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The effect of formulation variables on the stability of nebulized aviscumine

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Abstract

The pulmonary drug delivery of proteins present an alternative to parenteral and oral administration. Nebulization of aqueous protein solutions is an ideal method for pulmonary application of therapeutic proteins considering the difficulties of their formulation as MDIs or DPIs. This research presents the effect of variable excipients on the stability of freeze-dried aviscumine after reconstitution and nebulization. Formulations containing different lyoprotectants have been lyophilized and reconstituted with isotonic salt solution. The loss of aviscumine activity in the nebulizer reservoir and after nebulization with a PariBoy® air-jet nebulizer, a Multisonic® ultrasonic nebulizer and a Systam® ultrasonic nebulizer was determined by a binding assay. The effect of variable lyoprotectants such as 8% (w/v) Dextran T1, HES130, HES450, HP-β-CD and 6% (w/v) HES450 plus 2% (w/v) mannitol on the stability of aviscumine to air-jet and ultrasonic nebulization has been evaluated. Only 50% of aviscumine activity was retained after 20 min nebulization, where 8% (w/v) HES450 was shown to be the best stabilizer. Stabilization of aviscumine by the addition of variable surfactants as 0.01 and 0.1% (w/v) Poloxamer 188, 0.03 and 0.1% (w/v) PEG 8000, 0.03 and 0.1% (w/v) Solutol HS15 and 0.03 and 0.1% (w/v) octanoyl-*N*-methyl-glucamide to the reconstitution solution has also been studied. By the addition of 0.03% (w/v) octanoyl-*N*-methyl-glucamide, 70% of the activity was retained after 20 min nebulization.

Keywords: Nebulizer; Octanoyl-*N*-methyl-glucamide; Stability of proteins; Lyoprotectants; Aviscumine

1. Introduction

Mistletoe extracts have been used for decades for non-specific stimulation of the immune system in cancer therapy. Mistletoe lectin (ML) was identified as the active principle with cytotoxic and immunomodulatory potencies (Hajto et al., 1989; Burger et al., 2001). After the proof of activity of ML in cancer therapy, its therapeutic use faced with two problems: the optimization of ML concentration in extract and its instability in aqueous solution. To solve these problems, recombinant ML (aviscumine, a dimeric 57-kDa protein) has been produced in *E. coli* (Langer et al., 2002) and the stabilization of aviscumine has been achieved in an aqueous solution using different excipients (Witthohn et al., 2002) as well as by lyophilization (Gloger et al., 2002). After the success to formulate aviscumine for parenteral application for cancer therapy, new efforts to develop alternative, non-invasive delivery systems have been started. Drug delivery via the respiratory tract presents an alternative to parenteral and oral administration of

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proteins. The slow mucociliary clearance from the lower lung tissue, presumably low metabolic activity, and bypassing metabolism by the liver make the pulmonary route a viable route of application (Abu-Dahab, 2001). Nevertheless, the development of a pharmaceutical aerosol product of aviscumine requires appropriate techniques for product manufacture (Gupta and Adjei, 1997). Nebulization is an easy method for producing an aerosol mist from a protein solution considering the difficulties accompanied by its formulation as DPI or MDI. However, the integrity and activity of proteins should not be affected or otherwise be influenced by nebulization (Ip et al., 1995). The aim of this study was to evaluate the effect of formulation variables on the stability of aviscumine to air-jet as well as to ultrasonic nebulization.

2. Materials and methods

2.1. Materials

Aviscumine, precipitated in 2.67 mol/l ammonium sulfate (storage: 2–8 °C) and the anti-aviscumine antibodies were provided by VISCUM AG (Zwingenberg, Germany). All stability studies were conducted using the same raw material batch of aviscumine 5 mg/ml (batch: 01212/SUG). All aviscumine solutions were buffered to pH 8.0 with 100 mmol/l Tris-(hydroxymethyl)-aminomethane with a final pH adjustment with 1 N HCl. Dextran T1 was obtained from Amersham Pharmacia (Upsala, Sweden). HP-β-cyclodextrin was obtained from Cerestar, (Krefeld, Germany). Hydroxyethylstarch (MW 130000 and 450000) was supplied by Fresenius KABI (Linz, Austria). Tris-buffer, Na_2-EDTA, octanoyl-*N*-methylglucamide, HCl and NaCl were purchased from Merck (Darmstadt, Germany). Polysorbate 80 was obtained from Uniquema (Euperberg, Belgium). Poloxamer 188 and Solutol HS15 were purchased from BASF (Ludwigshafen, Germany), while PEG 8000 was obtained from Hoechst (Frankfurt, Germany). BSA, OPD-tablets, asialofetuin and anti-mouse-IgG-POD were received from Sigma–Aldrich (Steinheim, Germany). The water used was of double-distilled quality. The vials were 3 ml vials and supplied by Forma Vitrum (St. Gallen, Switzerland). The stoppers were obtained from The West Company (Aachen, Germany).

2.2. Methods

2.2.1. Preparation of a solution for freeze-drying

For the freeze-drying of aviscumine five formulations have been studied, which have to contain a buffer (Eckhardt, 1992). Because of the higher stability of aviscumine in the alkaline region, the solution has been adjusted at pH 8 with Tris-buffer due to its low pH-shift during freezing which may cause denaturation of the protein (Gloger and Müller, 2000). Polysorbate 80 has been added to prevent the degradation of the protein on the ice–protein-interface (Chang et al., 1996) and prevent the irreversible defolding of the protein due to the rapid reconstitution (Webb et al., 2002). Na_2-EDTA was used here as an antioxidant to protect the protein against metal ions (Woog, 1993). Variable lyoprotectants have been used because of their main role in the stabilization of proteins through there amorphous character by embedding the protein and preventing its degradation on the protein–ice-interface (Lee and Timasheff, 1981) and collapsing of the cakes during freeze-drying by raising the glass point (T_g') (Johnson et al., 2002). Lyoprotectants can also replace the water after drying by binding hydrogen bonds on the protein surface (water replacement theory; Timasheff, 1992).

Tris-buffer, surfactant and the different polysaccharides were dissolved in double distilled water. This solution was adjusted to pH 8 with 1 N HCl. Then, Na_2-EDTA and aviscumine were added and homogenized. The volume was adjusted with water and the solution was shaken with a Vortex Genie 2 (Bender and Hobein, Zürich, Switzerland). Table 1 summarizes the formulations of aviscumine that have been freeze-dried.

