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Determination of amphotericin B, liposomal amphotericin B, and amphotericin B colloidal dispersion in plasma by high-performance liquid chromatography

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Abstract

Amphotericin B is a potent polyene antifungal drug for intravenous treatment of severe infections. It is used as amphotericin B-deoxycholate and in order to reduce amphotericin B toxicity as lipid-formulated complex (liposomal or colloidal dispersion). A sensitive and specific analytical method is presented for the separation of lipid-complexed and plasma protein-bound amphotericin B in human heparinized plasma. This separation, which is required for pharmacokinetic studies, is achieved by solid-phase extraction (SPE) via Bond Elut C_{18}. The protein-bound amphotericin B has a higher affinity to the SPE material and is therefore retained, whereas the lipid-complexed amphotericin B is eluted in the first step. The recovery of the SPE was >75% for high concentrations and >95% for low concentrations. Quantification was performed by reversed-phase HPLC using a LiChrosorb-RP-8 column, UV detection (λ=405 nm) and a mixture of acetonitrile–methanol–0.010 *M* NaH_2PO_4 buffer (41:10:49, v/v) as mobile phase. The retention time for amphotericin B under the given conditions was 6.7 min. The calibration curves were found to be linear ($r \geq 0.999$) in two different ranges (5.0–0.50 μg/ml and 0.50–0.005 μg/ml). Intra- and inter-day precision and accuracy fulfilled the international requirements. No interference from other drugs (typical broad medication for intensive-care patients) or common plasma components was detected in >400 samples analyzed.

Keywords: Amphotericin B; Antibiotics

1. Introduction

Amphotericin B is an antifungal antibiotic [1] produced as a fermentation by-product of *Streptomyces nodosus*, a soil actinomycete. The chemical structure (Fig. 1) shows a large lactone ring of 37 carbon atoms in which one side of the ring is a hydrophobic conjugated heptaene chain and the other side with seven hydroxyl groups is hydrophilic. The macrolide ring also contains a six-membered ketalic ring to which the aminosugar mycosamine is bonded through an α-glycosidic linkage. The amphipathic nature of amphotericin B (hydrophobic tail and hydrophilic head) allows it to be complexed with other moieties to alter its pharmacokinetics and pharmacodynamics. Amphotericin B binds to sterols in the cell membranes of both fungal and human

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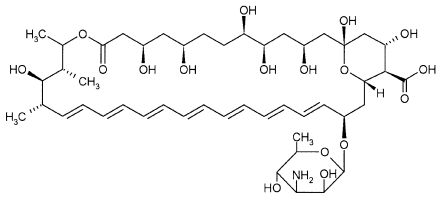

(1R, 3S, 5R, 6R, 9R, 11R, 15s, 16R, 17R, 18S, 19E, 21E, 23E, 25E, 27E, 29E, 31E, 33R, 35S, 36S, 37S - 33 - (3 - amino - 3, 6 - dideoxy - b - D - mannopyranosyloxy) - 1, 3, 5, 6, 9, 11, 17, 37 - octahydroxy - 15, 16, 18 - trimethyl - 13 - oxo - 14, 39 - dioxabicyclo [33.3.1] nonatriaconta - 19, 21, 23, 25, 27, 29, 31 - heptaene - 36 - carboxylicacid

Fig. 1. The chemical structure of amphotericin B with its nomenclature.

cells. Its higher affinity for ergosterol, the sterol found in fungal cell membranes, over cholesterol, the sterol found in human cell membranes, allows amphotericin B to be used systemically [2–5].

