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DETERMINATION OF AMPHOTERICIN B IN RAT BLOOD PLASMA. COMPARISON OF ANTIFUNGAL ACTIVITY AND PHARMACOKINETICS PROFILE OF CONVENTIONAL AMPHOTERICIN B AND ITS CONJUGATE WITH POLY(ETHYLENE GLYCOL)

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New intravenous non-covalent conjugate of amphotericin B (AMB)-poly(ethylene glycol)(PEG) (M = 10 000 g mol^{-1}) is described. This well water soluble conjugate contains 4 % (w/w) free AMB. A simple and sensitive HPLC-UV method was developed and validated for the quantification of free amphotericin B in rat plasma. A simple mobile phase consisting of 10 mM ammonium acetate (pH 3.60) and acetonitrile (56:44, v/v) was pumped at a flow rate of 0.3 ml min^{-1} through a reverse phase column maintained at 30 °C. Rifampicin was used as an internal standard (IS). Rapid sample preparation involved the addition of 500 μl acetonitrile-methanol mixture and 10 μl IS to 200 μl plasma to precipitate plasma

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proteins. Supernatant was evaporated to dryness under the stream of nitrogen and dissolved in 125 μl of the mobile phase and injected onto column. The procedures were validated within the linear concentration range from 0.06 to 2.5 μg ml^{-1} with good reproducibility and linear response (r^2 = 0.9988). The method described is cost-effective and has necessary accuracy and precision for the rapid quantitative determination of AMB in rat plasma. A two-compartment model described the plasma drug concentration-time profiles.

Introduction

Amphotericin B (AMB) is a polyene macrocyclic compound, which was isolated in the second half of the 20th century from *Streptopmyces nodus* [1]. This membrane-active antifungal antibiotic belongs among life-saving drugs in treatment of systemic fungal infections [2] which are a major cause of morbidity and mortality in immunodeficient patients (AIDS), in transplant recipients or tumor patients undergoing immunosuppressive chemotherapy [3,4].

The mechanism of its biological action is not fully understood yet [5]. It is generally known that AMB action results from the selective interaction with ergosterol, which is a component of fungal cell membranes. AMB forms with ergosterol barrel-stave channels which allow escaping of K^+ ions and small molecules. This disruption of cell membrane leads to death of the fungal cell [6,7]. However, clinical application of AMB is limited by its poor solubility and potential serious organ toxicities, particularly nephrotoxicity, which mainly appear to be dose-dependent [8]. With the aim of increasing the therapeutic index of AMB, a number of formulations and conjugates have been adopted. These formulations enable solubilisation of AMB in aqueous media and ensure continuous dosage usually accompanied by lowered toxicity [9]. The oldest formulation of AMB, which is used for intravenous administration, is a colloidal dispersion of AMB with sodium deoxycholate (Fungizone) [10,11]. Fungizone (often called conventional formulation) exhibits broad spectrum of antifungal activity but unfortunately it is often ineffective for immunocompromised patients [12]. However, over 30 % of patients treated with Fungizone show signs of serious kidney disorders, in some studies [13] this number is close to 50 %. Recently, nanospheres, liposomes and microspheres were used to create new formulations of AMB. Among clinically utilized formulation belong AmBisome, Abelcet and Amphotec. These formulations ensure smoother release of AMB accompanied by its restricted distribution in the kidneys, thereby lowering its nephrotoxicity [9]. Other methods for increase in solubility and therapeutic index of AMB were also studied. Thus, Domb *et al*. conjugated AMB to a water-soluble polysaccharide arabinogalactan, which is reported to overcome many limitations of AMB such as its insolubility and toxicity [14,15]. Over several years, research work using poly(ethylene

