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Development of a high-performance liquid chromatography method for the determination of caspofungin with amperometric detection and its application to in vitro microdialysis experiments

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Abstract

Microdialysis is an increasingly employed technique for the determination of tissue pharmacokinetics. A high-performance liquid chromatography method for the quantitative determination of caspofungin in human microdialysates with amperometric detection is described. Since microdialysis of caspofungin is performed with a 100,000 molecular mass cut-off membrane, microdialysates contain protein that was precipitated at pH 4 with acetonitrile. Addition of 1-propanol (33%, v/v) to the sample extract improved the analytical recovery to 81–89%. Caspofungin and the internal standard clarithromycin were separated isocratically on a cyanopropyl silica column using acetonitrile–0.05 M citrate (33:67, v/v), adjusted to an apparent pH of 6.9, at a flow rate of 1.0 ml/min, and amperometric detection at +950 mV oxidation potential. Within-day and between-day imprecision and inaccuracy were <11%. The lower limit of quantification was 0.07 μg/ml. The method was applied to in vitro microdialysis experiments. Ringer's solution containing 1% (w/v) human albumin was used for the perfusing and surrounding medium, respectively. Albumin did not entirely prevent adsorption of caspofungin to the surface of membrane and/or tubing. When the binding-sites were saturated with albumin plus caspofungin prior to the start of sampling, the percentage of drug appearing in the microdialysate ("recovery") remained stable over the concentration range tested.

Keywords: Caspofungin; Echinocandin; Microdialysis; High-performance liquid chromatography; Amperometric detection

1. Introduction

Caspofungin (MK-0991), a semi-synthetic cyclic lipopeptide, is a parenteral antifungal agent belonging to the echinocandin/pneumocandin family. It inhibits the synthesis of 1,3-β-D-glucan, which is an essential constituent of the cell wall of pathogenic fungi including *Candida* spp., *Aspergillus* spp. and *Pneumocystis jiroveci* [1,2]. As for all antimicrobial agents, caspofungin concentrations in the target tissue are considered most important for clinical efficacy. In rats, radiolabeled caspofungin is extensively distributed throughout the body, with highest levels of radioactivity in homogenates of liver, kidney, lung and spleen [3]. Analysis of homogenized tissue, however, cannot provide information about the continuous concentration–time profile, nor information about the partitioning of the drug between the histological compartments, i.e. the intravasal, interstitial, and intracellular space. In contrast, the microdialysis technique is a well established in vivo method for the monitoring of drug concentrations in the interstitial space of tissues, which is the major site of fungal infections [4]. In brief, a probe with a semi-permeable hollow fibre connected to an inlet and outlet tubing, is implanted into the tissue of interest and constantly perfused with a physiological solution. Drug molecules diffuse along their concentration gradient from the interstitial space fluid into the perfusion fluid (dialysate) and reach a diffusion equilibrium that for most compounds is incomplete, i.e. the concentration in the dialysate is lower than that in the interstitial space fluid. The ratio of these two concentrations, termed "recovery", is determined by the flow rate as well as the physical and

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chemical characteristics of the drug and the microdialysis membrane. It can be explored in in vitro microdialysis experiments which are a prerequisite for the utilization of the microdialysis technique for a given drug in human pharmacokinetic studies [5].

For the analysis of caspofungin in microdialysates, a sensitive method suitable for small sample volumes (typically 20–30 μl) is required. Previously described high-performance liquid chromatography methods for the quantification of caspofungin in plasma or urine with either fluorescence or mass spectrometric detection are unsuitable for use on microdialysates because they include solid-phase extraction of 0.5–1 ml sample and injection of 75–100 μl of the extract [6–9]. The lower limits of quantification (LLOQs) of these methods vary between 0.01 and 0.15 μg/ml, dependent on the sample volume subjected to solid-phase extraction. Our aim was to develop an alternative method that allows the analysis of the small sample volumes obtained by microdialysis with a sensitivity comparable to that of existing methods, yet without the need of the expensive and technically demanding mass spectrometer.

We here describe an assay for the quantitative determination of caspofungin in microdialysates based on amperometric detection and report the results of in vitro microdialysis experiments on caspofungin which were performed in view of planned pharmacokinetic studies in vivo.