Distribution of amphotericin B is believed to be multicompartmental in plasma. Amphotericin B is more than 90% protein-bound, primarily to lipoproteins. Metabolism of amphotericin B is unknown. Despite having many well-known side effects and toxicities [6] and the introduction of several antifungal agents of the imidazole class, amphotericin B remains the drug of choice for many serious systemic fungal infections [7]. Because of side effects and certain dose-limiting toxicities associated with conventional amphotericin B, lipid-based formulations [8] were developed to increase the tolerability of the drug without compromising its antifungal effects. These formulations contain novel drug delivery systems [9–11] designed to increase the therapeutic index. Available amphotericin B lipid formulations include liposomal amphotericin B (AmBisome), and amphotericin B colloidal dispersion (ABCD, Amphotec). Liposomal amphotericin B is amphotericin B intercalated into a unilamelar bilayer liposomal membrane [12], which has a diameter of less than 100 nm and consists of hydrogenated soy phosphatidylcholine, cholesterol, distearoylphosphatidylglycerol, and α-tocopherol [13]. ABCD is amphotericin B complexed in a 1:1 molar ratio with cholesteryl sulfate to form a colloidal dispersion [9].

The interpretation of serum or tissue amphotericin B concentrations is complicated by the fact that many assays to measure amphotericin B concentrations do not distinguish protein-bound amphotericin B from amphotericin B that is lipid-formulated. The pharmacokinetics of amphotericin B following administration of amphotericin B lipid formulations are nonlinear and vary substantially depending on the lipid formulation administered [14]. Varying rates of release of amphotericin B from the carriers may account for the differences between the products. Plasma amphotericin B concentrations attained following administration of lipid-based amphotericin B generally are higher and the volume of distribution is lower than those reported for similar doses of conventional amphotericin B [15]. There are very few clinical studies comparing these formulations, and pharmacokinetic parameters for a given amphotericin B lipid formulation should not be used to predict the pharmacokinetics of any other amphotericin B lipid formulation (i.e., conventional or lipid based). The clinical relevance of pharmacokinetic differences between the various amphotericin B lipid formulations, however, has not been determined.

The present study describes the development of a liquid chromatographic method able to determine simultaneously concentrations of amphotericin B and liposomal amphotericin B or ABCD at relevant concentrations for pharmacokinetic studies and therapeutic monitoring.

2. Experimental section

2.1. Chemical and reagents

Acetonitrile, methanol and water (LiChrosolv; Merck, Darmstadt, Germany) were of HPLC gradient grade. Monobasic sodium phosphate was purchased from Sigma (Vienna, Austria). The Bond Elut C_{18} (100 mg) solid-phase extraction (SPE) cartridges were from Varian (Vösendorf, Austria). Amphotericin B (50 mg per vial) was obtained from Bristol-Myers Squibb (New York, NY, USA), ABCD (100 mg per vial) from Sequus Pharmaceuticals (Menlo Park, CA, USA) and liposomal amphotericin B (100 mg per vial) from NeXstar Pharmaceutical (Boulder, CO, USA).

2.2. Instrumentation

High-performance liquid chromatography (HPLC) was performed with a modular system HP 1100 (Hewlett-Packard, Wilmington, DE, USA) consisting of a variable-wavelength UV–visible detector (Model G1314A), an autosampler (Model G1313A), a quaternary pump (Model G1311A) and an on-line degasser (Model G1322A). Output data from the detector were integrated via Hewlett-Packard software (HP ChemStation Rev. A.06.03, Palo Alto, CA, USA).

A LiChrosorb RP-8 column (200×4.6 mm; 5 μm; Agilent Technologies, Vienna, Austria) connected to a Zorbax SB-C_8 (12.5×4.6 mm; 5 μm; Agilent Technologies) precolumn was used at ambient temperature. The mobile phase, delivered at a flow-rate of 0.7 ml/min, consisted of a mixture of acetonitrile–methanol–10 m*M* NaH_2PO_4 buffer (41:10:49; v/v). The injection volume was set to 100 μl.

2.3. Preparation of external standards

The stock solutions with concentrations of 5.0 mg/ml were prepared by diluting each drug in HPLC gradient grade water. They were stored at −75°C. The stock solutions were added to 500 μl heparinized plasma (supplied by healthy volunteers) to provide concentrations of 5.0, 1.0, 0.5, 0.05, 0.025, 0.005 μg/ml of amphotericin B and were treated as described below.

