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Research paper

Effect of cryoprotectants on the stability and aerosol performance of nebulized aviscumine, a 57-kDa protein

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Dedicated to Prof. Dr. Dr.h.c. Bernd W. Müller on the occasion of his 60th birthday.

Abstract

Nebulization of aqueous drug solutions is a suitable delivery system for pulmonary application of proteins because it can easily produce droplets small enough to reach the alveolar region. However, proteins are sensitive to nebulization. Therefore, stabilizers need to be added which on the other hand influence the aerosol performance, such as average droplet size or mass output. This research presents the effect of various cryoprotectants such as Na-polyphosphate, $CaCl_2 \times 6H_2O$ and $MgSO_4 \times 7H_2O$ on the stability and aerosol performance of freeze-dried aviscumine after reconstitution and nebulization using three different nebulizers. Formulations containing Tris-buffer, polysorbate 80, Na_2-EDTA and HES450 were lyophilized and reconstituted with a buffered isotonic solution containing 100 mmol/l Tricine-buffer pH 8, 0.03% (w/v) octanoyl-*N*-methylglucamide, 150 mmol/l NaCl and a cryoprotectant. The aviscumine activity was determined by a binding assay. The addition of 0.2% Na-polyphosphate to the reconstitution medium led to retention of approx. 73% of the aviscumine activity after 20 min nebulization with the Systam® ultrasonic nebulizer. It has been observed that 84 and 72% of the activity were retained by the addition of 10 mmol/l $CaCl_2 \times 6H_2O$ using PariBoy® air-jet and Multisonic® ultrasonic nebulizer, respectively. In addition, a decrease in the mean droplet size with increasing the cryoprotectant concentration has been observed. A relationship between the average droplet size, surface tension and viscosity depending on the used cryoprotectant type and concentration could be established.

Keywords: Nebulization; Cryoprotectants; Stability of proteins; Droplet size; Aviscumine

1. Introduction

Mistletoe extracts have been used for non-specific stimulation of the immune system in cancer therapy for many decades. Mistletoe lectin (ML) which belongs to the class of type II ribosome inactivating proteins (RIPs) was identified as the main therapeutic component and is composed of a catalytically active A-chain with rRNA *N*-glycosidase activity and a B-chain with carbohydrate binding properties [1]. After verifying the activity of ML in cancer therapy, its therapeutic application was limited because of its low stability and because of the difficulties in optimizing its concentration in the plant extract [2,3]. To circumvent these problems, aviscumine heterodimer (a 57-kDa protein) can now be produced in *Escherichia coli* by cloning, expression and co-association of the ML's A- and B-chain [4,5]. Meanwhile the anticancer activity of aviscumine has been demonstrated [6]. So far, stable formulations of aviscumine for the parenteral use have been prepared in aqueous solution [7] as well as by lyophilization [8]. In addition, further efforts have been started to find an alternative, non-invasive route of administration. One attractive alternative to treat lung cancer is to deliver aviscumine directly to the lungs taking advantage of the slow mucociliary clearance from the lower lungs, the presumably low metabolic activity and the bypassing of the liver metabolism [9,10]. However, the aerosol size distribution is an important variable in defining the efficiency of aerosolized drugs [11]. Since the nebulizers represent the simplest means of producing an aerosol in the respirable range, they were used to deliver aviscumine for this study. However, the high shear force in the air-jet nebulizer as well

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as ultrasonication in ultrasonic nebulizers could potentially damage a labile drug molecule such as aviscumine as reported earlier for other protein drugs [12,13]. The potential degradation can be reduced by the addition of excipients such as surfactants, buffer salts and cryoprotectants, but the addition of any stabilizing excipient also affects droplet formation. Therefore, careful selection of additives is required [14]. In this study, the effect of various cryoprotectants on the stability and aerosol performance of aviscumine during air-jet as well as during ultrasonic nebulization is investigated.

