Available online at www.sciencedirect.com
SCIENCE DIRECT®
International Journal of Pharmaceutics 254 (2003) 3–5
international journal of pharmaceutics
www.elsevier.com/locate/ijpharm

Note

Plasma protein adsorption patterns on surfaces of Amphotericin B-containing fat emulsions

Sven Schmidt, Rainer H. Müller*

Pharmazeutische Technologie, Biopharmazie und Biotechnologie, Institut für Pharmazie, Freie Universität Berlin, Kelchstraße 31, D-12169 Berlin, Germany

Received 28 August 2002; accepted 11 November 2002

Abstract

Nephrotoxicity of the conventional Amphotericin B formulation Fungizone® is the most common side effect in treatment of systemic fungal infections. Lipid formulations of Amphotericin B including fat emulsions showed a reduced nephrotoxicity. In vivo distribution studies of lipid formulations have shown an accumulation of Amphotericin B in liver and spleen, while concentration in the kidneys is reduced. Blood proteins adsorbed onto particles after intravenous administration are regarded as the key factors determining their in vivo fate. Two-dimensional polyacrylamid gel electrophoresis is a powerful tool for analysis of protein adsorption patterns. This paper deals with the question if there is any correlation between proteins adsorbed on surfaces of AmB fat emulsions produced with a new production technique and the potentially organ distribution of this formulation.

Keywords: Amphotericin B; Emulsions; High pressure homogenization

Amphotericin B (AmB) has been available for more than 30 years and remains the drug of choice for the treatment of systemic fungal infections. Literature describes many side effects by infusion of the conventional formulation Fungizone® like fever, chills, headache, hypotension, bronchospasm or allergic reactions. However, the most common problem and limiting factor in treatment is nephrotoxicity (Walker, 1998).

It is possible to reduce nephrotoxicity by mixing Fungizone® with fat emulsions such as Intralipid or Lipofundin (Caillot et al., 1993). Organ distribution studies showed a reduced concentration of AmB in the kidneys for AmB emulsions and other lipid formulations, this could be the main reason for reduced nephrotoxicity of this formulation. But the mixture is physically not stable which leads to precipitation of AmB, so the formulation is not safe for intravenous (i.v.) application (Shadkhan et al., 1997).

AmB is a drug which is poorly soluble in water and in oily phases, so the only way to incorporate it into the formulation is integration of molecules in the stabilizing lecithin layer. The principle method to localize a drug in the interfacial lecithin layer of emulsions is dissolution of lecithin and the drug in an organic solvent, removal of the solvent by evaporation and using the remaining lecithin-drug mixture for emulsion production (Davis and Washington, 1988; Lance et al., 1995). Now it is possible to localize the drug directly

* Corresponding author. Tel.: +49-30-83850696; fax: +49-30-83850616.
E-mail address: mpharma@zedat.fu-berlin.de (R.H. Müller).

0378-5173/02/$ – see front matter
doi:10.1016/S0378-5173(02)00667-1

in the lecithin layer by high pressure homogenization (Müller, 2000).

The mechanisms of AmB incorporation could lead to changes in the physical properties of the emulsions surfaces, which are an important factor for protein adsorption after i.v. application. Blood proteins adsorbed onto the droplet surface after i.v. administration are regarded as the key factors determining their in vivo fate. Adsorbed proteins play an important role in recognition of body foreign particles by the RES. Two-dimensional polyacrylamide electrophoresis (2-DE) assays have been developed to assess the interaction of colloidal drug carriers and plasma proteins and was used to investigate AmB loaded emulsion.

Drug-loaded emulsions were produced by adding the powdered drug to the emulsion Lipofundin N 20% by stirring (Ultra-Turrax, Jahnke & Kunkel, Staufen, Germany). The obtained dispersions containing oil droplets and drug crystals were than subjected to a high pressure homogenization process using a Micron LAB 40 (APV Deutschland GmbH, Unna, Germany). Production was performed typically at 45 °C applying 1500 bar and 5–20 homogenization cycles.

Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) is a powerful tool for the simultaneous detection of up to 10,000 proteins at the same time. The 2-D PAGE separates the proteins according to two different parameters. In the first dimension isoelectric focusing (IEF) is carried out for protein separation due to their isoelectric points (pI). The second dimension, a SDS-PAGE separates the proteins according to their molecular weights. The used protocol enables the determination of proteins with an isoelectric point ranging from pH 4–10 and a molecular weight between 250 and 6 kDa.