2.2.2. Freeze-drying procedures

Vials were filled with 500 μl of 100 μg/ml aviscumine solution and loaded at room temperature into a freeze-dryer GFT6 (Klein Vakuumtechnik, Freudenberg, Germany). The product was cooled to −32 °C within 300 min. After that the primary drying was initiated and continued for 26 h. Primary drying is followed by secondary drying for 10 h. The utilized program is detailed in Table 2.

2.2.3. Preparation of a solution for nebulization

The lyophilisates were reconstituted with 5 ml isotonic solution containing 150 mmol/l NaCl and the

Table 1
Aviscumine formulations for freeze-drying

Active substance	Buffer	Surfactant	Antioxidant	Lyoprotectants
Aviscumine [0.1 g/l]	Tris-buffer [0.1 mol/l]	Tween® 80 [1 g/l]	Na_2-EDTA [0.1 g/l]	Dextran T1 [80 g/l]
Aviscumine [0.1 g/l]	Tris-buffer [0.1 mol/l]	Tween® 80 [1 g/l]	Na_2-EDTA [0.1 g/l]	HES130 [80 g/l]
Aviscumine [0.1 g/l]	Tris-buffer [0.1 mol/l]	Tween® 80 [1 g/l]	Na_2-EDTA [0.1 g/l]	HES450 [80 g/l]
Aviscumine [0.1 g/l]	Tris-buffer [0.1 mol/l]	Tween® 80 [1 g/l]	Na_2-EDTA [0.1 g/l]	HP-β-CD [80 g/l]
Aviscumine [0.1 g/l]	Tris-buffer [0.1 mol/l]	Tween® 80 [1 g/l]	Na_2-EDTA [0.1 g/l]	HES450 [60 g/l] + Mannitol [20 g/l]

Table 2
Freeze-drying program used for drying of the aviscumine formulations

Step	Time (min)	Starting temperature (°C)	End temperature (°C)	Pressure (mbar)
1. Freezing	300	Room temperature	−32	40
2. Primary drying	600	−32	−30	0.8
	600	−30	−15	0.8
	180	−15	−10	0.8
	180	−10	−5	0.8
3. Secondary drying	300	−5	15	0.1
	300	15	15	0.1

obtained solution has been adjusted at pH 8 with Tris-buffer. To study the effect of variable surfactants on the stability of aviscumine during nebulization, the different surfactants have been added to the reconstitution medium.

2.2.4. Air-jet and ultrasonic nebulization

All nebulization experiments were performed using a PariBoy® air-jet nebulizer (Pari-Werk, Starnberg, Germany), a Multisonic® ultrasonic nebulizer and a Systam® ultrasonic nebulizer (Otto Schill, Probstzella, Germany). The technical data of these nebulizers provided by the manufacturer are given in Table 3. The air-jet nebulizer was operated normally for 20 min while the ultrasonic nebulizers were operated for only 20 s followed by a 20 s cutout to prevent the degradation of aviscumine by heating (Cipolla et al., 1994). These cycles have been repeated for 20 min so that the temperature in the reservoir did not exceed 35 °C within the nebulization time. The temperature has been monitored using a Data-Logger thermometer 306 (Conrad Electronic, Hamburg, Germany). The starting reservoir volume used for all studies was 5 ml. An internal standard of carboxyfluoresceine sodium (CF) was added to the initial volume in a concentration of 2 μg/ml. The aerosol mist was then collected and samples of 100 μl/5 min were removed from the reservoir as well as from the collection system and then diluted with a dilution factor of 100. The samples have been stored at −70 °C until analysis.

Table 3
Technical data of the nebulizers used in the study

	PariBoy® air-jet nebulizer	Multisonic® ultrasonic nebulizer	Systam® ultrasonic nebulizer
Total output[a] (mg/min)	460	750	Between 200 and 1000
Residual volume (g)	1	0.4	0.6
MMD[b] (μm)	4.1	4.7	3.5
Frequency (MHz)	–	1.7	2.4
Compressor pressure (bar)	1.3	–	–

[a] Total output measured for an isotonic saline solution (0.9% (w/v) NaCl).
[b] MMD: Mass median diameter.

2.2.5. Description of the collection apparatus

Collecting aerosol on a filter or other collecting system is considered as a problem because it does not reflect the amount of active protein which reaches the lung. Cipolla et al. have considered the difficulties of the dry collection of proteins due to its denaturation during collection and drying. They have demonstrated an approach to collect "wet" protein aerosols so that problems arising through collection of a "dry" aerosol can be avoided (Cipolla et al., 1994). For similar reasons, Ip et al. have used a simple technique that allow the aerosol emerging from a collision nebulizer to be collected in a wet or dry state using a condenser column at 1 °C or dry filtered air at approximately 30 °C, respectively (Ip et al., 1995). In the present study, a newly developed apparatus to ensure the collection of the active protein has been used (Fig. 1). The nebulizer (A) containing the test solution and 2 μg/ml CF was operated and an air flow of 12 l/min was drawn through the device by means of a vacuum pump. At the same time water was nebulized from a second nebulizer (B) ensuring water vapor saturation of the outgoing air. The aerosol mixture was passed into a glass condenser (C), where the droplets were condensed onto the internal wall of the coil. A mixture of 50% ethylenglycol and 50% water precooled to −5 °C was used to cool the condenser. The first and the second flask (D and E) were filled with 5 ml washing solution containing 100 mmol/l Tris-buffer pH 8 and 0.01% (w/v) Polysorbate 80. The tested solution was nebulized for 20 min and every 5 min the apparatus was rinsed with 10 ml washing solution and the collected volume was adjusted with the same washing solution to 25 ml. A sample was taken every 5 min and analyzed for the carbohydrate binding activity of aviscumine and the concentration of CF (internal standard). This method ensures a collection of approximately 60–70% of the nebulized solution with the rest being collected on the filter (F). However, a quantitative collection of the nebulized protein was not necessary as the analyzed collected protein is always referenced to the CF concentration (see Section 2.2.7).