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 (bat.: 01212/SUG) and all aviscumine solutions were buffered to pH 8.0. Hydroxyethylstarch (MW 450 000) was supplied by Fresenius KABI (Linz, Austria). Tris-(hydroxymethyl)-aminomethane, Na-polyphosphate, $CaCl_2 \times 6H_2O$, $MgSO_4 \times 7H_2O$, octanoyl-*N*-methylglucamide, HCl, NaOH and NaCl were purchased from Merck (Darmstadt, Germany). Polysorbate 80 was obtained from Uniquema (Euperberg, Belgium). Tricine, BSA, OPD-tablets, asialofetuin and anti-mouse-IgG-POD were received from Sigma–Aldrich (Steinheim, Germany). Principally, all the utilized chemicals were of analytical grade and 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 a formulation developed by Gloger for the stabilization of aviscumine during freeze-drying has been adopted [15]. Aviscumine formulations containing Tris-buffer, Polysorbate 80, Na_2-EDTA and HES450 have been freeze-dried. Because of the higher stability of aviscumine in the alkaline region, the solution was buffered to pH 8.0 with 100 mmol/l Tris-(hydroxymethyl)-aminomethane and the final pH adjustment was done with 1 N HCl. Tris-buffer has been chosen due to its low pH-shift during freezing which may denaturize the protein [16,17]. Polysorbate 80 has been added to prevent the degradation of the protein at the ice–protein interface [18] and the irreversible unfolding of the protein due to the rapid reconstitution [19]. Na_2-EDTA was used here as an antioxidant to protect the protein against metal ions [20]. A lyoprotectant as HES450 has also to be added because of its main role to stabilize the protein due to its amorphous character enabling an embedding of the protein and preventing its degradation at the protein–ice interface [21]. Lyoprotectants can also prevent the collapse of the cakes during freeze-drying by raising the glass transition temperature T'_g [22] and by replacing the water after drying by building hydrogen bonds to the protein surface (water replacement theory) [23]. Next, 100 mmol/l Tris-buffer, 0.1% (w/v) polysorbate 80 and 8% (w/v) HES450 were dissolved in water. This solution was adjusted to pH 8 with 1 N HCl. Then, 0.01% (w/v) Na_2-EDTA and 100 μg/ml aviscumine were added and homogenized. The volume was adjusted with water to volume and the solution was shaken.

2.2.2. Freeze-drying procedure

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 1.

2.2.3. Preparation of a solution for nebulization

The influence of different salts with cryoprotective properties such as (0.05 and 0.2% (w/v)) sodium polyphosphate, (1 and 10 mmol/l) $CaCl_2 \times 6H_2O$ and (0.05 and 0.2% (w/v)) $MgSO_4 \times 7H_2O$ on the stability and the aerosol performance of aviscumine has been investigated by the addition of these salts to a buffered isotonic solution containing 150 mmol/l NaCl, 0.03% (w/v) octanoyl-*N*-methylglucamide and 100 mmol/l Tricine-buffer. The pH value of these solutions was adjusted to pH 8.0 with 1 N NaOH. These solutions were used to reconstitute the lyophilisates. Finally, the reconstituted lyophilisates were nebulized.

2.2.4. Air-jet and ultrasonic nebulization

All nebulization experiments were completed 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

Table 1
Freeze-drying program used for drying of the aviscumine formulations

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

by the manufacturer, are detailed in Table 2. The air-jet nebulizer was operated continuously for 20 min, whereas the ultrasonic nebulizers have been operated discontinuously for only 20 s followed by a 20 s cut-out to prevent the degradation of aviscumine by heating [24]. These cycles have been repeated up to 20 min total nebulization time so that the temperature in the reservoir did not exceed 35°C during nebulization. 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 at a concentration of 2 μg/ml to act as a non-absorbable, non-denaturable marker [12]. The aerosol mist was then collected and samples were taken from the reservoir as well as from the collected aerosol and diluted with a dilution factor of 100. The samples have been stored at −70°C until analysis [32].

2.2.5. 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. The galactose specific lectin (B-chain) binds to the glycoprotein (asialofetuin) on the micro titer plate (Nunc, Wiesbaden, Germany) and the detection of the molecule was carried out by binding monoclonal anti-aviscumine antibody to the toxic A-chain. BSA 1% was used for saturation of empty spaces. Anti-mouse-IgG-peroxidase was used as secondary detection antibody. By the addition of *o*-phenylene-diamine [OPD], which is a peroxidase substrate, a soluble orange-brown color is produced which can be detected spectrophotometrically at 492 nm. The relative activity was determined related to a freshly prepared solution containing the same excipients as the sample [25].

The concentration of CF was determined using a Perkin–Elmer-Fluorimeter Model LS 50B (Perkin–Elmer, Buckinghamshire, England). Samples were placed in 10 mm path length polystyrol cuvettes (Sarsted, Nümbrecht, Germany) and the fluorescence intensity was detected at an excitation and emission wavelength of 490 and 515 nm, respectively [26,27]. The aviscumine concentration was calculated in relation to the concentration of CF in the initial solution before nebulization. The percentage of the remaining aviscumine activity in the reservoir and after collection of the aerosol cloud was referenced to the CF concentration using Eq. (1):

$$\text{Activity}_{\text{aviscu min } e} = A_r - (C_{CF} - A_n) \quad (1)$$

where A_r and A_n (all in percent) represent the aviscumine activity remaining after reconstitution and nebulization, respectively. C_{CF} is the concentration (in percent) of CF after nebulization.

2.2.6. 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 method as described in Section 2.2.5. The percentage of the 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.