2.4. Preparation of plasma samples

Blood samples from amphotericin B-, ABCD- or liposomal amphotericin B-treated patients were collected in heparin-containing test tubes (4 ml S-monovettes, lithium-heparin, 15 I.E. heparin/ml blood; Sarstedt, Nümbrecht, Germany), and plasma was separated at once with centrifugation at 350 *g* for 10 min. The plasma samples were then stored at −75°C until analysed.

The C_{18} SPE column was prerinsed with 1.0 ml methanol followed by 1.0 ml of water. Then the column was mounted to a new collecting tube. A 500-μl volume of heparinized plasma was pipetted onto the SPE columns. The remaining plasma was washed from the column with 500 μl of water. For a later analysis of the complexed amphotericin B the collecting tube (fraction A) was removed. The SPE C_{18} cartridge was washed with 500 μl of 45% aqueous methanol (for removing heparin and other maybe interfering compounds) with the help of centrifugation at 350 *g* for 2 min. The analyte was eluted from the column into a HPLC vial with 300 μl of 60% aqueous acetonitrile (fraction B) with the aid of centrifugation at 350 *g* for 2 min. Then 150 μl of water was added to the HPLC vial and vortex-mixed for a few seconds. The analysis of this HPLC sample gave the amount of uncomplexed, protein-bound amphotericin B (fraction B) in plasma.

Another SPE C_{18} column was prerinsed with 1.0 ml methanol followed by 1.0 ml of water. Fraction A was mixed with 1.0 ml of methanol (for breaking the lipid-formulated amphotericin B complex and for precipitating proteins), vortex-mixed for a few seconds and centrifuged at 7000 *g* for 15 min. The clear supernatant was removed completely and pipetted onto the cartridge. Subsequently the column was washed with 500 μl of 45% aqueous methanol with the aid of centrifugation at 350 *g* for 2 min. Amphotericin B was eluted from the column with 300 μl of 60% aqueous acetonitrile into a HPLC vial. A 150-μl volume of water was added and vortex-mixed for a few seconds. The analysis of this HPLC sample gave the amount of complexed amphotericin B (fraction A) in plasma.

2.5. Validation procedure

2.5.1. Linearity

The calibration curves were obtained by plotting the peak areas as a function of the respective concentrations (0.005, 0.025, 0.050, 0.50, 1.0, 5.0 μl/ml) for each analyte. The slope, intercept and correlation *r* of each calibration curve together with the mean, standard deviation (SD), precision relative standard deviation (RSD) and mean relative error (MRE) were determined. The MRE was calculated as follows: MRE=(mean measured value−theoretical value)/theoretical value. The minimum acceptable coefficient to establish linearity was set at 0.95 a priori.

2.5.2. Intra-day and inter-day precision

The intra-day repeatability of the method was determined by analysis of three concentrations (0.005, 0.5, 5.0 μg/ml) on the same day ($n=5$). Inter-day reproducibility was assessed on 5 different days with three concentrations (0.005, 0.1, 5.0 μg/ml).

2.5.3. Recovery

The SPE recovery of amphotericin B from human heparinized plasma was determined for high (5.0 μg/ml, 2.0 μg/ml), medium (0.050 μg/ml) and low (0.005 μg/ml) concentrations by comparison of the results ($n=5$) from SPE and from unextracted pure amphotericin B directly injected into the chromatographic system.

2.5.4. Separation of protein-bound and lipid-formulated amphotericin B via SPE

ABCD (0.50 μg/ml), or liposomal amphotericin B (0.50 μg/ml) was mixed with three different concentrations of amphotericin B (1.00, 0.50, 0.15 μg/ml) and the amount of complexed and uncomplexed, protein-bound amphotericin B was measured. The experiments were repeated three times on 3 consecutive days.