2. Experimental

2.1. Reagents and standard solutions

A caspofungin reference solution (7 mg/ml) was prepared by dissolving the pharmaceutical preparation "Cancidas™ 70 mg for injection" (MSD Inc., New Jersey, USA) according to the manufacturer's instruction with double-distilled water. The resulting solution contains caspofungin acetate corresponding to 7 mg/ml caspofungin, plus acetic acid, sodium hydroxide, 54 mg sucrose and 36 mg mannitol. Aliquots of this reference solution were stored at −80 °C. Working solutions (70 μg/ml) were prepared daily by dilution of the reference solution with Ringer's solution containing 1% (w/v) human albumin (HA). Calibration standards were prepared by further dilution of the working solution to final concentrations ranging from 0.07 to 35 μg/ml. Additional standards were prepared by spiking blank human microdialysates from soft tissue (obtained from a previous study). Clarithromycin (Abbott Laboratories, Illinois, USA) was dissolved in acetonitrile. Ringer's solution was purchased from Mayrhofer Pharmazeutica (Linz, Austria). All other solvents and reagents were purchased from Sigma–Aldrich (Steinheim, Germany).

2.2. Sample preparation

All study samples were stored at −80 °C until analysis. Samples were deproteinized by vortex-mixing of 20 μl sample with 60 μl ice-cold acetonitrile containing the internal standard clarithromycin and centrifuged at 12,000 × *g* for 10 min at 4 °C. The supernatant was evaporated under nitrogen gas to dryness and re-dissolved in 20 μl mobile phase.

Modification I The effect of acidification of the sample on the analytical recovery of the drug was tested. Five microlitres of 0.05 M citric acid in water was added to 20 μl sample (pH of the mixture approximately 4) prior to the deproteinization step.

Modification II Organic additives were investigated for their ability to reverse the well-known adsorption of caspofungin to the wall of polypropylene test tubes [6]. Twenty microlitres sample extracts were mixed with 5 or 10 μl each of methanol, ethanol or 1-propanol (final content of alcohols 20% or 33%, v/v) and vortex-mixed for 30 s. Twelve microlitres of the mixture was injected.

2.3. Chromatographic conditions

The HPLC system consisted of a Beckman System Gold 126 pump connected to a 508 autosampler (Beckman Coulter, Inc., Fullerton, CA, USA), an amperometric detector (BAS, West Lafayette, IN, USA) and a PC with Beckman 32 Karat integration software installed. Isocratic separation was carried out at ambient temperature on a cyanopropyl silica column LiChroCART CN, 250 mm × 4.0 mm, 5 μm particle size, which was protected by a 4 mm × 4 mm guard column of the same stationary phase (VWR Int., Darmstadt, Germany). The mobile phase was composed of a solution of anhydrous citric acid (0.05 M), adjusted to pH 6.3 with sodium hydroxide, and acetonitrile in a 67:33 volume ratio (apparent pH 6.9). The flow rate was 1.0 ml/min. The detector cell potential for oxidation was examined from +650 to +1100 mV to obtain voltage-signal relationships, using a working electrode of glassy carbon and an Ag/AgCl reference electrode (BAS).

2.4. Performance assessment

Ten-point calibration lines were constructed from the ratios of the peak area of caspofungin to internal standard versus the caspofungin concentrations of the calibration standards ranging from 0.07 to 35 μg/ml. Sample concentrations were calculated from the equation $y = ax + b$ as determined by the linear regression analysis of the calibration data. Recoveries of caspofungin are expressed as the ratio of peak areas of spiked and processed standard samples at all concentrations of the calibration line to corresponding peak areas from similarly processed blanks which were spiked post-extraction. The method was validated by analysis of calibration standards at four different concentrations (7, 3.5, 0.35 and 0.07 μg/ml) in six replicates on four different days. Imprecision was given as the relative standard deviation (R.S.D.). Inaccuracy was determined with independently prepared standard solutions and calculated as follows: Inaccuracy (bias) = (observed mean concentration − nominal concentration)/nominal concentration × 100. The lower limit of quantification (LLOQ) was defined as the lowest concentration at which the R.S.D. is ≤15%. Stability data were obtained by monitoring peak areas of standard solutions and processed sam-

ples at three different concentrations (3.5, 0.35 and 0.07 μg/ml) over a period of 24 h at room temperature and at 4 °C. Additionally, standard solutions were measured after each of three freeze–thaw cycles (−80 °C).