2.2.7. Determination of the droplet size

The volume median diameter (VMD; X_{50}) of the nebulized aerosol has been determined utilizing a HELOS Laser diffractometer (Sympatec, Clausthal-Zellerfeld, Germany). Assuming that the density of the nebulized droplets is of equal value, the VMD could be correlated to the mass median diameter (MMD) [28]. The filling volume was adjusted to 5 ml before each measurement and the measurements were done at 24 ± 2°C and a relative humidity of 45 ± 5%. An isotonic NaCl solution (0.9% (w/v) was used as a standard. The measuring conditions and other technical data are summarized in Table 3.

2.2.8. Determination of the surface tension

To study the influence of the surface tension of the drug solution on the droplet size, the surface tension of the reconstituted lyophilisates has been measured at room temperature utilizing a Processor Tensiometer K12 (Krüss, Hamburg, Germany). To ensure the validity of the results, each measurement was repeated 10 times ($n = 10$).

2.2.9. Determination of the viscosity

The dynamic viscosity of the drug solution has been measured according to Ph. Eur. 1997 using a capillary viscometer with a 0_c capillary (Schott, Darmstadt, Germany) at a temperature of 24°C.

Table 2
Technical data of the used nebulizers (information given by the supplier)

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

[a] MMD, mass median diameter.

Table 3
Laser diffraction parameters and corresponding technical data

Lens	20 mm
Measuring range	0.18 to 35 μm
Distance between nebulizer and laser beam	63 mm
Measuring time for every cycle	100 s
Measuring intervals	One measurement every 20 s (5 measurements/cycle)
Number of cycles	3
Flow rate at the nebulizer mouth piece	40 ml/min

3. Results and discussion

3.1. Influence of cryoprotectants on the aviscumine activity

Most native proteins are only marginally stable, with a free energy of stabilization of only about 50 ± 15 kJ/mol. A protein formulation in an aqueous solution is particularly susceptible to conformational changes in its native structure, often leading to physical or chemical degradation. Therefore, proteins are commonly lyophilized to achieve long-term stability [29,30]. However, the addition of excipients is generally required to yield high recoveries of native protein after lyophilization and reconstitution [19]. In this research, a fixed formulation for lyophilization as well as for reconstitution of aviscumine has been chosen. This standard reconstitution medium contained a surfactant (0.03% (w/v) octanoyl-*N*-methylglucamide (OMEGA)) which covers the stagnant boundary layer during dissolution and prevents the irreversible unfolding of the protein [19]. Surfactants also have the ability to protect the protein at the air–protein interface and to reduce the adsorption of proteins onto the surfaces of the nebulizer if the protein was unfolded during nebulization [12]. Nevertheless, in previous studies OMEGA showed a beneficial effect on aviscumine as compared with other surfactants, which can be explained considering its alkylglycosidic nature [31,32]. OMEGA possesses an acyclic sugar head which has high affinity for building hydrogen bonds with the hydrophilic surface of proteins and protects it at the air–protein interface during nebulization. However, inhalation of aqueous solutions of certain compositions may cause bronchoconstriction and coughing [33,34]. Neutral isotonic solution is the best compromise to minimize this bronchoconstriction or coughing while achieving acceptable stability with respect to degradation. These considerations led to the addition of 150 mmol/l NaCl and buffering this isotonic reconstitution medium with 100 mmol/l Tricine to pH 8. Tricine-buffer was selected because of the amino acid nature of this compound, which has the potential to interact directly with proteins, e.g. by hydrogen bonding to peptide groups, and so allowing a better stabilization [35]. However, to compare between the stabilizing effect of the used cryoprotectants, these were added to the standard reconstitution medium. The remaining aviscumine activity has been determined after reconstitution, in the nebulizer reservoir after nebulization as well as in the collected nebulized mist.

3.1.1. Determination of aviscumine activity after reconstitution

Zhang et al. have shown that the addition of different excipients such as amino acids, heparin sulphate or surfactants to the reconstitution solutions increased the recovered activity of proteins [36,37]. In this research, the effect of adding various cryoprotectants to the reconstitution medium on the activity of aviscumine was studied. The therapeutic dose of aviscumine in the nebulized solution was assumed to be 10 μg/ml; nevertheless, it was lyophilized in a concentration of 100 μg/ml. The choice of this higher concentration of aviscumine is based on the experience that increasing the protein concentration stabilizes proteins during freeze-drying [29]. On the other hand, the lyophilisates must be reconstituted with a dilution factor of 10. This relatively high dilution factor and the rapidly dissolving character of the lyophilisates increase the probability of irreversible defolding of this protein and cause the denaturation of aviscumine, so the addition of stabilizers as cryoprotectants was necessary. The various cryoprotectants were added to the reconstitution medium directly before reconstitution and its effects on aviscumine stability were then analyzed. No significant stabilizing or destabilizing effect of the various added cryoprotectants on the stability of aviscumine after reconstitution could be observed. Nevertheless, the addition of Na-polyphosphate showed a tendency to improve the aviscumine stability after reconstitution and the remaining activity was increased with increasing polyphosphate concentration (approx 94.6 and 96.8% remaining activity after reconstitution with 0.05 and 0.2% (w/v) Na-polyphosphate, respectively, compared with 94% for the control formulation). This stabilizing effect could be attributed to the lowering of the solubility of proteins by raising the salt concentration as well as to the higher viscosity of the Na-polyphosphate solution as compared to other salts, which delay the dissolution of the lyophilisates and prevent the irreversible denaturation of the protein [19,38]. On the other hand, the addition of 1 mmol/l $CaCl_2 \times 6H_2O$ showed no improvement of aviscumine stability (approx. 92.1% remaining activity), while at the higher concentration of 10 mmol/l a tendency to stabilize