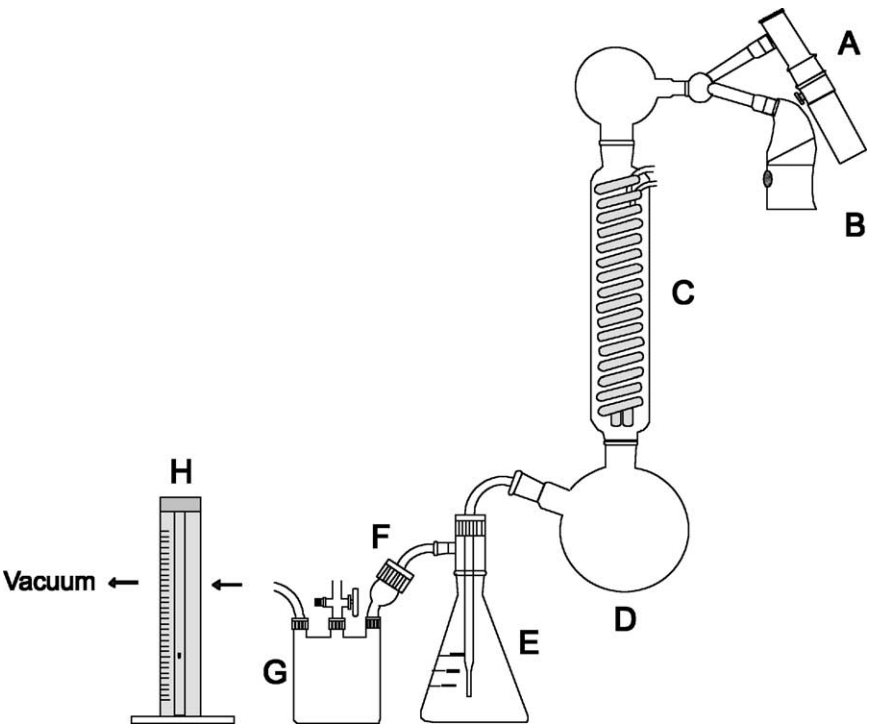


Fig. 1. A description of the system used to collect the aviscumine: (A) nebulizer containing the tested solution, (B) nebulizer containing water, (C) condenser column, (D) first collection flask, (E) second collection flask, (F) filter, (G) regulator flask, and (H) flow meter.

2.2.6. Assays

The carbohydrate binding activity of aviscumine after reconstitution and nebulization was measured by an Enzyme Linked Lectin assay (ELLA) which determines the intact protein by specific binding of the lectin (B-chain) and detecting the A-chain. Asialofetuin, which has a galactose binding site, was bound to the micro titre plates (Nunc, Wiesbaden, Germany) and the empty spaces were saturated with BSA (1%). Anti-aviscumine (A-chain) IgG (mouse) and anti-mouse-IgG-peroxidase conjugate were used for detection. By addition of *o*-phenylene-diamine [OPD], which is a peroxidase substrate, a soluble orange-brown color is produced and can be detected spectrophotometrically at 492 nm. The relative activity was determined related to freshly prepared solution which contained the same excipients as the sample (Poppe, 2001).

The concentration of carboxyfluorescein sodium (CF) was determined using a Perkin-Elmer-Fluorimeter Model LS 50B (Perkin-Elmer, Buckinghamshire, UK). Samples were placed in 10 mm pathlength polystyrol cuvettes (Sarstedt, Nümbrecht, Germany) and the fluorescence intensity was detected at excitation and emission wavelength of 490 and 515 nm, respectively (Grignon et al., 1989). The relative concentration was related to the concentration of CF in the initial solution before nebulization. The percentage of remaining activity of aviscumine in the reservoir and after nebulization was calculated using the Eq. (1).

$$\text{Aviscumine activity} = A_r - (C_{CF} - A_n) \quad (1)$$

where A_r is the percentage of the remaining aviscumine activity after reconstitution, A_n is the percentage of the remaining aviscumine activity after nebulization and C_{CF} is the percentage of the CF concentration after nebulization.

2.2.7. Solution concentration analysis

The increase of the drug concentration within the nebulization time has been determined using an internal standard of CF. The concentration of CF was determined with the same method as described in Section 2.2.6. The percentage relative concentration was related to the concentration of CF in the initial solution before nebulization (Eq. (2)).

$$\text{Concentration}_{CF} = \frac{F_t}{F_0} \times 100 \quad (2)$$

where F_t and F_0 represent the fluorescence intensity of CF at the time t and before nebulization, respectively.

3. Results and discussion

3.1. Influence of variable lyoprotectants on aviscumine activity

To compare the stabilizing effect of variable lyoprotectants the remaining aviscumine activity has been determined after reconstitution in the nebulizer reservoir as well as after the condensation of the nebulized mist.

3.1.1. Determination of the aviscumine activity after reconstitution

The therapeutic dose of aviscumine in the nebulized solution was assumed to be 10 μg/ml. However, it has been lyophilized from an aqueous solution with a concentration of 100 μg/ml. The choice of a higher protein concentration is based on the observation that an increasing protein concentration increases its stability (Carpenter and Crowe, 1988; Arakawa et al., 1993). On the other hand, the lyophilisates must be reconstituted with a dilution factor of 10. Therefore, 100 μg/ml aviscumine was lyophilized in vials, each vial containing 500 μl and then reconstituted to a final volume of 5 ml using isotonic solution (150 mmol/l). Formulations containing 100 mmol/l Tris-buffer, 0.01% (w/v) Na_2-EDTA, 0.1% (w/v) Polysorbate 80 combined with 8% (w/v) Dextran T1, HES130, HES450, HP-β-cyclodextrin or 6% (w/v) HES450 plus 2% (w/v) mannitol, respectively, have been lyophilized and the stabilizing effect of the variable excipients for aviscumine after reconstitution has been examined. The results are demonstrated in Fig. 2. It has been observed that about 15% of aviscumine activity is lost after freeze-drying and reconstitution with that volume. However, a significant difference in the stabilization effect of the added lyoprotectants after reconstitution could not be observed.