2.5. Microdialysis experiments

In vitro microdialysis experiments were performed according to Ståhle et al. by use of the "delivery method" and the "reverse dialysis method" [10]. Commercially available microdialysis probes with membrane length 16 mm and a molecular mass cut-off of 100,000 (CMA/custom made, CMA Microdialysis AB, Solna, Sweden) were used. Three microdialysis probes were placed in test-tubes containing Ringer's solution with 1% (w/v) human albumin (HA) that was gently agitated by a magnet stirrer. The probes were constantly perfused with Ringer's solution containing 1% HA at a flow rate of 1 μl/min by use of a precision pump (CMA/100 microinjection pump; CMA Microdialysis AB). For the "delivery method", caspofungin was added to the test-tubes at appropriate amounts to achieve a final concentration of 0.5 μg/ml in the fluid surrounding the tip of each probe ($C_{surround}$). After manual flushing of the probe followed by equilibration for 30 min at constant perfusion, eight dialysate samples were collected at 30 min intervals from the outlet lines. This procedure was repeated with caspofungin concentrations of 5 and 50 μg/ml in ascending order. Caspofungin concentrations were analyzed in the dialysate samples as described above ($C_{dialysate}$). Microdialysates obtained from the 50 μg/ml test concentration were 1:4 diluted with blank matrix before analysis. The "recovery" was calculated by the formula: Recovery (%) = $C_{dialysate}/C_{surround} \times 100$. (For clarification: Here the term "recovery" means the percentage of drug appearing in the dialysate, in contrast to the analytical term in the HPLC method description). For the "reverse dialysis method", which is based on the fact that drug diffusion occurs in both directions through the membrane, the experimental set-up was equal with the exception that caspofungin was added to the perfusion fluid ($C_{perfusate}$), while the surrounding fluid remained drug-free. The recovery was calculated from the drug disappearance through the membrane by the formula: Recovery (%) = $100 - (C_{dialysate}/C_{perfusate} \times 100)$.

In a second experiment, the system was perfused with Ringer's solution containing 1% (w/v) HA plus 50 μg/ml caspofungin for 120 min prior to the start of sampling. The experimental set-up was modified as follows: caspofungin test concentrations in the fluid surrounding the tip of the probe and the perfusion fluid, respectively, were 50, 15, 5 and 0 μg/ml and were tested in descending order.

3. Results

3.1. Development of the HPLC method

3.1.1. Sample preparation and recovery of the analyte

The analytical recovery of caspofungin from Ringer's solution containing 1% (w/v) HA after precipitation of protein at neutral pH was poor and irreproducible (<30%, data not shown).

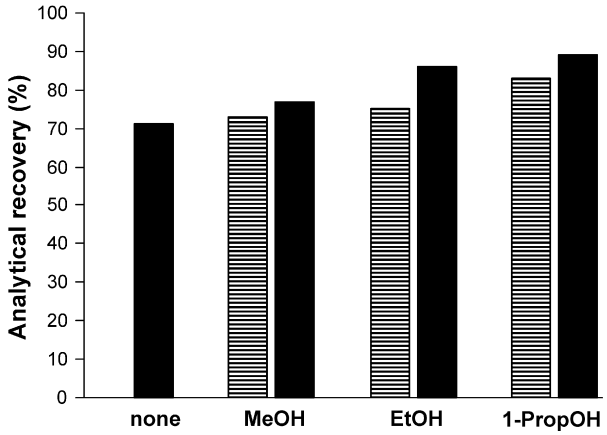

Fig. 1. Optimization of the analytical recovery (highest values shown) of caspofungin from Ringer's solution containing 1% human albumin. Deproteinized sample extracts were modified by the addition of methanol (MeOH), ethanol (EtOH) or 1-propanol (1-PropOH; final content 20% (striped bars) or 33% (solid bars)).

Apparently, considerable amounts of drug were retained in the protein pellet and on the surface of the polypropylene test tube. Acidification of the sample with citric acid to pH 4 prior to the deproteinization step (modification I) leading to stronger ionisation of the caspofungin molecule, increased the recovery of caspofungin to 66–71%. Further improvement was achieved by the addition of organic solvents to the sample extract (modification II), suggesting that adsorbed caspofungin was detached from the wall of the test tube. Highest recovery values were achieved with ethanol and 1-propanol at a final content of 33% (v/v) in the sample extract without any deterioration of the peak shape (Figs. 1 and 2). Following these observations, the sample preparation procedure for further applications included modification I as well as modification II with 33% 1-propanol. Under these conditions, the analytical recovery of caspofungin was 81–89% which is comparable to the values for plasma of an earlier described method using solid-phase extraction [6]. The recovery of the internal standard clarithromycin was 86–92%.