the aviscumine could be observed (remaining activity of approx. 96.3% compared to 94% for the control). This observation could be explained by the possible binding of Ca^{2+} to the protein which increases the net charge of the protein and causes a repulsive force between the protein molecules. This electrostatic repulsive force should prevent the protein association or aggregation and hence stabilize the aviscumine [38]. Moreover, the addition of $MgSO_4 \times 7 H_2O$ did not lead to any improvement in the stability of aviscumine. (Fig. 1)

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

The remaining aviscumine activity in the nebulizer reservoir as well as in the collected aerosol mist after 20 min nebulization time is shown in Fig. 2(a, b), respectively. Generally, ultrasonic nebulization led to a higher extent of denaturation of aviscumine than air-jet nebulization. These observations are in accordance with the experiments done on rhDNase (an approved protein for treatment of cystic fibrosis). These experiments also have shown that rhDNase can be aggregated by ultrasonic nebulization if the melting transition temperature is approached or exceeded in the solution during nebulization [24]. So, the British Thoracic Society has suggested in 1997 to use only jet nebulizers for the delivery of rhDNase [39]. The heating effect of ultrasonic nebulizers on the stability of lactate dehydrogenase (LDH) also has been studied and a correlation between the increasing temperature in the medicament reservoir and the stability of LDH was found [13]. However, control of the temperature in the medicament reservoir below the melting transition temperature of aviscumine ($T_m = 50°C$) [25] still led to a high loss of its activity, suggesting that other factors can contribute as well to the degradation. Sato et al. have attributed the degradation of alpha-interferon in an ultrasonic nebulizer to the formation of cavitations in the nebulizing solution which descended from the transduced ultrasonic waves [40]. In addition, it might also be possible that the electric energy causing the vibration of the piezoelectric crystal could result in a change in the net charge of the proteins and so affects their stability. However, these changes in the net charge could also be affected by the ion concentration in the reconstitution solution and, hence, by the type and concentration of the added cryoprotectant; while the addition of 0.05% (w/V) Na-polyphosphate showed no destabilizing effect utilizing the PariBoy® air-jet nebulizer, a 20 and 10% decrease of the aviscumine activity in the medicament reservoir after 20 min nebulization time could be observed with the Multisonic and Systam® ultrasonic nebulizers (Fig. 2a). This destabilizing effect could be attributed to the increase in the net charge of the protein due to the binding of the Na^+ ions to the protein surface. Thus, an increase of the free energy of the protein should be induced and the resulting repulsive forces cause decrease in the stability of the protein. Moreover, the loss of aviscumine activity was higher with the Multisonic than with the Systam® ultrasonic nebulizer because of the difference in the method of energy transmission between the two nebulizers. In the Multisonic® ultrasonic nebulizer the piezoelectric crystal is in a direct contact with the nebulizing solution, which allows a higher electrostatic effect as compared to the Systam® ultrasonic nebulizer where water is used as a transducer medium. By increasing the Na-polyphosphate concentration from 0.05 to 0.2% (w/v) an increase of the aviscumine activity has been achieved (from approx. 79 to 87%, 66 to 82% and from 68 to