3.1.2. Determination the stability of aviscumine to ultrasonic and air-jet nebulization

The loss of the activity after nebulization has been determined assuming the activity before nebulization to be 100%. The loss of aviscumine activity in the

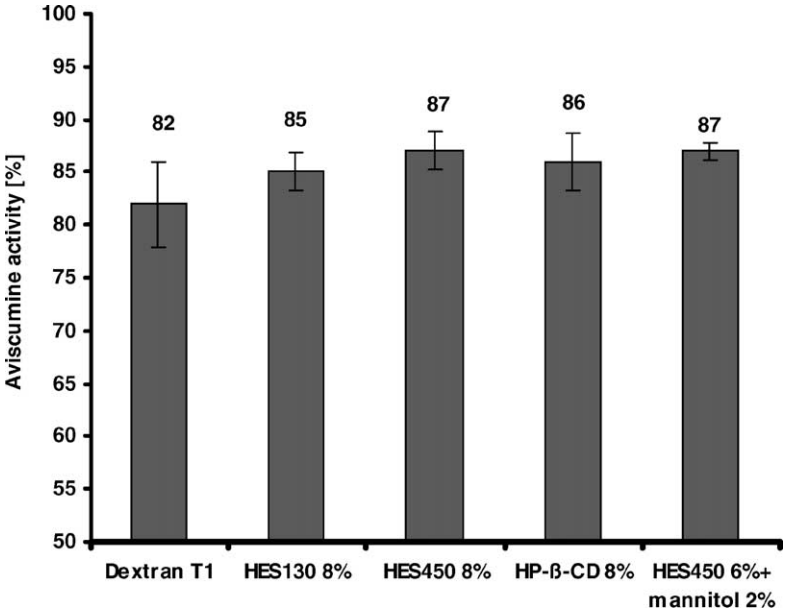


Fig. 2. Influence of lyoprotectants on aviscumine activity after reconstitution ($n = 3$; mean ± S.D.).

nebulizer reservoirs as well as for the condensed mist is shown in Fig. 3a and b, respectively. Considering the loss of aviscumine activity in the medicament reservoir, ultrasonic nebulization showed a higher destabilizing effect on aviscumine as compared to air-jet nebulization except for the formulation containing both HES450 6% (w/v) and mannitol 2% (w/v). Here the loss of the aviscumine activity is only approximately 1.5% in the Multisonic® ultrasonic nebulizer compared to approximately 12.4% in the PariBoy® air-jet nebulizer. Nevertheless, the denaturation was high (approximately 48.5%) in the case of the Systam® ultrasonic nebulizer. No significant denaturation of aviscumine has been observed in the medicament reservoir of the PariBoy® air-jet nebulizer for the formulations containing 8% (w/v) Dextran T1, 8% (w/v) HES130 and 8% (w/v) HP-β-CD compared to approximately 29, 43 and 8% in the Multisonic® ultrasonic nebulizer reservoir and approximately 52, 51 and 28% in the Systam® ultrasonic nebulizer reservoir, respectively. Nevertheless, for the formulation containing HES450, a 5% loss of the aviscumine activity could be observed in the reservoir of the PariBoy® air-jet nebulizer while approximately 17 and 39% loss of the aviscumine activity could be determined in the reservoir of Multisonic and Systam® ultrasonic nebulizer, respectively. These findings support the recommendations of the British Thoracic Society to use only the jet nebulizer for the delivery of proteins (in that case rhDNase which is an approved protein now clinically used for the treatment of cystic fibrosis and delivered by air-jet nebulizer). The society asserts that ultrasonic nebulization may cause thermal coagulation of rhDNase (Conway and Watson, 1997). Cipolla et al. have noted that rhDNase can be aggregated by ultrasonic nebulization if the melting transition temperature is approached or exceeded in the solution during nebulization (Cipolla et al., 1994). Niven et al. have shown that the destabilizing effect of ultrasonic nebulization can be reduced by preventing heating of the nebulizer solution during operation. They have controlled the temperature in the medicament reservoir using a cooling coil (Niven et al., 1994). In this study, the temperature of the nebulizer solution was kept below the melting transition temperature of aviscumine, $T_m = 50\,^{\circ}C$ (Poppe, 2001). Nevertheless, the loss of aviscumine activity in ultrasonic nebulizer was high suggesting that other factors may have an influence on the protein stability. The effect of ultrasonic waves on the stability of alpha-interferon has been studied by Satoe et al. using two ultrasonic nebulizers operating