2.6. Pharmacokinetics

Plasma concentration–time curves after injection of amphotericin B, ABCD or liposomal amphotericin B were evaluated with a noncompartmental model using Kinetica-2000 (InnaPhase, Champs-sur-Marne, France). The area under the concentration–time curve from time zero to time n of the last sample (AUC_{0-n}) was computed using the log linear method, trapezoidal when $C_n > C_{n-1}$. AUC_{0-n} is defined as AUC_{0-n} from $t=0$ to t_{last} (AUC_{0-n} is $AUC_{0-24\ h}$).

3. Results and discussion

3.1. Solid-phase extraction recovery

After comparison of amphotericin B levels of extracted plasma standards and pure, unextracted water standards, the SPE recovery was found to be >75% for high concentrations and >95% for medium and low concentrations (Table 1).

Table 1
Validation for protein-bound, lipid-unformulated amphotericin B

Concentration (μg/ml)	% Recovery±SD (mean)	RSD (%)	
Recovery of the solid-phase extraction column ($n=5$)			
5.000	76.2±3.5	4.6	
2.000	75.2±1.5	2.0	
0.050	95.9±2.6	2.7	
0.005	97.9±1.1	1.1	
Theoretical concentration (μg/ml)	Concentration found (mean) (μg/ml)		MRE (%)
Intra-day precision and accuracy ($n=5$)			
5.000	4.660	3.1	−6.8
0.500	0.509	1.8	1.8
0.005	0.005	1.6	−0.6
Inter-day precision and accuracy ($n=5$)			
5.000	4.832	6.0	−3.4
0.100	0.106	4.7	6.0
0.005	0.005	4.5	−2.8

RSD and MRE were calculated from the average value of duplicates.

3.2. Validation assay precision

In the described chromatographic method the retention time for amphotericin B in heparinized plasma was 6.7 min. The standard curves showed linearity over the two selected concentration ranges (5.0–0.5 μg/ml and 0.5–0.005 μg/ml). The divided calibration curve is a result of the two different SPE recoveries. The intra- and inter-day precision for low (0.005 μg/ml), medium (0.1 μg/ml) and high (5.0 μg/ml) concentrations are summarized in Table 1. The lowest concentration of material in samples used for validation was 5 ng/ml. No interference from other drugs or common plasma compounds (Fig. 2) was detected in >400 samples analyzed. The samples were collected from intensive-care patients with broad medication such as antibiotics (vancomycine, teicoplanin, β-lactame-anitbiotics including piperazillin, new generation cephalosporine and carbapenemes), gancyclovir, acyclovir and cyclosporin A, prograf, proton-pump-inhibitors (omeprazole),