glycol)s (PEG)s for modification of properties of AMB has been described. These polymers are non-toxic and well soluble in aqueous solution [16]. PEGs are known to be non-biodegradable and readily excretable after administration into living organisms. These compounds also exhibit good pharmacokinetic and biodistribution behaviour [17] and show low accumulation in reticuloendothelial system (RES) organs, liver and spleen. In research paper [18], synthesis of a conjugate, in which 50 mol % AMB is bound by covalent carbamate link to methoxy(polyethylene glycol) (mPEG) (M = 5000 g mol^{-1}), was described, the remaining AMB is linked non-covalently. The covalent part of the conjugate operates as a carrier of free AMB. The *in vitro* test had shown similar effect as that of conventional AMB. In the case of pH-sensitive conjugate [19], the linkage of AMB and PEG (M = 5000, 10 000, 20 000 g mol^{-1}) is formed with the help of imino bond. These conjugates are relatively stable in phosphate buffer at pH = 7.4 (37 °C) but in acidic phosphate buffer at pH = 5.5 (37 °C), hydrolysis of imino bond causes unlocking of free AMB [20]. Analogous conjugate was prepared by reaction of amphotericin B with polyethylene Glycol-b-poly(L-lysine). Twelve molecules of AMB were associated with a copolymer block *via* pH-sensitive imino bond again. The half-life was two minutes and the obtained LD_{50} value was 45 mg kg^{-1} (determined *in vivo* — mice) [20]. The above conjugates were created with the aid of linear poly(ethylene glycol) in contrast to the AMB-star poly(ethylene glycol) conjugate [21] and the nystatin-star poly(ethylene glycol) conjugate [22]. These conjugates contain a β-D-glucopyranoside molecular switch sensitive to β-glucosidases (E.C.3.2.1.21), which are specifically present in the enzyme outfit of fungal pathogens.

The aim of this work was characterisation and identification of pharmacokinetic properties and also efficacy determination of a new amphotericin B conjugate with poly(ethylene glycol) (M = 10 000 g mol^{-1}). A new HPLC method coupled with a UV/VIS detector was developed and validated.

Materials and Methods

Chemicals and Reagents

Amphotericin B (> 80 %), ammonium acetate and acetic acid (99 %) were purchased from Sigma-Aldrich (Darmstadt, Germany) and rifampicin (Rif) (> 97 %) as the internal standard was supplied by Fluka (Darmstadt, Germany). HPLC grade acetonitrile (ACN) and methanol (MeOH) Merck (Darmstadt, Germany) were used during the analysis. Amphotericin B Squibb (Fungizone), purchased from Bristol-Myers Squib (Epernon, France), was used for pharmacokinetic distribution studies in rats. Chemicals and solvents used for synthesis of conjugate were obtained from Fluka or Aldrich.

Stock solutions of AMB (0.4 mg ml^{-1}) and rifampicin (0.08 mg ml^{-1}) were

prepared by dissolution in methanol and stored in freezer at –20 ºC; under these conditions, the drug was found to be stable for at least one month. The AMB and Rif working solutions were prepared by dilution with methanol (0.04, 0.004, 0.0004, 0.00004 mg ml^{-1} for AMB, and 0.008 mg ml^{-1} for Rif).

Synthesis of PEG-2AMB Conjugate

Poly(ethylene glycol)-α,ω-bis-(4-nitrophenyl carbonate) (1 g, 0.1 mmol) and 4-dimethylaminopyridine (13 mg, 0.1 mmol) were dissolved in dry dimethylformamide (6 ml). The solution was mixed (in a stirrer, protected from light and under inert argon atmosphere) with an amphotericin B suspension (184 mg, 0.2 mmol) in dimethylformamide (2 ml). AMB passed gradually into the solution during the stirring of reaction mixture. The whole process proceeded at room temperature. After 21 days, a solution of ethanolamine (6 mg, 0.1 mmol) in dimethylformamide (0.5 ml) was added to the mixture. The reaction mixture was filtrated, and the pure solution was poured into the ether (500 ml). The obtained product was filtered and dried with diethyl ether (3×40 ml). After that the dried crude conjugate was dissolved in methanol (5 ml) and precipitated with diethyl ether (500 ml) again. The yield was 1.1 g (80 %) of pale yellow coloured product after another filtration and drying under vacuum. The UV-VIS spectrum of PEG-2AMB (in methanol) shows peaks at 410, 386, 367, 346 nm. The IR (KBr) spectrum of PEG-2AMB shows peaks at 3439, 2945, 2887, 2741, 1710, 1467, 1360, 1343, 1280, 1201, 1161, 963, 845, 532 cm^{-1}. ^{1}H NMR and GPC were used for characterization of PEG-2AMB. The PEG-2AMB preparation scheme is depicted in Fig. 1.