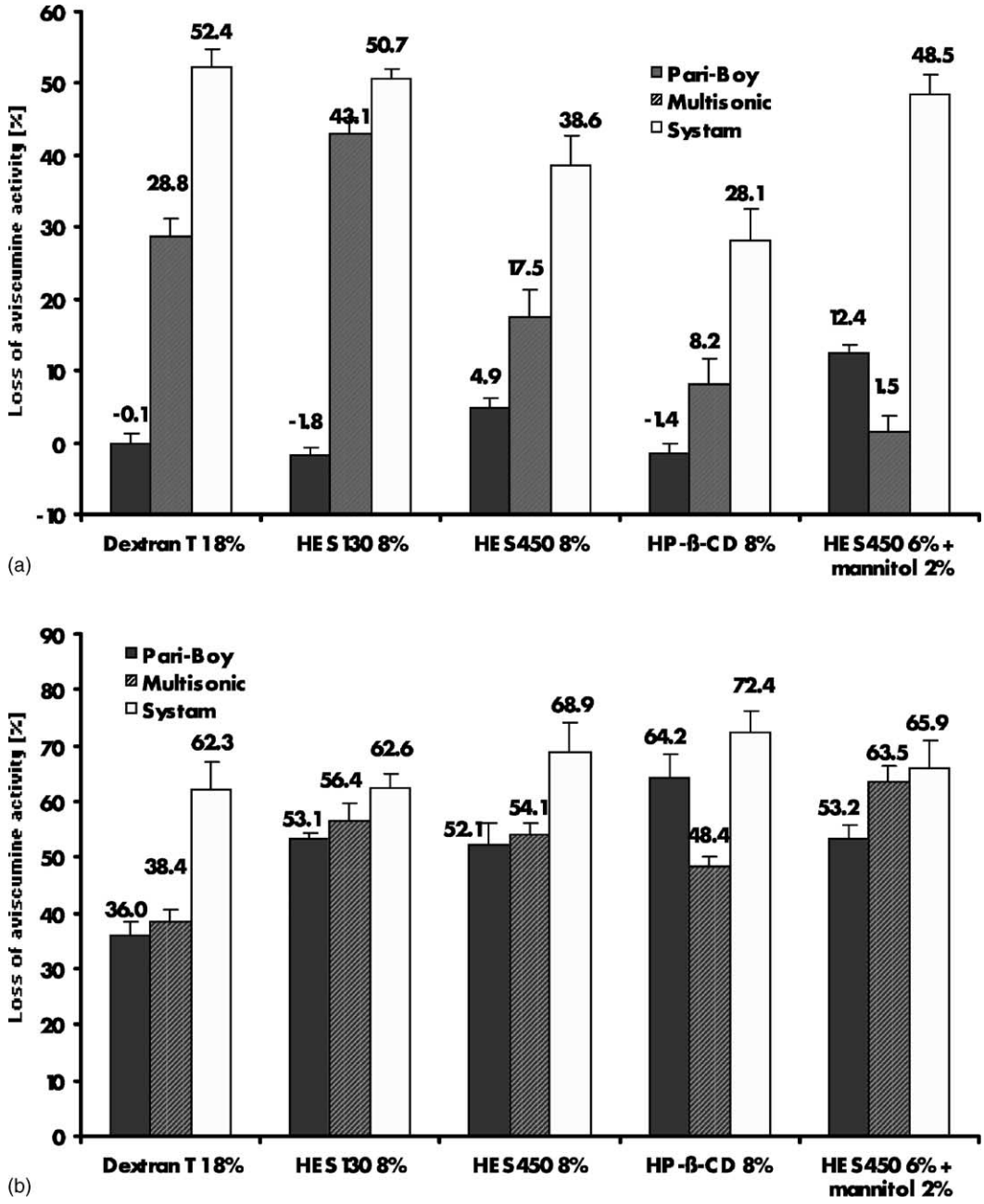


Fig. 3. Influence of lyoprotectants on the loss of aviscumine activity (a) in the medicament reservoir and (b) in the condensed aerosol mist after 20 min nebulization time ($n = 3$; mean ± S.D.).

at 80 and 160 kHz. They showed that the protein was inactivated utilizing the lower frequency but not at the higher and suggested that the formation of cavitations was responsible for that loss of activity (Sato et al., 1992). These observations oppose the data shown here where the Systam® ultrasonic nebulizer (operating at 2.4 MHz) exhibited a higher destabilization of aviscumine than the Multisonic® ultrasonic nebulizer

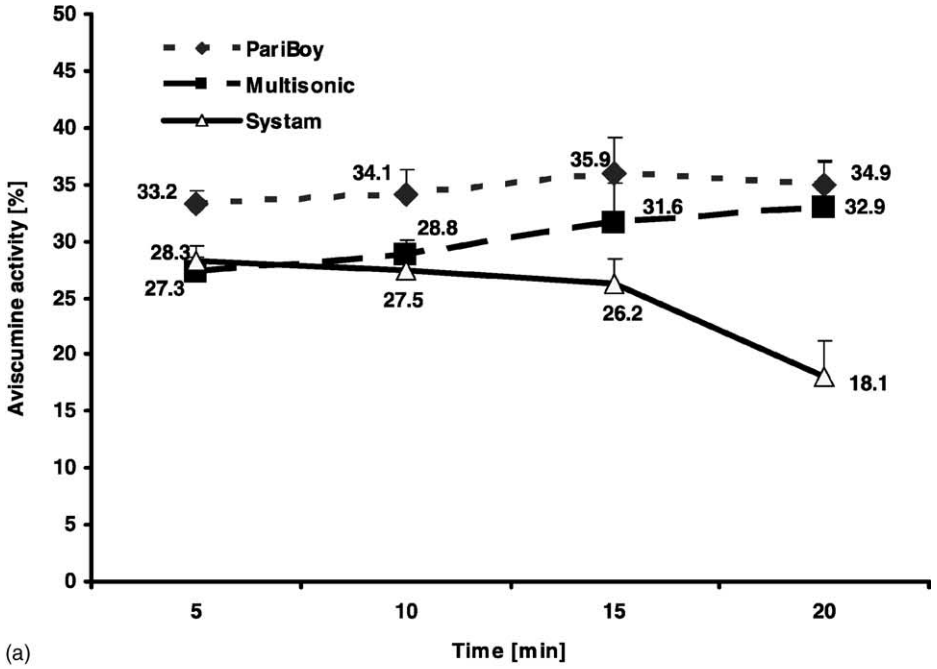
(operated at 1.7 MHz). The loss of aviscumine activity reached 72% using the System® ultrasonic nebulizer compared to 63% using the Multisonic® ultrasonic nebulizer which could be related to the System's higher energy input. HES450 and HP-β-cyclodextrin showed a relatively good stabilization effect for aviscumine. The stabilizing effect of HES can be linked to its relatively high glass transition temperature (T_g) of about 139 °C and its high T_g', which allow a better drying of the lyophilisates (Carpenter et al., 1999). This stabilizing effect can also be related to the higher viscosity of the freeze-drying solution, which in turn increases the dissolution time of the lyophilisate and hence prevent the irreversible defolding of aviscumine during the reconstitution process (Webb et al., 2002). The combination of HES450 with mannitol showed a negative effect on the stability of aviscumine which most likely is due to the crystallization of mannitol during freeze-drying and storage (Izutsu et al., 1994). On the other hand, HP-β-cyclodextrin showed a stabilizing effect of aviscumine, especially during nebulization with the Multisonic® ultrasonic nebulizer. Brewster et al. have shown the solubilizing and stabilizing effect of HP-β-CD on IL-2 (Brewster et al., 1991). It is supposed that aviscumine is embedded in the cyclic amorphous structure of HP-β-CD and so allow a stabilizing effect. Dextran T1 was a good stabilizer for aviscumine in PariBoy® air-jet nebulizer as well as in Multisonic® ultrasonic nebulizer, which can be attributed to the preferential binding of Dextran T1 to aviscumine (Gloger et al., 2002). Nevertheless, this formulation had a low T_g' (about −38 °C) which negatively affects the drying of the lyophilisates and by this decreases its long-term stability (Steckel et al., 2002).