For the analysis of the in vitro plasma protein adsorption patterns on emulsions droplets with different surface charge 2 ml of each emulsion were incubated with 6 ml citrate stabilized human plasma. Incubation of the samples took place for 5 min at a temperature of 37 °C. Emulsion droplets were separated from bulk plasma by centrifugation with 15,000 × *g* (Cryofuge 20-3, Hereaus, Germany). Afterwards, they were washed three times with phosphate buffer pH 7.4. Protein desorption was carried out with a SDS-solution according to (Blunk et al., 1993; Bjellquist et al., 1998). For the first dimension immobilized non-linear pH gradients (IPGs) ranging from 3.5 to 10 (Amersham Pharmacia, Sweden) were used. Sample entry was performed by sample-rehydration in a custom-made reswelling tray (Sanchez et al., 1997).

The slab gels were cast with a Model 395 gradient former and multigel casting chamber, containing 9–16% acrylamide gradient and 2.6% piperazine diacrylamide (PDA) as cross-linker.

After 2-D PAGE, the gels were silver stained according to Blunk (1994) and scanned with an ImageScanner (Amersham Pharmacia, Sweden). Protein identification was carried out by matching the stained spots to a master map of human plasma which is accessible on the Expasy server (Appel et al., 1994). Semi-quantitative analysis of protein spots is possible with the Melanie III software (Bio-Rad, Germany).

Fig. 1 shows the comparison of silver stained gels of Lipofundin N 20% and the AmB-containing emulsion produced by high pressure homogenization. Lipofundin N 20% was used for the production of the drug loaded emulsion. AmB is located in the lecithin layer and influences the protein pattern.

Apolipoproteins were the most dominant protein species of the detected protein patterns (Table 1). For these species quantitative changes were detectable. Comparing the percentages of ApoA-IV shows 12.4% for drug free emulsion versus 8.3% drug loaded emulsion. A similar decrease from 16.8 to 8.7% is detectable for ApoC-III. Another decrease is shown for Albumin with an amount of 6% for drug free formulation and 3.4% for drug loaded. On the other hand on the AmB-containing formulation different Fibrinogen chains are accumulated but not for pure Lipofundin N 20%.

Table 1
Plasma protein adsorption (vol.%) on surfaces of Lipofundin N 20% and AmB-containing fat emulsion based on Lipofundin N 20%

Proteins	Lipofundin N 20%	Lipofundin N 20% + AmB
Albumin	6.0	3.4
ApoA-I	17.6	15.5
ApoA-VI	12.4	8.3
ApoC-III	16.8	8.7
ApoJ	2.5	5.9
Fibrinogen-α	0	1.2
Fibrinogen-β	0	2.0
Fibrinogen-γ	0.4	3.9