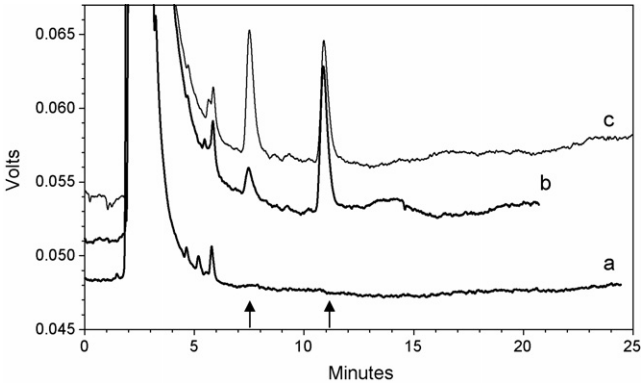

Fig. 2. Typical chromatograms of spiked microdialysates (skeletal muscle) from a male healthy volunteer. Arrows indicate the retention times of caspofungin (7.3 min) and the internal standard clarithromycin (11.1 min). (a) Blank; (b) caspofungin at the LLOQ (0.07 μg/ml); (c) caspofungin 0.35 μg/ml.

3.1.2. Chromatography

Under the HPLC conditions described, the both caspofungin and clarithromycin gave peaks of good symmetry with retention times of 7.3 and 11 min and were well separated from matrix compounds. Blank human microdialysates from soft tissues gave no late-eluting peaks which would make a column wash step necessary. Signals of both substances increased with increasing detector cell potential from +650 to +950 mV. Above 950 mV, the gain of peak area of clarithromycin was negligible and the baseline became increasingly unstable. Therefore, +950 mV was chosen for our assay.

Typical chromatograms of spiked microdialysates from a male healthy volunteer are shown in Fig. 2.

3.1.3. Performance assessment

The calibration curves were linear from 0.07 to 35 μg/ml ($R^2 > 0.998$) with a slope of 0.147 ± 0.014 and an intercept of 0.026 ± 0.017 (mean ± standard deviation; $n = 8$). The LLOQ was 0.07 μg/ml in both Ringer's solution containing 1% (w/v) HA and microdialysate from soft tissue. Blank microdialysates from soft tissue of a total of eight healthy male volunteers showed no interfering peaks.

The use of an internal standard was indispensable. Since derivatives of caspofungin with its unique cyclic lipopeptide structure are not commercially available, we chose the macrolide clarithromycin for the internal standard. As mentioned above, the analytical recovery of clarithromycin and caspofungin was similar. Imprecision and inaccuracy of the method were with <11% in the acceptable range [11]. We conclude that clarithromycin, though not an analogue of caspofungin, is an appropriate internal standard and compensates for the inevitable loss of detector sensitivity during the working-day.

Values of within-day and between-day imprecision and inaccuracy are summarized in Table 1.

3.1.4. Stability

Caspofungin in Ringer's solution containing 1% (w/v) HA, in human microdialysates, and in processed samples was stable at 4 °C and at room temperature for at least 24 h. Likewise, in standard solutions after one freeze–thaw cycle the deviation of measured concentrations from the nominal ones were within the imprecision of the assay. After the second and third freeze–thaw cycle, measured caspofungin concentrations were reduced to 75–83% and 68–72%, respectively, of the nominal concentrations.

Table 1
Within-day and between-day precision (relative standard deviation; R.S.D.) and accuracy (bias)

Caspofungin concentration (μg/ml)		R.S.D. (%)	Bias (%)
Nominal	Measured (mean)		
Within-day ($n = 6$)			
7.000	7.032	1.2	+ 0.4
3.500	3.461	2.4	− 1.1
0.350	0.334	4.4	− 4.6
0.070 (LLOQ)	0.066	9.7	− 5.7
Between-day ($n = 24$)			
7.000	6.983	0.9	− 0.2
3.500	3.444	3.1	− 1.6
0.350	0.321	6.9	− 8.3
0.070	0.065	10.3	− 7.1