3.1.3. Influence of the nebulization time on the activity of aviscumine

Aiming to determine the effect of the nebulization time on the stability of aviscumine and the factors affecting it, the aviscumine activity was determined after 5, 10, 15 and 20 min of nebulization. Fig. 4a presents (as one example) the effect of nebulization time on the activity of nebulized aviscumine after condensation for the formulation containing HES450. No significant effect could be detected using the PariBoy® air-jet nebulizer, while a significant decrease in the aviscumine activity over the nebulization time could be established in the case of the System® ultrasonic nebulizer. Opposite to this, a non-expected increase in activity could be observed using the Multisonic® ultrasonic nebulizer. Trying to explain these results, the changes in the concentration of CF as an internal standard have been followed over the same time interval (Fig. 4b). Evaporation of solvent during nebulization leads to a gradual increase in the concentration of the drug solution in the reservoir. This increasing drug solution concentration in the Multisonic reservoir (approximately 28% after 20 min) was higher than in the other two nebulizers, which is attributed to the direct contact of the piezoelectric crystal with the drug solution, allowing an increase of the temperature in the reservoir and, thus, a higher solvent evaporation. Considering this concentration effect, the results of the increasing aviscumine activity over the nebulization time could be explained as follows: an increasing nebulization time causes a decrease in the activity of the protein due to the longer exposure to stress conditions as heat and ultrasonic waves in the ultrasonic nebulizers or shearing forces due to the recycling of large droplets in the jet nebulizer. Opposing this fact, the increase in protein concentration has a well known stabilizing effect (Carpenter and Crowe, 1988; Arakawa et al., 1993; Khatri et al., 2001), and, therefore, the increase in the remaining activity during nebulization in the case of the Multisonic® ultrasonic nebulizer could be attributed to the increase in concentration (approximately 28% within 20 min). On the other hand, the concentration increase in the case of the System® ultrasonic and the PariBoy® air-jet nebulizers (approximately 109 and 119%, respectively) was not high enough to oppose the destabilizing factors during nebulization.

3.2. Influence of surfactants on aviscumine activity

Because of the stabilizing effect of HES450 to aviscumine during freeze-drying and after nebulization, it has been chosen as lyoprotectant for further studies. A formulation containing 100 mmol/l Tris-buffer, 0.01% (w/v) Na_2-EDTA, 0.1% (w/v) Polysorbate 80 and 8% (w/v) HES450 has been lyophilized and then reconstituted with 5 ml isotonic buffered solution (100 mmol/l Tris-buffer and 150 mmol/l NaCl), resulting in 0.01% (w/v) Polysorbate 80 being present in the nebulizing solution. The influence of surfactants



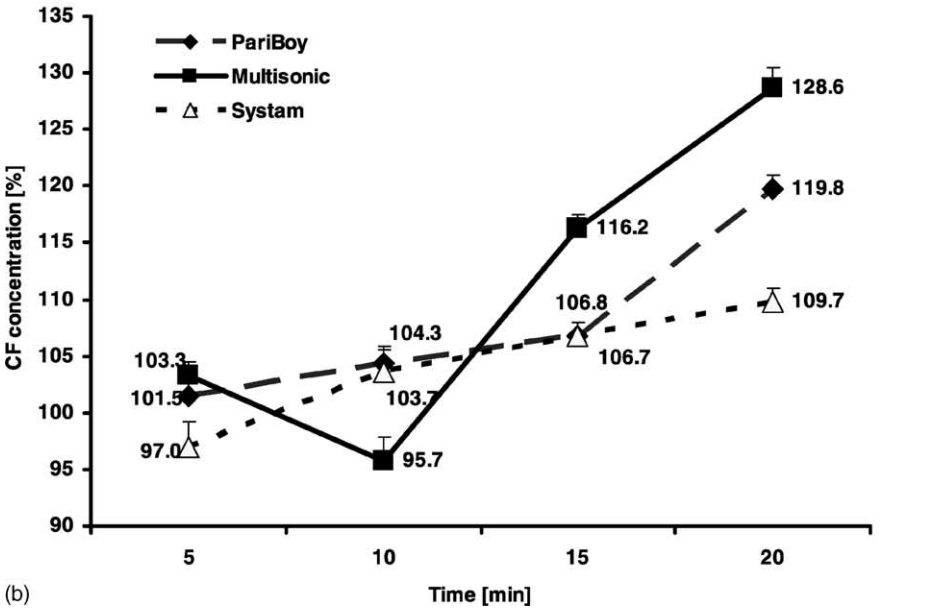


Fig. 4. Influence of nebulization time on (a) aviscumine activity and (b) on the CF concentration for the HES450 formulation ($n = 3$; mean ± S.D.).

on the stability of aviscumine after reconstitution as well as after nebulization has been studied by adding the variable surfactants to the reconstitution medium.

3.2.1. Determination of the aviscumine activity after reconstitution

The Polysorbate 80 already present in the aqueous solution for freeze-drying could not sufficiently stabilize the aviscumine after reconstitution where already about 13% of aviscumine activity was lost. This observation can be related to the lower concentration of Polysorbate 80 in the stagnant boundary layer during dissolution, which was not sufficient to prevent aviscumine denaturation (Webb et al., 2002). On the other hand, increasing its concentration is not recommendable because of its high foaming effect at higher concentrations, which makes the solution not suitable for nebulization. So, the addition of other surfactants as poloxamers, PEG 8000, Solutol HS15 and octanoyl-*N*-methyl-glucamide [OMEGA] with a lower foaming effect can be helpful. The results are given in Fig. 5. In general, the surfactant addition has improved the stability of aviscumine after reconstitution. These results are in accordance with the results of Zhang et al. who have shown a stabilizing effect of poloxamer, PEG 300 and Polysorbate 20 on Keratinocyte Growth Factor [KGF] (Zhang et al., 1995). However, significant differences could hardly be detected due to the high analytical variability of the binding assay.

