software, a recommended pharmacokinetic program in China, version 2.1.1). The individual C_{max} (maximum plasma concentration) and t_{max} (time to reach C_{max}) were directly obtained from the plasma concentration versus time data. The other pharmacokinetic parameters were obtained by the software.

3. Results and discussion

The present study described a specific, sensitive, simple and reliable as well as low-cost HPLC-UV method based on the current conditions in our laboratory. Although the liquid–liquid extraction was time-consuming, the common chromatographic conditions and inexpensive procedure for sample preparation made this method widely suitable for routine monitoring. Furthermore, the low LLOQ obtained with a UV detector in this method was able to allow us to avoid the use of fluorescence detector, which required expensive equipment. On the other hand, more stable responses can be acquired under UV detector than fluorescence detector, particularly when the fluorescence intensity of moxifloxacin varied with pH values of solution [8,16]. The practical applicability of this method has been demonstrated in the pharmacokinetic study on healthy volunteers.

During the development of method, severe tailing that would impair the resolutions, sensitivity and precision had been observed. This tailing was probably attributed to the amphoteric property and silanophilic interaction of moxifloxacin [8]. The primary approaches for solving this problem were the application of special analytical columns, pre-column derivatization and ion-pair reagents as well as gradient elution [3–6,8,13]. In our study, the common Kromasil C_8 analytical column was screened and peak shape with symmetry factor more than 0.85 was obtained. The improvement of symmetry would benefit both sensitivity and precision of the method, and the LLOQ of this developed methods reached 0.05 μg/ml. Compared to the complicated compositions of mobile phase and/or gradient elution ways used in some HPLC methods [3,5,14], one of the advantages of our method was the application of an isocratic mobile phase with simpler composition. The lower volume ratio of phosphate buffer solution and no tetrabutylammonium salts in the solvent system would benefit the protection of the HPLC flow system [17]. By adjusting the relative proportion of methanol and acetonitrile, reasonable retention time for moxifloacin and I.S. was obtained with good peak shape.

For the analysis of biological samples, low extraction yields and more endogenous substances would influence the practical applicability of HPLC methods, especially when UV detector was used. To achieve better extraction efficacy and less interference, various sample processing approaches including direct protein precipitation and liquid–liquid extraction with different solvents were respectively investigated. The chromatographs from different sample processing ways were compared, and subsequently the extraction with dichloromethane was chosen and further studied. The previous study showed that pK_a of moxifloxacin was changeable in different solutions [8], thus, besides investigating other influencing factors, the effects of pH value on extraction efficacy were specially evaluated in this study. The alkalization of

Table 1
The intra- and inter-day precision and accuracy of moxifloxacin spiked in human plasma ($n = 6$).

Concentration (μg/ml)	Precision (CV, %)		Accuracy (mean ± SD, %)	
	Intra-day	Inter-day	Intra-day	Inter-day
0.05	8.98	9.61	104.58 ± 9.39	105.86 ± 10.18
0.1	6.01	6.83	104.50 ± 6.28	108.17 ± 7.39
1.6	4.58	4.53	101.03 ± 4.63	103.02 ± 4.67
5.0	3.84	5.43	99.37 ± 3.82	100.38 ± 5.43

Table 2
The stability of moxifloxacin under different conditions (%, $n = 3$[a]).

Concentration (μg/ml)	Freeze–thaw stability	Long-term stability	Short-term stability	Extracted sample stability
0.1	97.26 ± 5.13 (102.73)	102.14 ± 8.45 (95.61)	99.25 ± 4.15 (96.51)	99.91 ± 2.02 (97.95)
1.6	101. 47 ± 5.33 (95.38)	97.84 ± 4.48 (96.31)	96.56 ± 4.87 (93.21)	98.83 ± 2.59 (97.46)
5.0	98.79 ± 3.98 (95.55)	97.72 ± 4.79 (95.23)	100.16 ± 5.78 (95.13)	99.05 ± 1.97 (97.13)

The determined concentrations at 0 h were defined as 100%. The ratio of determined concentration each time versus concentration at 0 h was calculated. Values were mean ± SD, with the last determined mean values in the parenthesis.

[a] n: number of replicates of each concentration level.

the samples was found to benefit the extraction recovery. After comprehensively evaluating extraction efficiency and total processing time, 5 min of one step liquid–liquid extraction with 5 ml of dichloromethane was used. In this study, the extraction yields over 80% were much higher than that reported by Srinivas et al. [15].

Under the optimized chromatographic conditions and sample processing procedure, the retention times of moxifloxacin and I.S. were approximately 7.6 min and 5.7 min, respectively. Each run can be completed within 10 min. The chromatograms showed a clear and excellent separation between moxifloxacin, I.S. and endogenous interferences from plasma. No interfering peaks were detected at the retention time of either moxifloxacin or internal standard. Representative chromatographs were shown in Fig. 1. The calibration curve for moxifloxacin exhibited a good linearity in the concentration range of 0.05–5.0 μg/ml. The linear equation (mean ± SD, $n = 3$) was $y = (2.1382 \pm 0.0109)x + (0.0067 \pm 0.0153)$ ($r = 0.9997 \pm 0.0001$) (y: peak area ratio of moxifloxacin to I.S., x: concentrations of moxifloxacin). The deviations of standards from nominal concentration all were within ±15%. LOD and LLOQ for moxifloxacin were 0.015 μg/ml and 0.05 μg/ml, respectively. The moxifloxacin concentration in human plasma at 48 h (about five half lives) after oral dose 400 mg can be detected, suggesting that the sensitivity of this developed HPLC method was satisfactory. The precision and accuracy of the developed method, including those of lower limit of quantity concentration, were within the specified ranges (Table 1) and satisfied with the requirements of guideline of SFDAC. The extraction recoveries (mean ± SD) for moxifloxacin in human plasma sample were 80.48 ± 4.63%, 90.30 ± 3.31% and 91.25 ± 3.91% at low, medium and high concentrations, respectively. Moxifloxacin in human plasma sample was stable during the storage, freeze–thaw cycles, processing and analysis (Table 2).