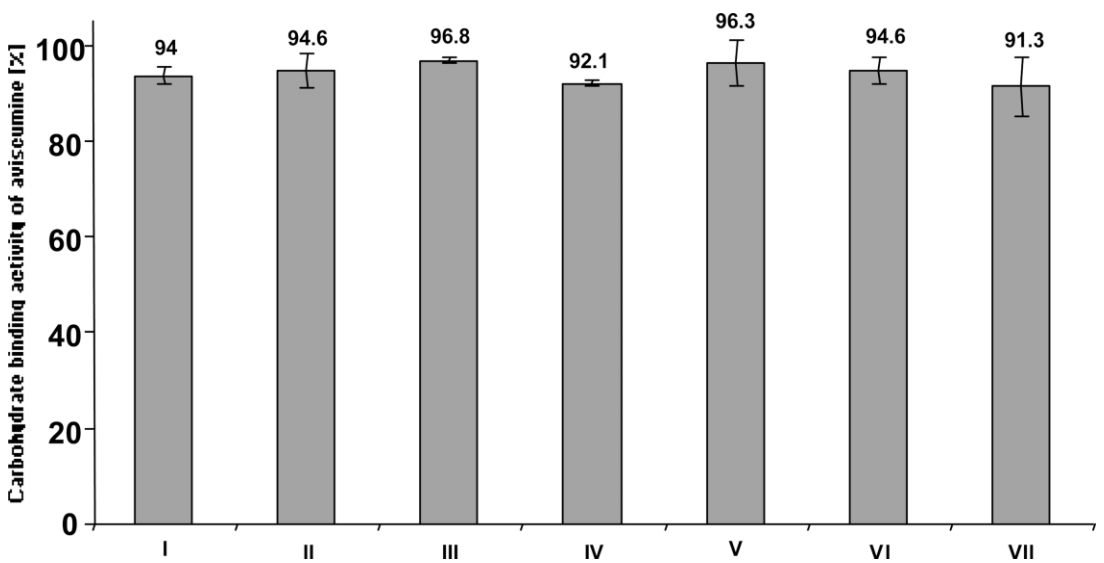

Fig. 1. Influence of cryoprotectants in the reconstitution solution on the carbohydrate binding activity of aviscumine after reconstitution of the standard freeze-dried product (error bars = SD) [I = Control without a cryoprotectant; II = 0.05% (w/v) Na-polyphosphate; III = 0.2% (w/v) Na-polyphosphate; IV = 1 mmol/l $CaCl_2 \times 6H_2O$, V = 10 mmol/l $CaCl_2 \times 6H_2O$, VI = 0.05% (w/v) $MgSO_4 \times 7H_2O$, VII = 0.2% $MgSO_4 \times 7H_2O$].