Apparatus and Chromatographic Conditions

The HPLC analysis was performed using a Shimadzu (Kyoto, Japan) system consisting of two LC-10ADvp pumps, a SPD-M10Avp UV/VIS detector, a DGU-14A degasser, a CTO-10ASvp column oven and a SLC-10Avp system controller. LC/MS solution software was used for data collection and aquisition. The used analytical column was 5 μm particle size LiChrospher® 125 RP-18e (Merck, Darmstadt, Germany) protected by a compatible guard column Phenomenex C_{18} (4×3.0 mm I.D.) (Phenomenex, Torrance, USA). The mobile phase consisted of 10 mM ammonium acetate pH 3.60 (adjusted with acetic acid) and acetonitrile (56:44, v/v). The flow rate was 0.3 ml min^{-1}. The injected sample volume was 20 μl. The wavelengths for detection were 409 nm for AMB and 254 nm for rifampicin as internal standard.

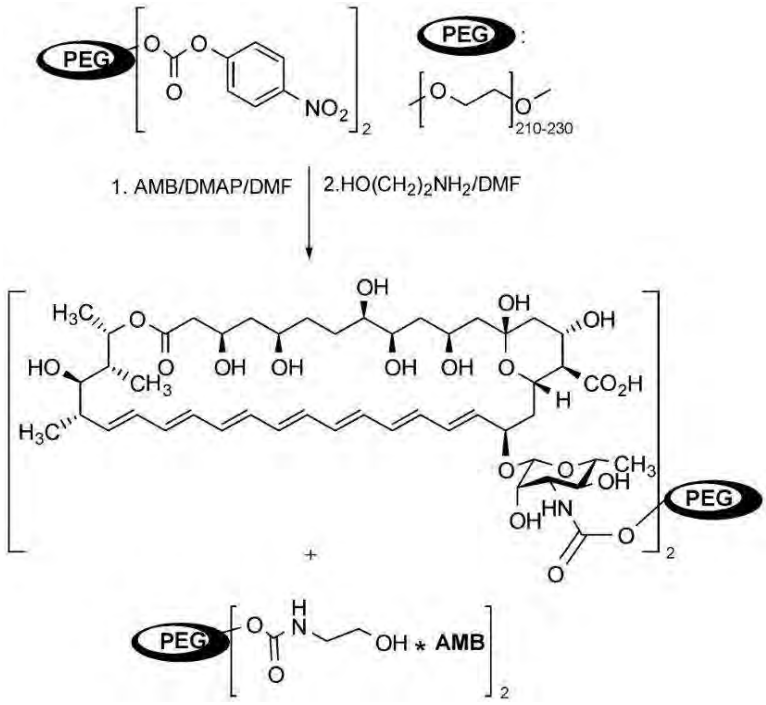

Fig. 1 PEG-2AMB conjugate preparation scheme and its final structure

Method Validation

A seven-point calibration curve was established between 0.06 and 2.5 μg ml^{-1} of AMB in plasma. Therefore, blank plasma was spiked with the appropriate amount of AMB and rifampicin (internal standard). Each measurement was made in triplicate and the average values were used to the calibration curve evaluation. The method precision and accuracy were tested at three levels (0.06, 0.3 and 2.5 μg ml^{-1}). Precision was evaluated as the percentage relative standard deviation (% *RSD*) for both repeatability (within-day) and reproducibility (between-day and different analysts) for a selected compound and level. The maximum allowed tolerance for reproducibility and repeatability can be calculated from the Horowitz equation

$$RSD_{\max} = 2^{(1-0.5\log C)}$$

where C is concentration in μg ml$^{-1}\times10^{-6}$. These values are 2/3 $RSD_{\max}$ and $RSD_{\max}$, respectively.

The accuracy (expressed as mean error in percents) was defined as the difference between the calculated amount and specified amount of AMB. The limit of quantification (*LOQ*) of the method was defined as the lowest concentration where acceptable reproducibility and accuracy could be guaranteed.

In vivo Animal Studies

The study was performed with wistar male rats from BioTest Konarovice. The study protocol was approved by the ethical committee of Faculty of Pharmacy in Hradec Kralové, Charles University in Prague. Wistar male rats were purchased from BioTest (Konárovice, The Czech Republic). Two groups of rats were tested. The first group of rats was administered with conventional amphotericin B (Fungizone®) and the second with PEG-2AMB. First, pentobarbital was administered and the drug was applied to the *vena saphnena*. The blood was collected at the following times: 5, 10, 30, 60, 120 and 180 min *via* cardiac puncture. Blood samples were put into tubes Tapval (containing EDTA) and plasma was separed by centrifugation. Finally, plasma samples were frozen at –20 °C and stored till analysis.