3.2.2. Determination of the aviscumine stability during ultrasonic and air-jet nebulization

The aviscumine activity in the medicament reservoir as well as in the condensed aerosol mist after 20 min nebulization has been determined and is shown in Fig. 6a and b, respectively. In general, the addition of surfactant has improved the stability. This can be explained by the ability of the surfactants to protect the protein on the air–protein interface by a complex formation with micelles. On the other hand, the addition of surfactants can reduce the adsorption of proteins onto hydrophobic surfaces if the protein was unfolded during nebulization (Ip et al., 1995). Niven has attributed the stabilizing effect of PEG on rhG-CSF during nebulization to steric hindrance of PEG to the aggregation of rhG-CSF. He supported his suggestion by the higher stabilizing effect which was achieved by increasing the PEG concentration as well as by increasing the molecular weight of the PEG (Niven, 1997). In the present study it was also observed that

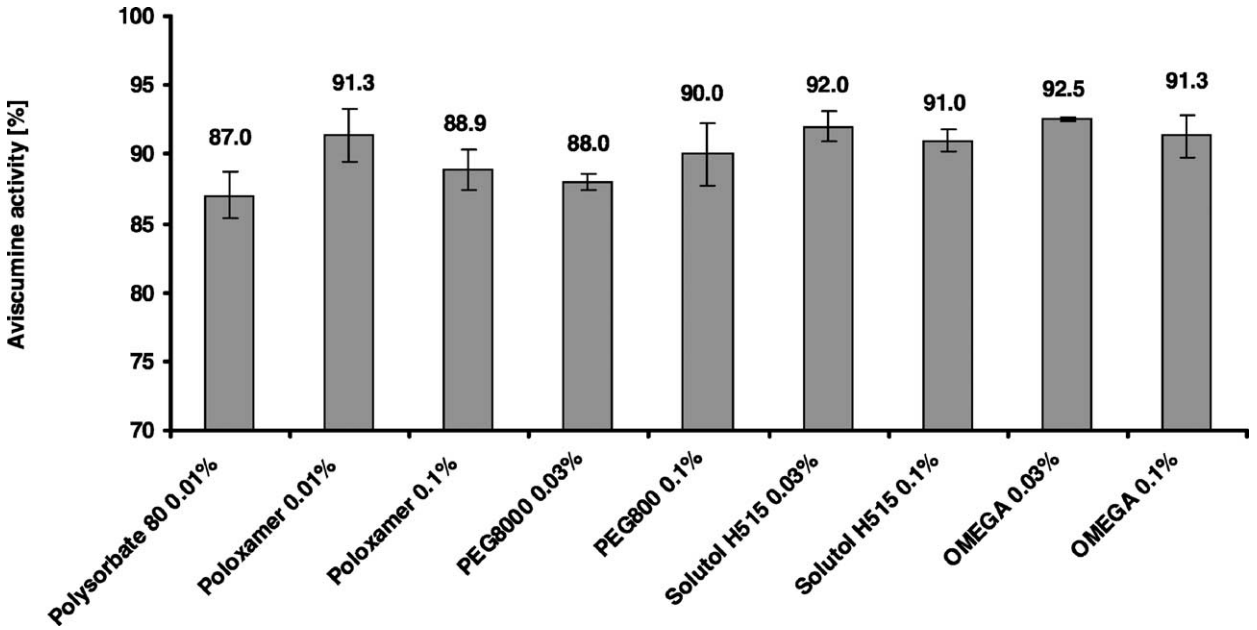


Fig. 5. Influence of surfactants on aviscumine activity after reconstitution ($n = 3$; mean ± S.D.).

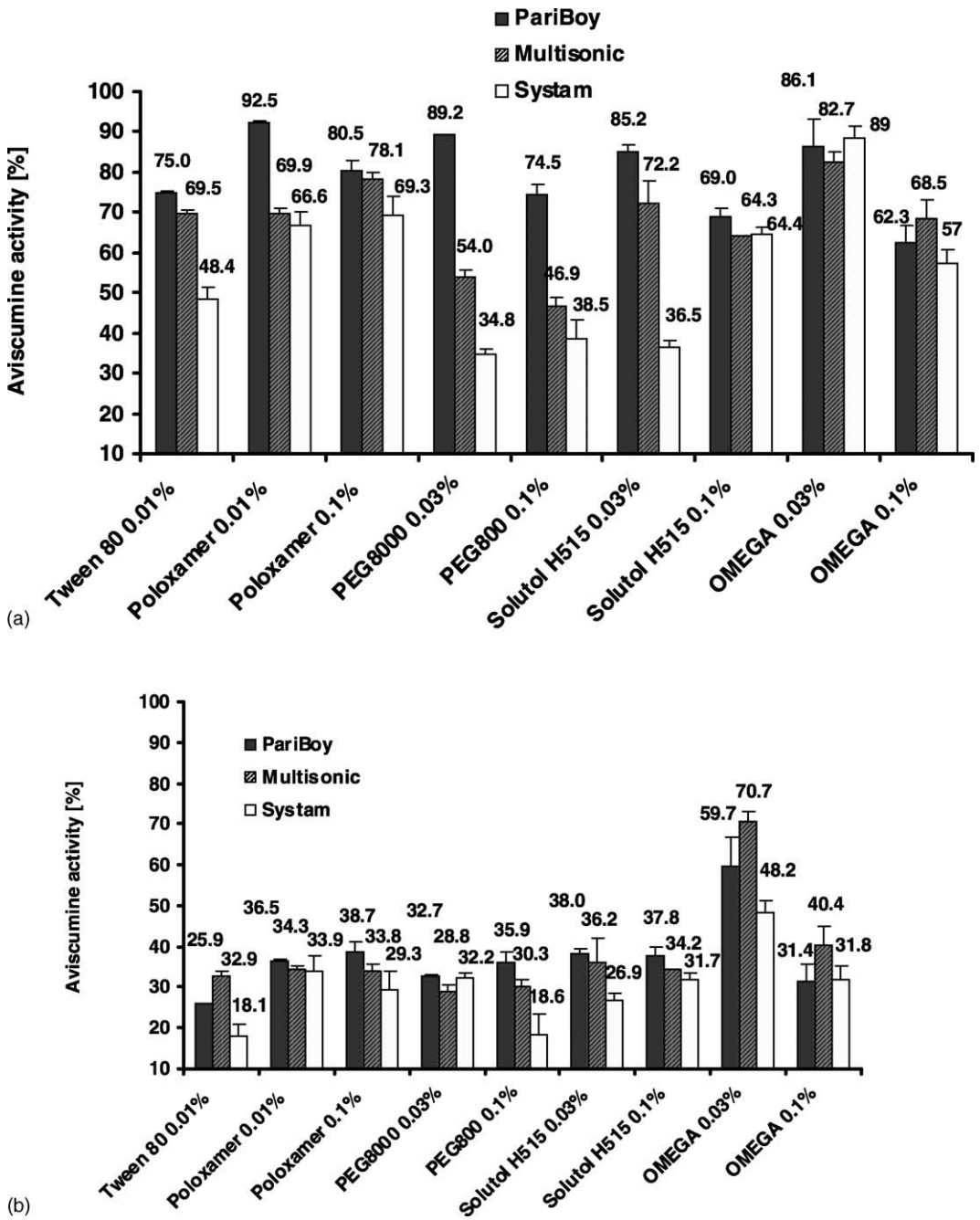


Fig. 6. Influence of surfactants on aviscumine activity (a) in the medicament reservoir and (b) in the condensed aerosol mist after 20 min nebulization time ($n = 3$; mean ± S.D.).