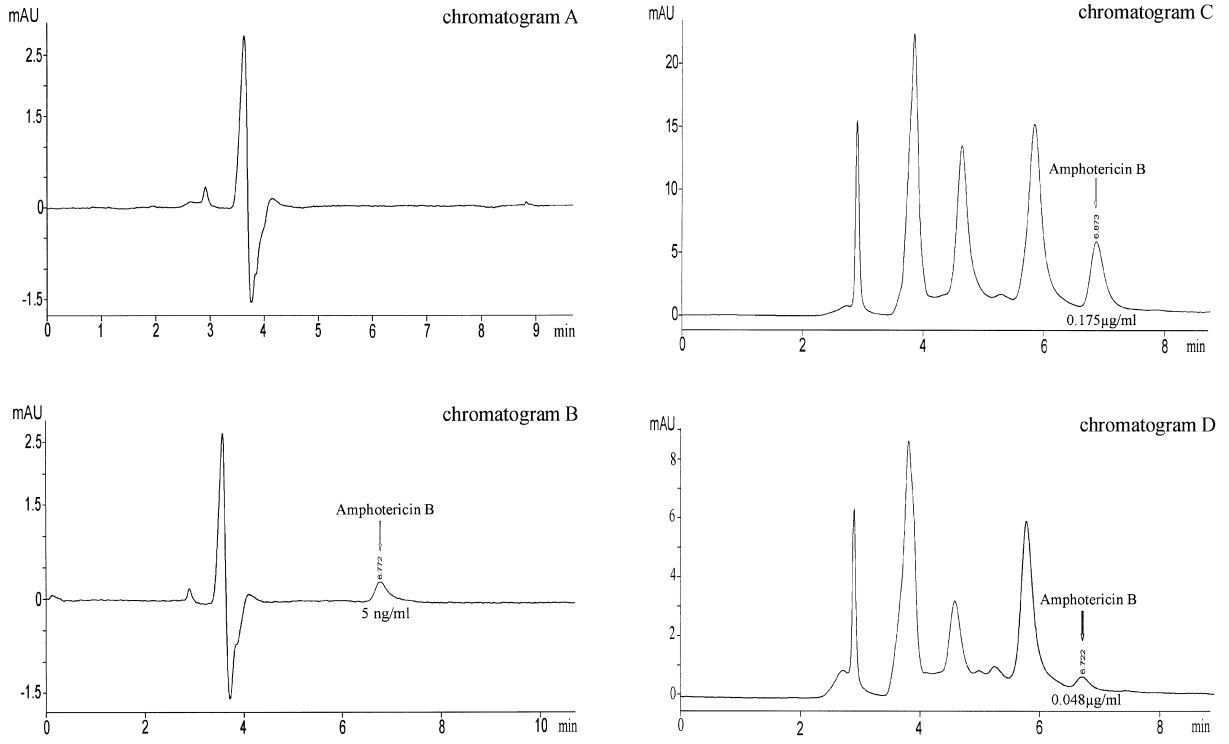

Fig. 2. Comparison of HPLC chromatograms of blank human plasma (chromatogram A) and human plasma containing 0.005 μg/ml amphotericin B (chromatogram B). A real patient sample is given in chromatogram C (0.175 μg/ml protein-bound amphotericin B) and chromatogram D (0.048 μg/ml complexed amphotericin B).

ranitidine, low-molecular-weight heparins, and vasopressor agents (noradrenaline).

3.3. Validation of the SPE separation

After intravenous injection of lipid-formulated amphotericin B in humans, the active substance can exist in two forms (I) as colloidal dispersion/liposomes or (II) plasma-dissolved, mostly associated with lipoprotein. Amphotericin B cannot exist as free entity in plasma. We have found a way to separate the two forms. The lipid-formulated substance is not retained on a C_{18} SPE cartridge, while the protein-bound amphotericin B is retained there. In two sets of experiments with ABCD and liposomal amphotericin B, we have shown the correct separation of the two forms via Bond Elut columns with RSD≤ 11.2% (Table 2).

3.4. Application to pharmacokinetic studies of intensive-care patients

The method was further validated by comparing our pharmacokinetic data obtained in a persistently febrile neutropenic patient with normal renal function on day 2 of empiric antifungal treatment receiving intravenous infusions of 60 mg (1 mg/kg body mass) of conventional amphotericin B daily with the published pharmacokinetic literature. Very good agreement between previously published data and pharmacokinetic results using the method of amphotericin B measurement described here was observed, with peak level of 1.74 μg/ml (t_{max}=0.5 h) and, after a rapid initial fall, plateau level of 0.81 μg/ml [15] (Fig. 3).