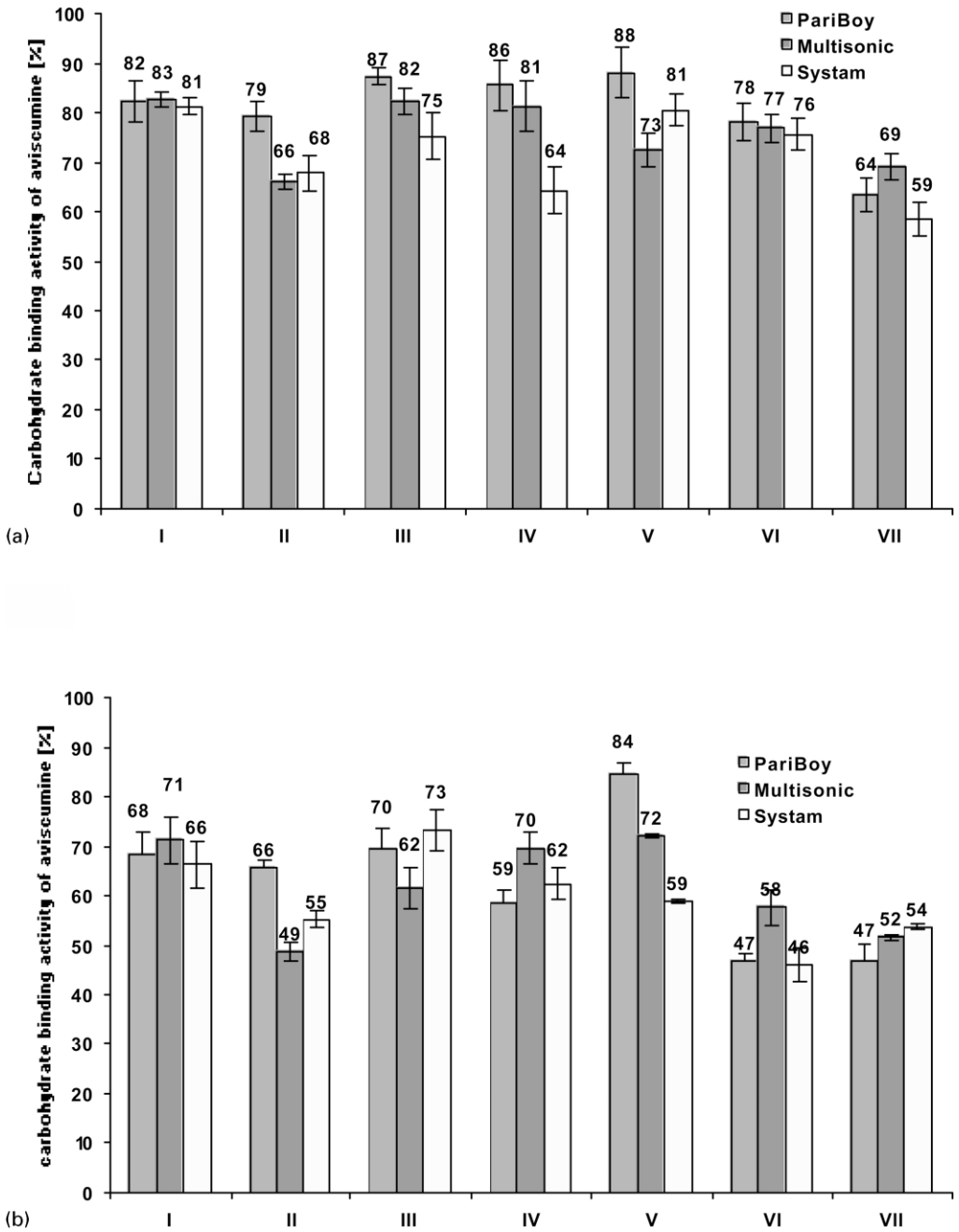

Fig. 2. Influence of cryoprotectants on the carbohydrate binding activity of aviscumine (a) in the reservoir and (b) in the nebulized solution after 20 min nebulization time (error bars = SD) [I = Control without a cryoprotectant; II = 0.05% (w/v) Na-polyphosphate; III = 0.2% (w/v) Na-polyphosphate; IV = 1 mmol/l $CaCl_2 \times 6H_2O$, V = 10 mmol/l $CaCl_2 \times 6H_2O$, VI = 0.05% (w/v) $MgSO_4 \times 7H_2O$, VII = 0.2% $MgSO_4 \times 7H_2O$].

75% with PariBoy® air-jet, Multisonic and Systam ultrasonic nebulizers, respectively), probably due to the higher viscosity of the resulting solution because of its polymeric nature which can decrease the repulsive forces between the protein molecules (Fig. 2a). This trend was also observed in the analyzed collected aerosol for these two formulations (Fig. 2b).

By adding 1 mmol/l $CaCl_2 \times 6H_2O$ to the reconstitution medium a 10% decrease of aviscumine activity after 20 min nebulization has been observed in the aerosol mist with the PariBoy® air-jet nebulizer, while no significant degradation has been observed in the reservoir. Opposite to that, an approx. 20% decrease in activity has been observed in the medicament reservoir of the Systam® ultrasonic nebulizer and no significant change in the activity was observed in the condensed aerosol mist. It is suggested that Ca^{2+} ions could also bind to the protein (preferential binding theory) [23] but due to its low concentration and the absence of electric excitation no repulsion occurs until droplet formation, which has a high surface area and allow a higher repulsion

between the protein molecules. By increasing the concentration of $CaCl_2 \times 6H_2O$ to 10 mmol/l, an approx. 24% increase of activity in the condensed aerosol after jet nebulization was achieved (Fig. 2b). The stabilizing effect of $CaCl_2$ in this concentration is suggested to be due to the stabilizing effect of calcium ions for the disulfide bond which is the main binding site between A- and B-chain of aviscumine [41]. On the other hand, $MgSO_4 \times 7H_2O$ in both concentrations showed a negative effect on the stability of aviscumine activity.

The results did not reveal a relationship between the preferential exclusion theory and the stabilizing effect of $CaCl_2$ and $MgSO_4$, although the preferential exclusion of salts increases in the order $Cl^- < SO_4^{-2}$ for anions and $Mg^{2+} = Ca^{2+}$ for cations. Thus, $MgSO_4$ should be more excluded from the protein surface and allow a better stabilization. A possible explanation to this contradiction can be found considering the surface tensions of the investigated solutions. A decreasing surface tension of the nebulizing solution allows the production of smaller droplets by the nebulizers and so decreases the stress conditions occurring due to the recycling of the large droplets. Consequently, the total residence time of the nebulizing solution in the medicament reservoir will decrease (decreasing the exposure time to the thermal stress in the ultrasonic nebulizer) and the exposure to shear stress due to the recycling will decrease as well. In addition, $CaCl_2$ can decrease the surface tension of the nebulized solution more than $MgSO_4$ within the used concentration ranges: by the addition of 10 mmol/l $CaCl_2 \times 6H_2O$ 11 mN/m decrease of the surface tension has been measured. On the other hand, the addition of 0.05% (w/V) $MgSO_4 \times 7H_2O$ led to a 9 mN/m decrease in surface tension while a further increase in the $MgSO_4$ concentration to 0.2% (w/v) did not further decrease the surface tension significantly (Fig. 4). Consequently, the lower surface tension of the $CaCl_2 \times 6H_2O$ containing solutions allowed a better stabilizing condition for aviscumine during nebulization. These results could also be explained by the presence of 0.06% (w/v) ammonium sulfate in the nebulized solution that is carried over from the bulk solution of aviscumine. It is that this sulfate ion concentration in the solution is high enough to cause the expected preferential exclusion and any additional increase did not show an improvement of the aviscumine activity. The decrease in surface tension of the drug solution caused by the addition of $CaCl_2 \times 6H_2O$ resulted in the formation of smaller droplets which can bypass the baffle and decrease the degradation of proteins due to fewer recycling cycles in the reservoir. Consequently, the drug output will increase and degradation by shear stress and ultrasonic waves will be reduced [42].