a further increase of the surfactant concentration resulted in a destabilization of the protein in the nebulized solution. For example, by adding 0.01% (w/v) Poloxamer 188 an increase in the aviscumine activity from approximately 75 to 92% could be achieved in the nebulizer reservoir of PariBoy® air-jet nebulizer. Nevertheless, by further increasing the Poloxamer 188 concentration to 0.1% (w/v) the remaining aviscumine activity was only approximately 80%. This could be explained as follows: By increasing the surfactant concentration, the foaming effect is also increased leading to the formation of cavitations which may have a negative effect on the stability of aviscumine. On the other hand, this observation was not valid for the ultrasonic nebulizer in which, for example, no significant increase in the aviscumine activity in the medicament reservoir could be observed using the Multisonic® ultrasonic nebulizer by adding 0.01% (w/v) Poloxamer 188 while an increasing activity from approximately 48 to 67% was observed when delivering the solution with the Systam® ultrasonic nebulizer. By further increasing the poloxamer concentration to 0.1% the activity reaches approximately 78% in the case of Multisonic nebulizer while no further activity increase could be observed in the reservoir of the Systam nebulizer (Fig. 6a). This observation could be attributed to the capability of the jet nebulizer to form cavitations more than the ultrasonic nebulizers due to its mechanism of action. On the other hand, increasing the PEG 8000 from 0.03% (w/v) to 0.1% (w/v) did not show a significant decrease of the activity of aviscumine in the reservoir. For example, this increasing concentration causes a decrease in the aviscumine activity from approximately 89 to 74% and 54 to 47% utilizing the PariBoy® air-jet nebulizer and the Multisonic® ultrasonic nebulizer, respectively, while this decrease in activity was not significant utilizing the Systam® ultrasonic nebulizer. Opposing to that, increasing the concentration of the Solutol HS15 from 0.03% (w/v) to 0.1% (w/v) led to an increase in the activity of aviscumine from approximately 36 to 64%. However, the increase of the OMEGA concentration from 0.03% (w/v) to 0.1% (w/v) showed a significant decrease in the aviscumine activity in the reservoir of all three nebulizers (Fig. 6a).

Apart from that, considering the activity of the collected aviscumine after nebulization, 0.03% (w/v) OMEGA had a superior stabilizing effect on aviscumine [approximately 60, 70 and 48% utilizing the PariBoy® air-jet nebulizer, Multisonic® ultrasonic nebulizer and Systam® ultrasonic nebulizer, respectively] as compared to the other used surfactants(Fig. 6b). This can be explained considering the chemical structure of OMEGA. OMEGA is a member of the *N*-D-Gluco-*N*-methylalkanamide family which belongs to the alkylglycoside non-ionic surfactants. Due to the electrical neutrality of OMEGA, it avoids the alteration of the charge of the solubilized protein and therefore, does not show any negative effect on the stability of proteins (Hildreth, 1982). As can be seen in Fig. 7, the alkylglycoside surfactants possess a straight alkyl chain and a sugar polar head group. OMEGA is characterized by its acyclic sugar residue which allows the hydroxyl groups to change their conformation freely (Smith and Rassi, 1994). This freedom in changing the conformation could allow a much higher affinity for building hydrogen bonds with the hydrophilic surface of aviscumine, while the alkyl chains of the OMEGA molecules can build non-covalent bonds with themselves. Through that binding, the dissolution of aviscumine in the reconstitution medium could be delayed, and by this prevent its irreversible unfolding (Webb et al., 2002) and a better protection of aviscumine on the air–protein interface during nebulization can be achieved. However, 0.1% (w/v) OMEGA showed a lower stabilizing effect as 0.03% (approximately 31, 40 and 32% compared with 60, 71 and 48% utilizing PariBoy® air-jet nebulizer, Multisonic® ultrasonic nebulizer and Systam® ultrasonic nebulizer, respectively). This is attributed to the higher affinity of OMEGA molecules to bind to themselves and aggregate at a higher concentration as well as to the higher foaming effect. In the case of 0.03% Solutol HS15 an increase of the activity was observed from approximately 26 to 38%, from 33 to 36% and from 18 to 27% utilizing the PariBoy® air-jet nebulizer, Multisonic® ultrasonic nebulizer and

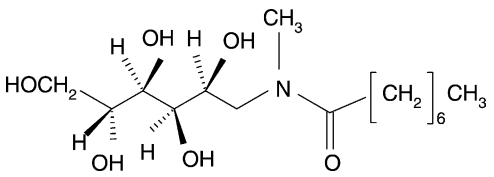


Fig. 7. Chemical structure of octanoyl-*N*-methyl-glucamide.

Systam® ultrasonic nebulizer, respectively. Increasing the concentration of Solutol HS15 to 0.1% did not result in a further significant change of the activity. That was to be observed also with PEG 8000 and Poloxamer 188, except that by increasing the concentration here from 0.03% (w/v) to 0.1% (w/v) led to a reduction in activity from 32 to 19% and from 34 to 29% utilizing the Systam® ultrasonic nebulizer.

4. Conclusion

These observations demonstrate that aviscumine stability *after* nebulization must be taken into consideration as well. Aviscumine has lost approximately 50% of its activity after nebulization. The ultrasonic nebulizers destabilized aviscumine to a higher extent than the air-jet nebulizer. The used lyoprotectants only showed minor stabilizing effects of aviscumine. However, the addition of surfactants to the reconstitution medium can reduce the aviscumine denaturation as well as its adsorption onto the inner surfaces of the nebulizer. The formulation requirements for ultrasonic nebulization are quite different from those found to be suitable for air-jet nebulization.

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