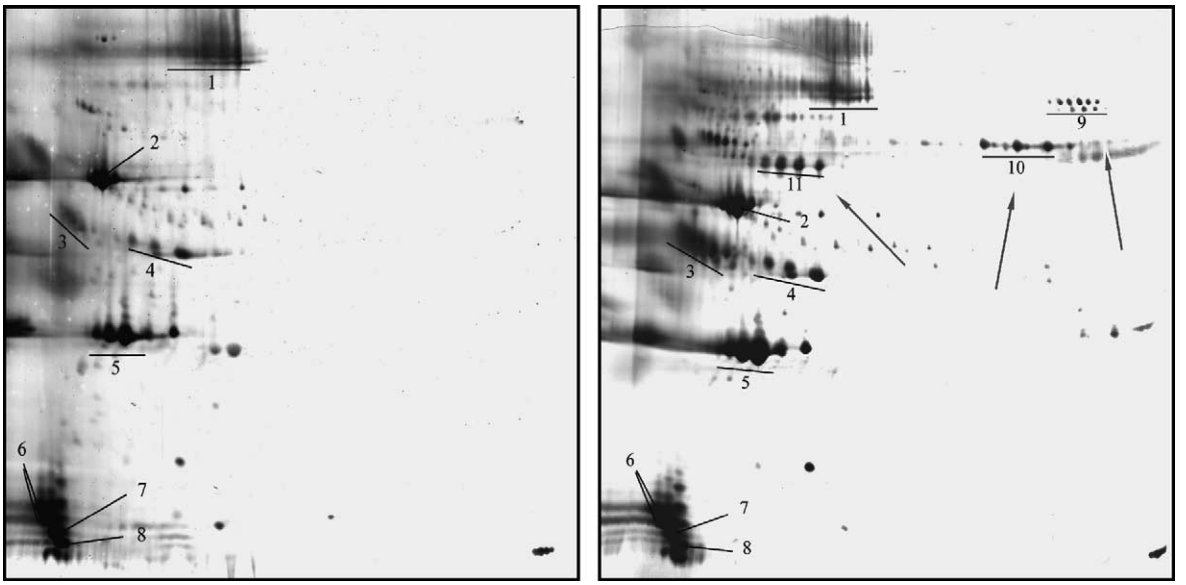

Fig. 1. Comparison of silver stained gels of Lipofundin N 20% (left) and the AmB-containing emulsion (right). Arrows mark the additional adsorbed fibrinogen chains. (1) Albumin, (2) ApoA-IV, (3) ApoJ, (4) ApoE, (5) ApoA-I, (6) ApoC-III, (7) ApoA-II, (8) ApoC-II, (9) Fibrinogen-α-chain, (10) Fibrinogen-β-chain, (11) Fibrinogen-γ-chain.

Apolipoproteins and Albumin are classified as dysopsonins which reduce the surface hydrophobicity. On the other hand Fibrinogen is an opsonin which enhances phagocytosis. The increase of opsonins and decrease of dysopsonins on the surface of i.v. injected particles can cause a faster uptake by MPS and stronger clearance from the blood stream when this take place to a sufficient high extent. This could lead to reduction of free AmB in blood circulation. The in vivo distribution is than potentially similiar to AmBisome® and other lipid formulations combined with reduced nephrotoxicity.

References

Appel, R.D., Sanchez, J.-C., Bairoch, A., Golaz, O., Min, M., Vargaz, J.R., Hochstrasser, D.F., 1994. SWISS-2-D PAGE: a database of two-dimensional gel electrophoresis images. Electrophoresis 14, 1232–1238.

Bjellquist, B., Pasquali, C., Ravier, F., Sanchez, J.-C., Hochstrasser, D.F., 1998. Non-linear immobilized gradient for two-dimensional electrophoresis and its definition in a relevant pH scale. Electrophoresis 14, 1357–1365.

Blunk, T., 1994. Ph.D. thesis, University of Kiel.

Blunk, T., Hochstrasser, D., Sanchez, J.-C., Müller, B.W., Müller, R.H., 1993. Colloidal carriers for intravenous drug targeting: plasma protein adsorption pattern on surface-modified latex particles evaluated by two-dimensional polyacrylamide gel electrophoresis. Electrophoresis 14, 1382–1387.

Caillot, D., Casasnovas, O., Solary, E., Chavanet, P., Bonnotte, B., Reny, G., 1993. Efficacy and tolerance of an amphotericin B lipid (Intralipid) emulsion in the treatment of candidaemia in neutropenic patients. J. Antimicrob. Chemother. 31, 161–169.

Davis, S.S., Washington, C., 1988. Drug emulsion. European Patent 0,296,845A1.

Lance, M.R., Washington, C., Davis, S.S., 1995. Structure and toxicity of amphotericin B/triglyceride emulsion formulations. J. Antimicrob. Chemother. 36, 119–128.

Müller, R. H., 2000. Dispersionen zur Formulierung wenig oder schwer löslicher Wirkstoffe, Deutsche Patentanmeldung Nr. 100 36 871.9.

Sanchez, J.-C., Rouge, V., Pisteur, M., Ravier, F., Tonella, L., Moosmayer, M., Wilkins, M.R., Hochstrasser, D.F., 1997. Improved and simplified in-gel sample application using reswelling of dry immobilized pH gradients. Electrophoresis 18, 324–327.

Shadkhan, Y., Segal, E., Bor, A., Gov, Y., Rubin, M., Lichtenberg, D., 1997. The use of commercially available lipid emulsions for the preparation of amphotericin B-lipid admixtures. J. Antimicrob. Chemother. 39, 655–658.

Walker, S., 1998. Amphotericin B in lipid emulsion: stability, compatibility and in vitro antifungal activity. Antimicrob. Agents Chemother. 42, 762–766.