3.2. Results of the in vitro microdialysis experiments

Caspofungin (molecular mass 1093) is almost entirely bound to protein, primarily to albumin (molecular mass 66,200), in human and animal plasma [12]. Therefore, in vitro microdialysis of caspofungin was performed with a 100,000 molecular mass cut-off membrane to allow for the diffusion of the whole protein–drug complex. HA was added to the test fluids to prevent net efflux of fluid through the pores of the microdialysis membrane [13], as well as to prevent adsorption of caspofungin to the plastic surfaces of the microdialysis system [6]. Values of recovery of caspofungin (percentage of the drug appearing in the dialysate) obtained in vitro are shown in Fig. 3A–D. With the "delivery method", the recovery increased from below the detection limit to around 18% in dependence of the ascending concentration of caspofungin added to the fluid surrounding the tip of the microdialysis probe (Fig. 3A). Conversely, in the "reverse dialysis" experiment, the recovery (as determined by the loss of caspofungin through the microdialysis membrane) decreased (Fig. 3B). This discrepancy let us assume that the albumin content of the fluids did not prevent adsorption of caspofungin to the material of the microdialysis membrane and/or the tubing. Consequently, recovery values will be falsified by adsorption and do not reflect the diffusion through the membrane as long as the binding-sites are unsaturated. Thus, the experiments were repeated with an initial period of saturation over 2 h with caspofungin at a concentration of 50 μg/ml in Ringer's solution containing 1% (w/v) HA, and the order of the concentrations tested descended over the experiment (0.5 μg/ml was excluded to ensure measurable values). The stable recovery values observed after the saturation procedure (Fig. 3C and D) support the hypothesis of adsorption of caspofungin to inner surfaces of the microdialysis system. An overall recovery range of 11.4–28.5% with relative small variability among the three probes was observed.

To investigate the reversibility of the saturation process, no caspofungin was added to the fluids during the last sampling period. Caspofungin was detected only in the microdialysates collected in the first sampling interval after the concentration change from 5 to 0 μg/ml (0.076–0.081 μg/ml), indicating only minimal removal of adsorbed caspofungin by the aqueous albumin-containing solution.

In an earlier study, albumin at a concentration of 0.25% was reported to be able to overcome adsorption of caspofungin to the surface of polypropylene labware [6]. The semi-permeable membrane of the microdialysis probes used in our experiments are made of polyether sulfone (PES), the tubing of fluorinated ethylene propylene (FEP, Teflon™). The affinity of caspofungin to one or both of these materials appears to be stronger, respectively caused by other physical/chemical mechanisms than that to polypropylene.

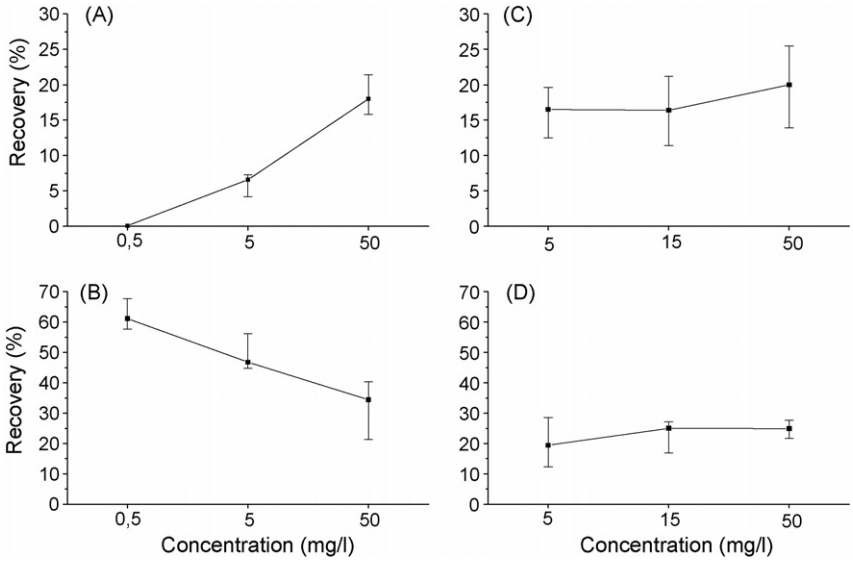

Fig. 3. Percentage of caspofungin (median and range) permeating a 100,000 molecular mass cut-off membrane in microdialysis ("recovery"). (A) Recovery obtained by the "delivery method"; (B) recovery obtained by the "reverse dialysis method"; (C) "delivery method" after pre-saturation of caspofungin binding-sites on membrane and tubing; (D) "reverse dialysis method" after pre-saturation of caspofungin binding-sites (for details see text).