200 μl plasma and 10 μl internal standard (rifampicin, 0.008 μg ml^{-1}) were mixed and then deproteinised. 500 μl acetonitrile-methanol mixture (1:1; v/v) was added to plasma samples, shaken for 120 s and centrifuged at 14 000 rpm (rcf 16 000 g) for 3 min. The supernatant was put into a glass conical tube and evaporated to dryness under the nitrogen stream (37 ºC). The residues were dissolved in 125 μl mobile phase in the case of rats injected with Amphotericin B Squibb. In the case of the rats to which the conjugate PEG-2AMB was applied, the residues were dissolved in the same volume of the mobile phase, but it was necessary to dilute the sample, because the concentration was above the highest calibration point.

Pharmacokinetic Analysis

The AMB concentration in blood was evaluated as the average value obtained from experiments, which were performed on six animals at each time point. The plasma concentration-time profile of AMB was determined using a two-exponential equation

$$C_t = A_0 e^{(\alpha t)} + B_0 e^{(\beta t)}$$

where C_t is concentration dependent on time, A and B are intersections with the Y axis, α and β are hybrid constants derived from rate constant. The data were processed using non-linear regression in the GraphPad Prism 5.0 program from GraphPad Software, Inc. (La Jolla, San Diego, CA, USA). Basic pharmacokinetics parameters such as area under curve (AUC) and the half-life ($t_{1/2}$) were calculated according to standard procedures using the coefficients and the exponents of the fitted function.

Mice Treatment with PEG-2AMB and with Conventional Amphotericin B

The potential therapeutic effect of PEG-2AMB conjugate was evaluated according to its comparison with conventional amphotericin B (Amphotericin B Squibb). Experimental systemic *candidiasis* was used as a model infection [23]. Cyclofosfamid (200 mg kg^{-1} intra peritioneal — *ip*) was administered to the ICR (Imprinting Control Region) mice and 4 days later, the mice were infected (intra venous — *iv*) with 1×10^5 CFU (colony forming units) *Candida albicans* H3988. Tested substances application started 48 hours after inoculation of *Candida* and it was repeated after 24 hours and 5 dosages were administered in total. The mice were observed and deaths were registered. The experiment was finished by euthanasia of mice 31 days after administration of *Candida* and then the autopsy was performed. Macroscopic examination of pathological changes (wrinkling parenchymatous organs, abscesses, nodes) was done and the organs were fixed for histological tests.

Results and Discussion

HPLC Method Validation

At first, the HPLC method [24] was optimized. The obtained retention times for AMB and Rif were 8.9 min and 12.2 min, respectively. The peak area ratio showed a linear dependency on the drug concentration in plasma within the studied range from 0.06 to 2.5 μg ml^{-1}. The obtained calibration curve was $y = 0.1698x + 0.01198$ (x = concentration of AMB, y = peak area ratio AMB/Rif). The correlation coefficient obtained from the calibration curve was 0.9988.

Table I Accuracy, repeatability, reproducibility and tolerance limits of the HPLC method at three concentration levels including the lowest, middle and the highest point of the amphotericin B calibration curve

Concentration μg ml^{-1}	Accuracy*) %	Repeatability**) %	Reproducibility*) %	RSD_{max} %	$2/3RSD_{max}$ %
0.06	9.8	9.0	13.0	24.4	16.3
0.3	–9.7	6.7	6.6	19.2	12.8
2.5	–4.5	2.9	2.8	13.9	9.3

*) $n = 18$
**) $n = 6$

As one can see in Table I, the obtained values had never exceeded those for both repeatability and reproducibility limits (15 %) at the concentration levels of 0.06, 0.3 and 2.5 μg ml^{-1}. The deviation between the measured concentration and the theoretical concentration (accuracy) of amphotericin B was below the acceptable 15 % threshold for all the levels in the range of calibration curve [25].

No interferences in retention times of AMB and Rif were detected during the analysis of different blank control plasma samples. The limit of quantification (*LOQ*) of the method was evaluated as 0.06 μg ml^{-1} for AMB.