3.2. Optimization of the aerosol performance

3.2.1. Determination of the droplet size

Particle sizes in the range of 1–5–μm are considered to be optimal for the delivery of therapeutic agents to the alveolar region. Nebulizers can theoretically produce droplets within that range. Nevertheless, the addition of excipients to the nebulizing solution may have a dramatic effect upon droplet formation; therefore, a careful selection of additives is required [14]. In this study, the effect of the cryoprotectants on the droplet size has been investigated. The results are demonstrated in Fig. 3. By increasing the Na-polyphosphate concentration from 20 to 100 mmol/l a 0.6 and 0.8 μm

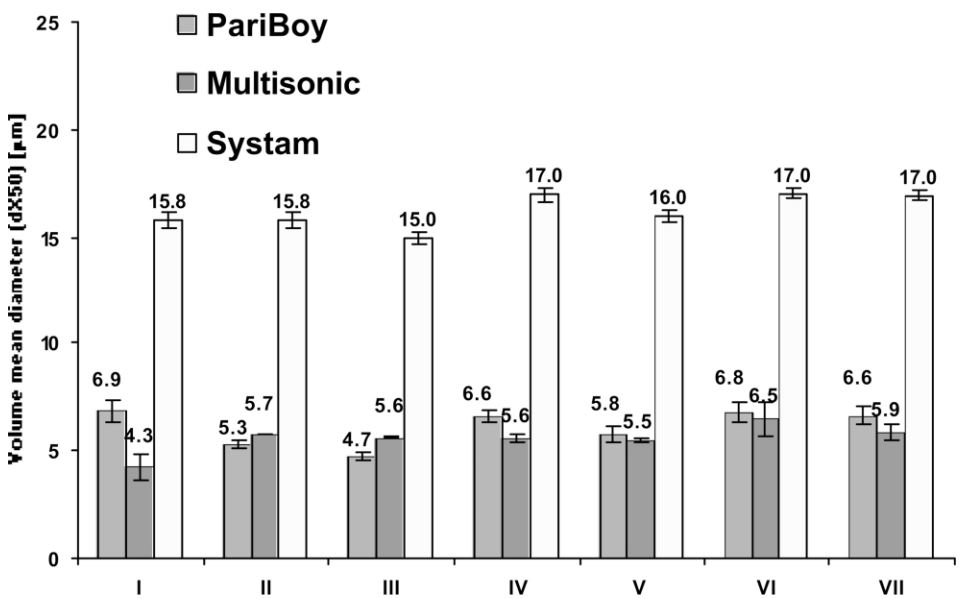

Fig. 3. Influence of cryoprotectants on the droplet size of nebulized aviscumine solution (error bars = SD) [I = 0.9% (w/v) NaCl; II = 0.05% (w/v) Na-polyphosphate; III = 0.2% (w/v) Na-polyphosphate; IV = 1 mmol/l $CaCl_2 \times 6H_2O$, V = 10 mmol/l $CaCl_2 \times 6H_2O$, VI = 0.05% (w/v) $MgSO_4 \times 7H_2O$, VII = 0.2% $MgSO_4 \times 7H_2O$].