The method described appears suitable for the simultaneous determination of the protein-bound and lipid-complexed fractions of liposomal amphotericin

Table 2
Separation of amphotericin B (A) and lipid-formulated amphotericin B (B) via SPE

	1.00 μg/ml A +0.50 μg/ml B	0.50 μg/ml A +0.50 μg/ml B	0.15 μg/ml A +0.50 μg/ml B
Amphotec			
Mean (A)±SD	0.952±0.034	0.504±0.005	0.143±0.005
Mean (B)±SD	0.483±0.024	0.429±0.035	0.431±0.045
RSD (%) (A)	3.6	0.9	3.3
RSD (%) (B)	4.9	8.2	10.5
AmBisome			
Mean (A)±SD	0.932±0.028	0.497±0.006	0.143±0.015
Mean (B)±SD	0.463±0.052	0.448±0.049	0.441±0.029
RSD (%) (A)	3.0	1.1	10.8
RSD (%) (B)	11.3	10.8	6.5

B or ABCD in plasma. In a persistently febrile neutropenic patient with impaired renal function of plasma creatinine levels around 2.0 mg/dl and concomitant potentially nephrotoxic medication, 300 mg of liposomal amphotericin B (4.5 mg/kg body mass) were administered daily over a period of 4 h; serial plasma samples were obtained on day 11 of treatment (Fig. 4). Peak plasma level of total amphotericin B which is the sum of liposomal and protein-bound fraction was 4.09 μg/ml (t_{max}=4 h), and the AUC_{0-n} was 36.53 mg h/l, which was similar to what was reported previously for total amphotericin B plasma levels [15]. Amphotericin B was circulating in both a protein-bound and a liposomal form, the clearances of which differed. Data support the concept of different compartmental distribution of the two forms of amphotericin B.

In a third patient, 300 mg of ABCD (4.0 mg/kg body mass) was administered daily over a period of 4 h; serial plasma samples were obtained on day 21 of treatment (Fig. 5). Here we found a lower total peak plasma level with 1.21 μg/ml (t_{max}=4 h), and

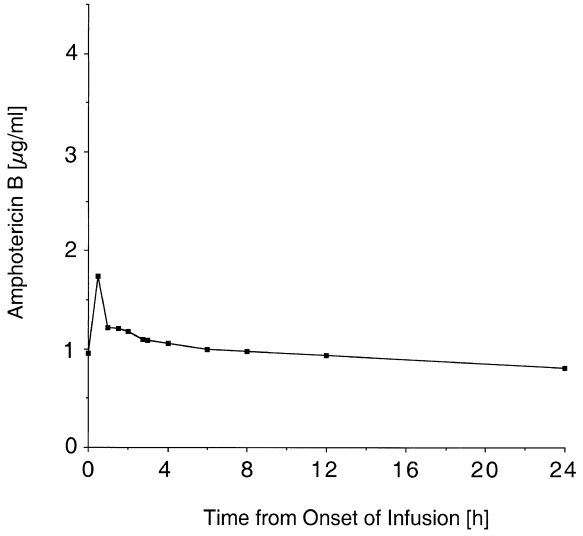

Fig. 3. Representative plasma concentration–time curve for an intensive-care patient after 60 mg of amphotericin B (d_2=2nd day of administration), where AmB is used as abbreviation for amphotericin B.

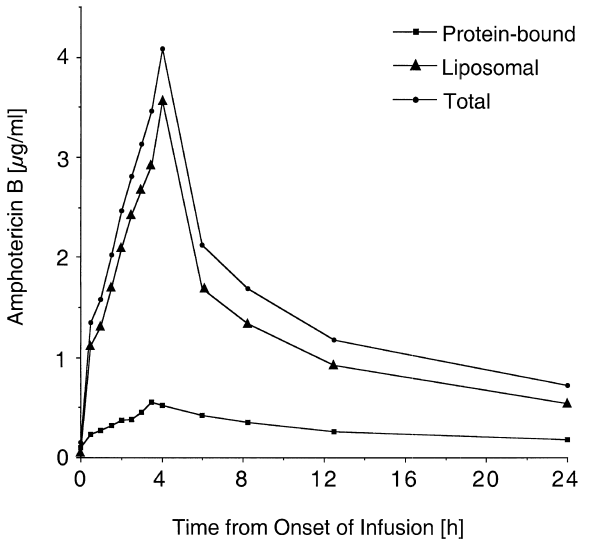

Fig. 4. Representative plasma concentration–time curve for an intensive-care patient after 300 mg liposomal amphotericin B (d_{11}=11th day of administration).