Pharmacokinetic Profile of Formulations in Blood

The pharmacokinetic profiles of AMB in blood after *iv* administration of conventional amphotericin B and PEG-2AMB conjugate are presented in Figs 2 and 3. Those plasma concentration vs. time curves of the drugs are evaluated by biexponetial equations

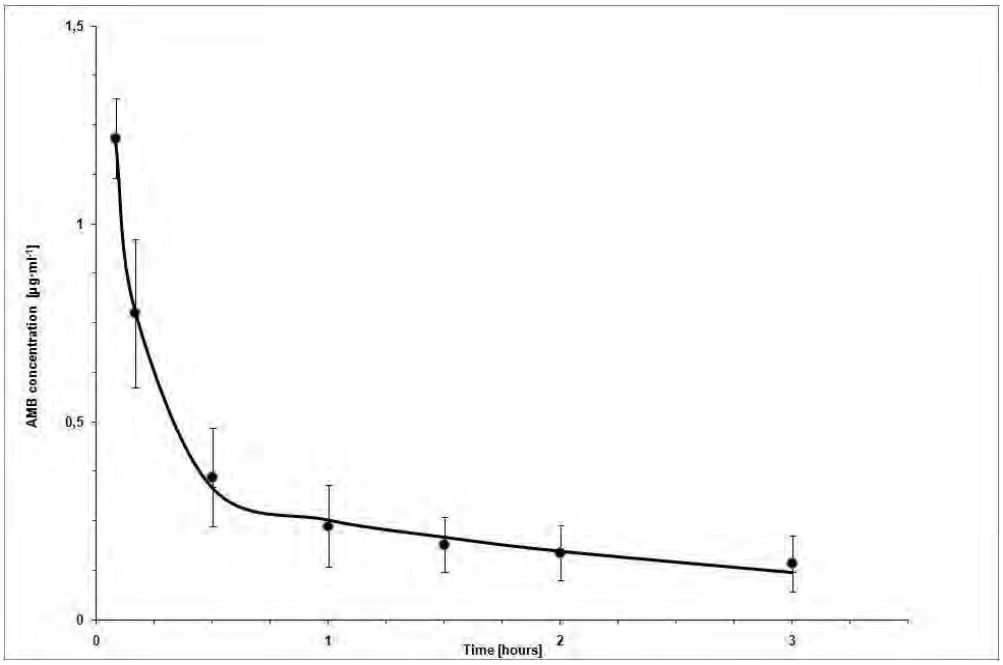

Fig. 2 Dependence of AMB plasma concentration vs. time after administration of single *iv* dose of conventional AMB

$$C = 1.6271\,e^{-8.009t} + 0.3655\,e^{-0.3718t}$$

for AMB and

$$C = 26.60\,e^{-12.63t} + 9.807\,e^{-1.222t}$$

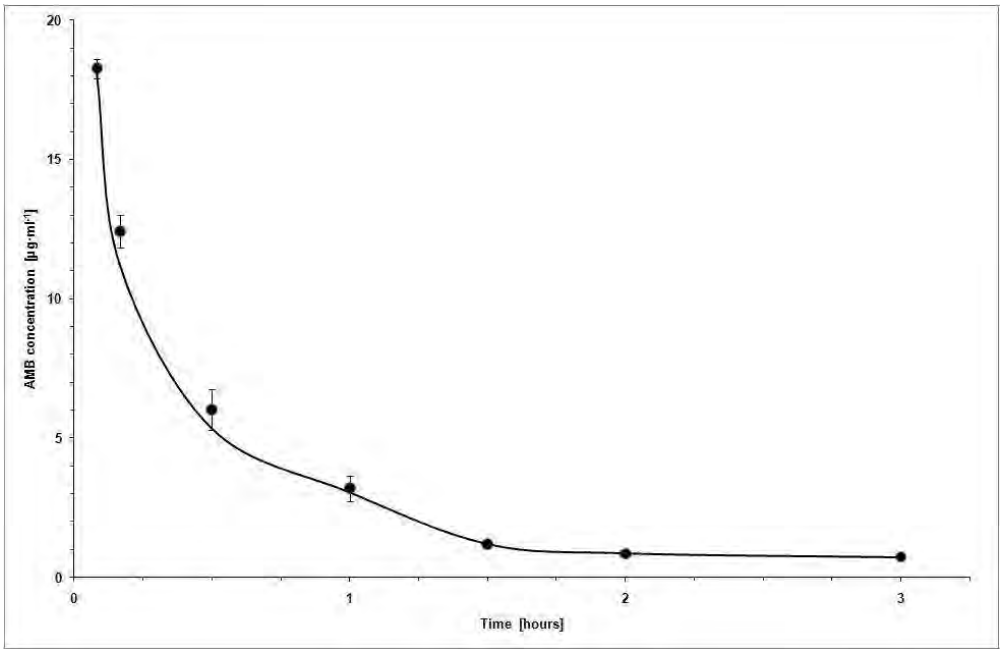

Fig. 3 Dependence of AMB plasma concentration vs. time after administration of single *iv* dose of PEG-2AMB