The plasma samples from 12 Chinese volunteers were analyzed with the validated method described above. The mean plasma concentration–time curve of moxifloxacin was illustrated in Fig. 2. Peak concentrations for individuals ranged from 2.60 to 3.79 μg/ml were able to be reached within 1–3 h (mean 1.75 h). The half-life was 10.14 h, while the $AUC_{(0-\infty)}$ was 39.83 mg h/l. In general, there were no significant differences between the major pharmacokinetic parameters in Chinese subjects and those in Caucasians [18–20].

In summary, a specific, sensitive, simple and widely applicable HPLC-UV analytical method for the determination of moxifloxacin in human plasma was developed. The chromatogram generates a well-resolved symmetric peak for moxifloxacin with good precision and accuracy. This method is economic, widely suitable for routine work and experimental conditions can be met in most healthcare settings. The method has been successfully applied to a pharmacokinetic study on moxifloxacin in Chinese subjects. And it could be used for the bioequivalence study of moxifloxacin in the future.

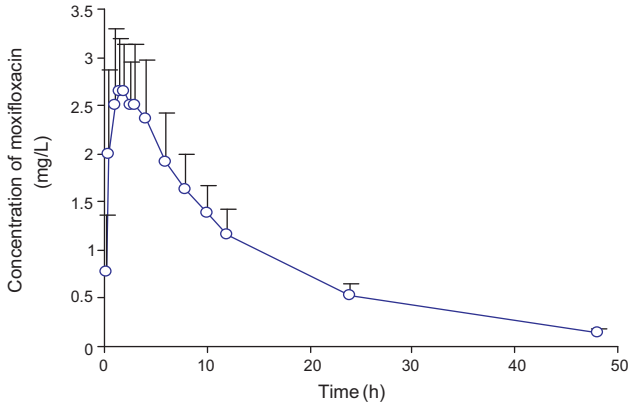

Fig. 2. Plasma concentration–time curve after a single oral dose of 400 mg moxifloxacin in 12 volunteers.

Acknowledgements

The authors gratefully thank Dr. Ying Chen for her help with the data processing. We also thank all volunteers who participated in this study and staff for supporting this study.

References

[1] G.M. Keating, L.J. Scott, Drugs 64 (2004) 2347.
[2] H. Stass, A. Dalhoff, J. Chromatogr. B 702 (1997) 163.
[3] K.P. Chan, K.O. Chu, W.W. Lai, K.W. Choy, C.C. Wang, D.S. Lam, C.P. Pang, Anal. Biochem. 353 (2006) 30.
[4] S. Schulte, T. Ackermann, N. Bertram, T. Sauerbruch, W.D. Paar, J. Chromatogr. Sci. 44 (2006) 205.
[5] J. De Smet, K. Boussery, K. Colpaert, P. De Sutter, P. De Paepe, J. Decruyenaere, J. Van Bocxlaer, J. Chromatogr. B 877 (2009) 961.
[6] S. Tatar Ulu, J. Pharm. Biomed. Anal. 43 (2007) 320.
[7] A.K. Hemanth Kumar, G. Ramachandran, J. Chromatogr. B 877 (2009) 1205.
[8] A. Laban-Djurdjević, M. Jelikić-Stankov, P. Djurdjević, J. Chromatogr. B 844 (2006) 104.
[9] H.A. Nguyen, J. Grellet, B.B. Ba, C. Quentin, M.C. Saux, J. Chromatogr. B 810 (2004) 77.
[10] S. Al-Dgither, S.N. Alvi, M.M. Hammami, J. Pharm. Biomed. Anal. 41 (2006) 251.
[11] K. Vishwanathan, M.G. Bartlett, J.T. Stewart, J. Pharm. Biomed. Anal. 30 (2002) 961.
[12] A.D. Pranger, J.W. Alffenaar, A.M. Wessels, B. Greijdanus, D.R. Uges, J. Anal. Toxicol. 34 (2010) 135.

[13] T. Lemoine, D. Breilh, D. Ducint, J. Dubrez, J. Jougon, J.F. Velly, M.C. Saux, J. Chromatogr. B 742 (2000) 247.
[14] H. Liang, M.B. Kays, K.M. Sowinski, J. Chromatogr. B 772 (2002) 53.
[15] N. Srinivas, L. Narasu, B.P. Shankar, R. Mullangi, Biomed. Chromatogr. 22 (2008) 1288.
[16] M. Kamberi, K. Tsutsumi, T. Kotegawa, K. Nakamura, S. Nakano, Clin. Chem. 44 (1998) 1251.
[17] H. Scholl, K. Schmidt, B. Weber, J. Chromatogr. 416 (1987) 321.
[18] H. Stass, A. Dalhoff, D. Kubitza, U. Schühly, Antimicrob. Agents Chemother. 42 (1998) 2060.
[19] J.T. Sullivan, M. Woodruff, J. Lettieri, V. Agarwal, G.J. Krol, P.T. Leese, S. Watson, A.H. Heller, Antimicrob. Agents Chemother. 43 (1999) 2793.
[20] J. Lettieri, R. Vargas, V. Agarwal, P. Liu, Clin. Pharmacokinet. 40 (Suppl. 1) (2001) 19.