It is planned to use the described pre-saturated system in pharmacokinetic studies in healthy volunteers and animal models. Since recovery values vary among the microdialysis probes, each probe has to be calibrated in vivo using the "reverse dialysis method" [10,14]. To date, no information is available about the concentration of echinocandins in the interstitial space fluid. However, the apparent volume of distribution of caspofungin corresponds to the volume of the extracellular fluid [9,15]. Normally, in this case the concentration curve in plasma provides a good reflection of the interstitial drug concentrations except for tissues with specialized vascular barriers. Plasma profiles of caspofungin in healthy male volunteers showed 12 h-concentrations of well above 1 μg/ml, even after sub-therapeutic doses of 35–40 mg [9]. The in vitro microdialysis recovery of caspofungin in our study was around 15%. Thus, considering the LLOQ of 0.07 μg/ml of the presented HPLC method, we expect measurable in vivo microdialysate concentrations over a sampling period of at least 8 h.

4. Conclusions

Simultaneous chromatography of caspofungin and clarithromycin in human microdialysates on a cyanopropyl silica column with amperometric detection is described. The method exerts good linearity, accuracy and precision. The known affinity of caspofungin to plastic surfaces turned out to be a disruptive factor in both the assay and the in vitro microdialysis experiments. The resolution of the problem in case of the assay was the addition of 1-propanol (33%, v/v) to the sample extract. In case of the microdialysis experiments, saturation of the binding-sites on membrane and/or tubing with caspofungin prior to the start of sampling was possible. The saturation procedure, as described above, is strongly recommended when commercially available microdialysis probes made of PES and FEP, respectively, are used in pharmacokinetic studies on caspofungin.

Taking into account the achieved recovery of caspofungin in in vitro microdialysis, the LLOQ of the described HPLC method and the known plasma pharmacokinetics of caspofungin, measurable in vivo microdialysate concentrations over a sampling period of at least 8 h can be expected.

References

[1] F.A. Bouffard, R.A. Zambias, J.F. Dropinski, J.M. Balkovec, M.L. Hammond, G.K. Abruzzo, K.F. Bartizal, J.A. Marrinan, M.B. Kurtz, D.C. McFadden, K.H. Nollstadt, M.A. Powels, D.M. Schmatz, J. Med. Chem. 37 (1994) 222.
[2] K. Bartizal, C.J. Gill, G.K. Abruzzo, A.M. Flattery, L. Kong, P.M. Scott, J.G. Smith, C.E. Leighton, A. Bouffard, J.F. Dropinski, J. Balkovec, Antimicrob. Agents Chemother. 41 (1997) 2326.
[3] J.A. Stone, X. Xu, G.A. Winchell, P.J. Deutsch, P.G. Pearson, E.M. Migoya, G.C. Mistry, L. Xi, A. Miller, P. Sandhu, R. Singh, F. DeLuna, S.C. Dilzer, K.C. Lasseter, Antimicrob. Agents Chemother. 48 (2004) 815.
[4] C. Joukhadar, M. Müller, Clin. Pharmacokinet. 44 (2005) 895.
[5] C. Buerger, C. Joukhadar, M. Müller, C. Kloft, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 796 (2003) 155.
[6] M. Schwartz, W. Kline, B. Matuszewski, Anal. Chim. Acta 352 (1997) 299.
[7] A.H. Groll, B.M. Gullick, R. Petraitiene, V. Petraitis, M. Candelario, S.C. Piscitelli, T.J. Walsh, Antimicrob. Agents Chemother. 45 (2001) 596.
[8] C.M. Chavez-Eng, M. Schwartz, M.L. Constanzer, B.K. Matuszewski, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 721 (1999) 229.
[9] J.A. Stone, S.D. Holland, P.J. Wickersham, A. Sterrett, M. Schwartz, C. Bonfiglio, M. Hesney, G.A. Winchell, P.J. Deutsch, H. Greenberg, T.L. Hunt, S.A. Waldman, Antimicrob. Agents Chemother. 46 (2002) 739.
[10] L. Ståhle, P. Arner, U. Ungerstedt, Life Sci. 49 (1991) 1853.
[11] FDA, Guidelines for Industry, Available at: http://www.fda.gov/cder/guidance/4252fnl.pdf, 2001.
[12] R. Hadju, R. Thompson, J.G. Sundelof, B.A. Pelak, F.A. Bouffard, J.F. Dropinski, H. Kropp, Antimicrob. Agents Chemother. 41 (1997) 2339.
[13] W.J. Trickler, D.W. Miller, J. Pharm. Sci. 92 (2003) 1419.
[14] M.R. Bouw, M. Hammarlund-Udenaes, Pharm. Res. 15 (1998) 1673.
[15] U. Theuretzbacher, Eur. J. Clin. Microbiol. Infect. Dis. 23 (2004) 805.