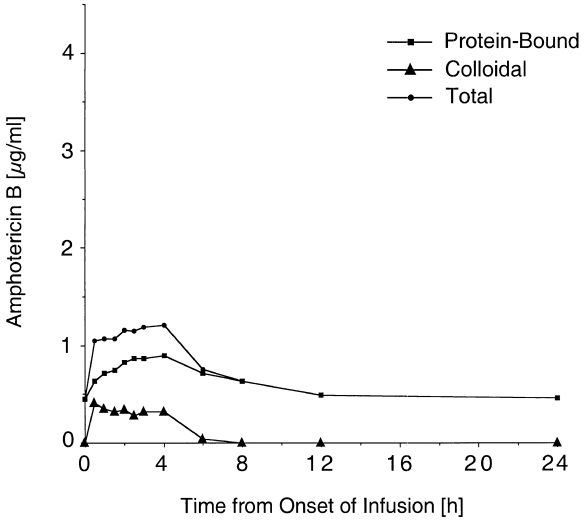

Fig. 5. Representative plasma concentration–time curve for an intensive-care patient after 300 mg ABCD (d_{21}=21st day of administration).

AUC_{0-n} was 12.85 mg h/l. We have determined lower colloidal share, which rapidly declined to zero. The protein-bound form showed higher values, which fall more gradually to plateau and even at 0.5 μg/ml, which is within the therapeutic range.

In conclusion, the data presented here provide an analytical means to determine the lipid-formulated amphotericin B concentrations in plasma and may allow investigation into their pharmcokinetics with respect to both efficiency and side effects related to amphotericin B therapy.

References

[1] K.M. Abu-Salah, Br. J. Biomed. Sci. 53 (1996) 122.
[2] V. Joly, R. Farinotti, L. Saint-Julien, M. Cheron, C. Carbon, P. Yeni, Antimicrob. Agents Chemother. 38 (1994) 177.
[3] I. Gruda, N. Dussault, Biochem. Cell Biol. 66 (1988) 177.
[4] A.S. Janoff, L.T. Boni, M.C. Popescu, S.R. Minchey, P.R. Cullis, T.D. Madden, T. Taraschi, S.M. Gruner, E. Shyamsunder, M.W. Tate, Proc. Natl. Acad. Sci. USA 85 (1988) 6122.
[5] J. Bolard, P. Legrand, F. Heitz, B. Cybulska, Biochemistry 30 (1991) 5707.
[6] V. Fanos, L. Cataldi, J. Chemother. 12 (2000) 463.
[7] S.H. Khoo, J. Bond, D.W. Denning, J. Antimicrob. Chemother. 33 (1994) 203.
[8] A. Wong-Beringer, R.A. Jacobs, B.J. Guglielmo, Clin. Infect. Dis. 27 (1998) 603.
[9] M.J. Ostro, P.R. Cullis, Am. J. Hosp. Pharm. 46 (1989) 1576.
[10] G. Lopez-Berestein, Ann. N.Y. Acad. Sci. 544 (1988) 590.
[11] G. Lopez-Berestein, Antimicrob. Agents Chemother. 31 (1987) 675.
[12] O. Ringden, J. Tollemar, G. Tyden, Lancet 339 (1992) 374.
[13] G. Gregoriadis, C. Davis, Biochem. Biophys. Res. Commun. 89 (1979) 1287.
[14] R. Patel, Mayo Clin. Proc. 73 (1998) 1205.
[15] V. Heinemann, D. Bosse, U. Jehn, B. Kahny, K. Wachholz, A. Debus, P. Scholz, H.J. Kolb, W. Wilmanns, Antimicrob. Agents Chemother. 41 (1997) 1275.