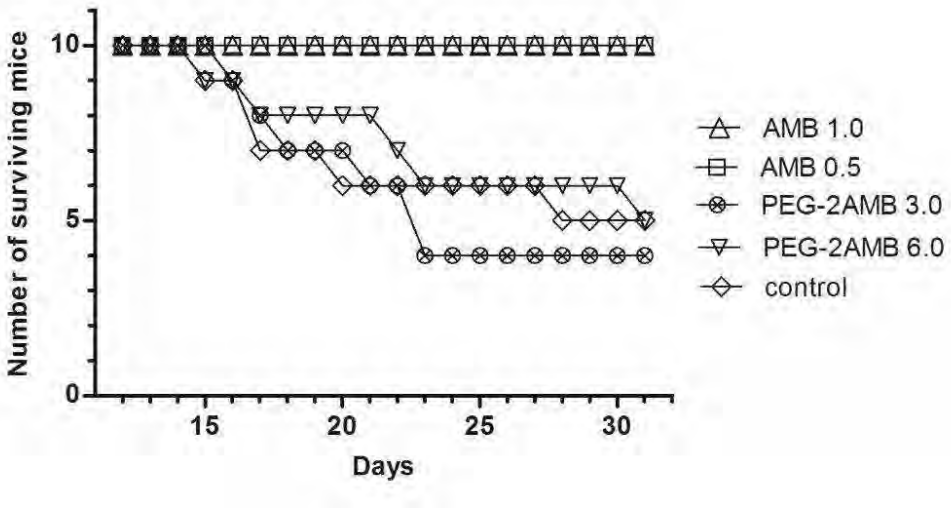

Fig. 4 Dependence of surviving mice on administered drug after inoculation of *Candida albicans*. AMB 1.0 – Amphotericin B Squibb at 1.0 mg kg^{-1}, AMB 0.5 – Amphotericin B Squibb at 0.5 mg kg^{-1}, PEG-2AMB 3.0 mg kg^{-1} – conjugate of amphotericin B with poly(ethylene glycol) at 3 mg kg^{-1}, PEG-2AMB 6.0 – conjugate of amphotericin B with poly(ethylene glycol) at 6 mg kg^{-1}, control – 5% glucose solution

for our conjugate. The obtained pharmacokinetic parameters for AMB were $t_{1/2\alpha}$ = 5.22 min, $t_{1/2\beta}$ = 1.86 h, $AUC_{0\to3}$ = 0.9157 μg h ml^{-1} and the extrapolated blood concentration at the time 0 min was 2.199 ± 0.125 μg ml^{-1}. The parameters for PEG-2AMB were $t_{1/2\alpha}$ = 3.3 min, $t_{1/2\beta}$ = 0.57 h, $AUC_{0\to3}$ = 10.83 μg h ml^{-1} and the extrapolated blood concentration at the time 0 min was 38.22 ± 3.81 μg ml^{-1}.

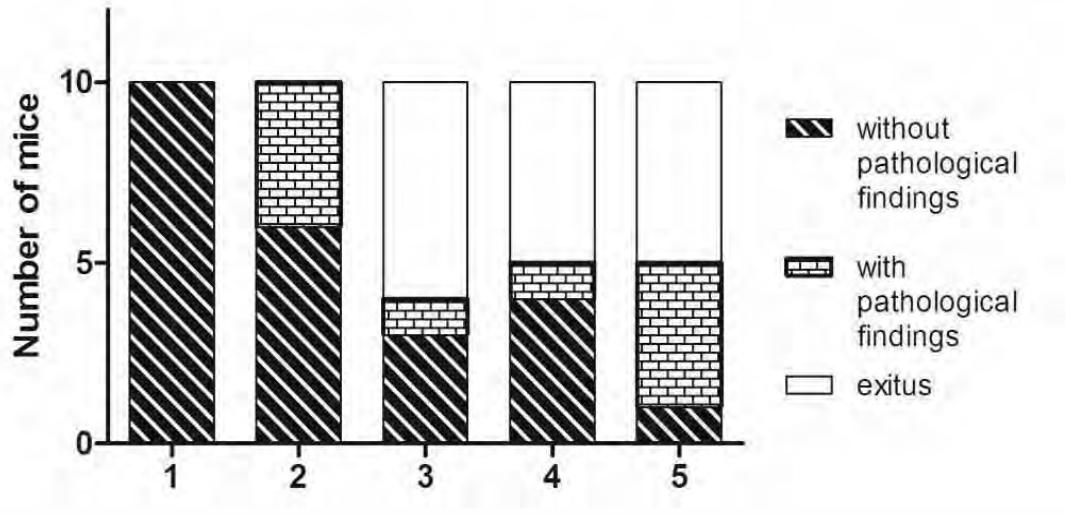

Fig. 5 Summarized results of substances administration effect on mice state on the 31st day after inoculation of *Candida albicans*. (1) AMB 1.0 – Amphotericin B Squibb at 1.0 mg kg^{-1}, (2) AMB 0.5 – Amphotericin B Squibb at 0.5 mg kg^{-1}, (3) PEG-2AMB 3.0 – conjugate of amphotericin B with poly(ethylene glycol) at 3 mg kg^{-1}, (4) PEG-2AMB 6.0 – conjugate of amphotericin B with poly(ethylene glycol) at 6 mg kg^{-1}, (5) control group

Mice Treatment with AMB-2PEG and with Conventional AMB

The therapeutic efficacy of PEG-2AMB conjugate was studied *via* mice infected with *Candida albicans*. Two dosages of conventional AMB (0.5 and 1.0 mg kg^{-1}) and two dosages of PEG-2AMB (3.0 and 6.0 mg kg^{-1}) with the similar content of AMB were used for treatment of an infected mice. The control group received placebo, which was 5 % glucose solution. The results of the treatment investigation are depicted in Fig. 4. As one can see, 100 % of mice treated with conventional preparation of amphotericin B at a dose of 0.5 and 1.0 mg kg^{-1} survived the whole experiment (31 days). However, approximately one half of control group and one half of mice treated with PEG-2AMB conjugate died between the 15th and 31st day of experiment. Figure 5 presents summarized results of substances administration effect on the mice state on the 31st day after inoculation of *Candida albicans*. Autopsy has shown that only mice treated with the dosage of 1.0 mg kg^{-1} of conventional amphotericin B were without pathological findings. The dose of 0.5 mg kg^{-1} of conventional preparation was sufficient for the treatment but there were registered pathological findings in 4 out of 10 mice. Only 1 of 10 control mice, as well as 1 tenth of those mice treated with PEG-2AMB 3 mg kg^{-1} were without macroscopic findings. In the group treated with 6 mg kg^{-1} of PEG-2AMB there were no pathological findings for 4 out of 10 mice.

Conclusion

A successful treatment of systemic mycoses with AMB is often complicated its toxicity, side effects or also by the high price of formulations. These disadvantages lead to preparation of new drugs with similar therapeutic effects, with lower toxicity and price. In the first part of this study, the synthesis of non-covalent amphotericin B with poly(ethylene glycol) conjugate was described. This conjugate shows more suitable solubility in water than conventional AMB. Moreover, poly(ethylene glycol) used as a drug carrier may reduce the preparation price. In PEG-2AMB conjugate, AMB is bound by non-covalent bond.

For the analysis of the free AMB in plasma, rapid and sensitive HPLC-UV method was used. This method was validated within the linear concentration range from 0.06 to 2.5 μg ml^{-1}. The pharmacokinetic profile and therapeutic efficiency of PEG-2AMB conjugate were compared with those of conventional amphotericin B. The concentration vs. time profiles show a decreasing course for both preparations described by two-exponential equation, and this trend is typical for a single *iv* administration. However, the initial AMB concentration after PEG-2AMB conjugate administration is approximately ten times higher. This effect may be caused by its higher solubility in water. Pharmacokinetic parameter half time ($t_{1/2}$) shows that conjugate PEG-2AMB has a higher elimination rate (0.57 h) than the conventional formulation (1.86 h). Figures 4 and 5 show a comparison of possible therapeutic effect of the conjugate PEG-2AMB and therapeutic effect of conventional amphotericin B. As one can see, the most efficient for the experimental *candidiasis* treatment was the dose of 1 mg kg^{-1} of conventional preparation. Unfortunately, the treatment with conjugate PEG-2AMB was not so efficient. However, the therapeutic index was evident.

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