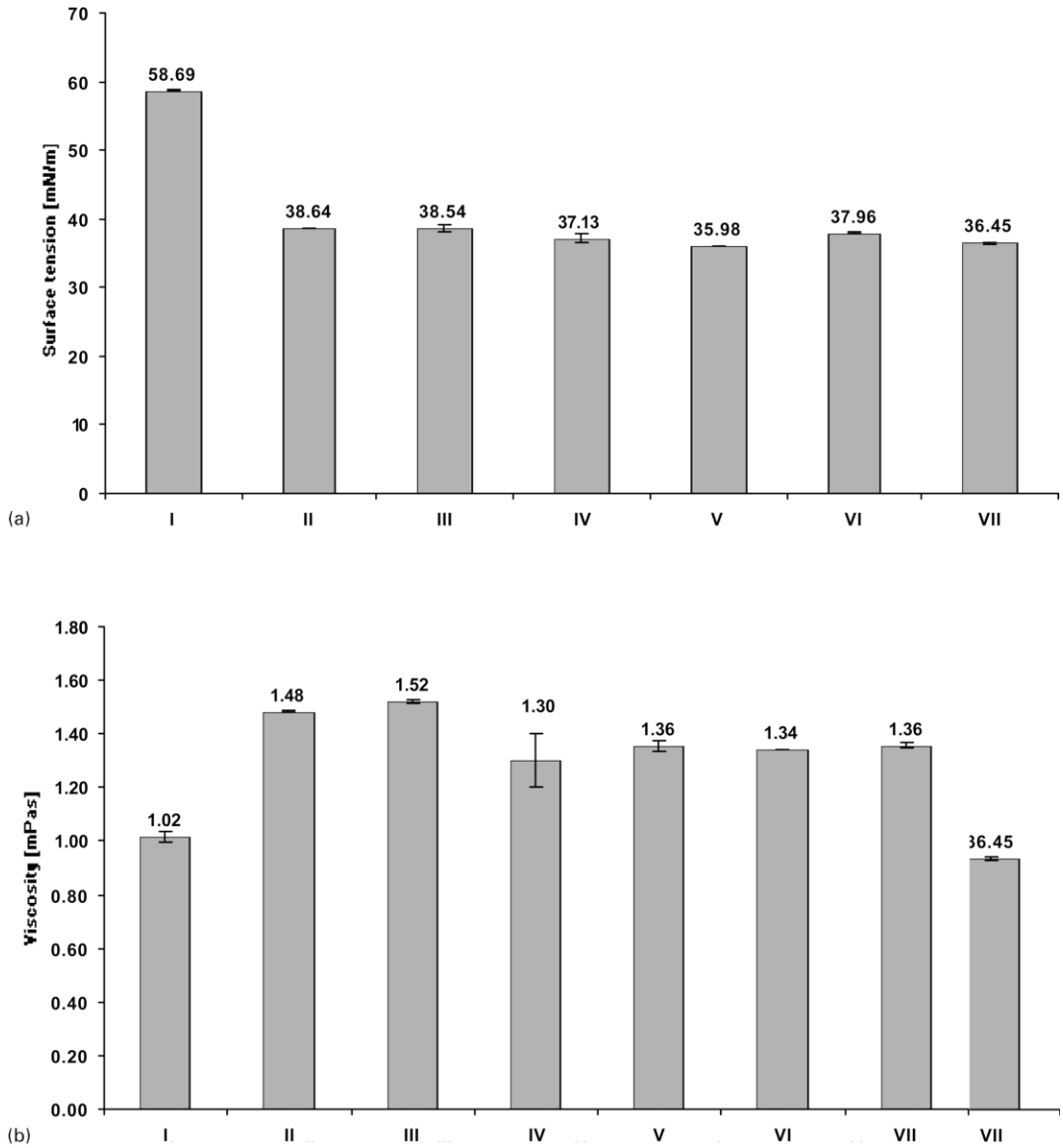

Fig. 4. Influence of cryoprotectants on (a) the surface tension and (b) the viscosity of the nebulized solution (error bars = SD) [I = 0.9% (w/v) NaCl; II = 0.05% (w/v) Na-polyphosphate; III = 0.2% (w/v) Na-polyphosphate; IV = 1 mmol/l $CaCl_2 \times 6H_2O$, V = 10 mmol/l $CaCl_2 \times 6H_2O$, VI = 0.05% (w/v) $MgSO_4 \times 7H_2O$, VII = 0.2% $MgSO_4 \times 7H_2O$].

decrease in the average droplet size has been achieved with the PariBoy® air-jet and the Systam® ultrasonic nebulizer, respectively, while no significant shift in the droplet size has been observed using the Multisonic® device. Increasing the $CaCl_2 \times 6H_2O$ concentration from 1 to 10 mmol/l led to a 0.8 and 1 μm decrease in the droplet size with the PariBoy® air-jet and the Systam® ultrasonic nebulizer, respectively. This decrease was also not significant with the Multisonic® ultrasonic nebulizer. In the case of $MgSO_4 \times 7H_2O$ the increasing concentration has not shown any significant effect on the droplet size.

3.2.2. Determination of surface tension and viscosity of the nebulizing solutions

Theoretically, the aerosol particle size produced by nebulization should be proportional to the surface tension of the drug solution but experimental work in this area has so far been contradictory [43,44]. Callaghan and Barry asserted that the primary droplet size is related to the surface tension but the baffles control the output size [42]. On the other hand, the viscosity of the drug solution should theoretically influence the mass flow rate of the liquid which also may affect the average primary droplet size [45]. McCallion et al. studied two ultrasonic nebulizers and found that the droplet size was proportional to the viscosity of the nebulized fluid [46]. In the present study these two factors were also analyzed with the aim to investigate their influence. The results of the measurements of the surface tension as well as the viscosity of the various cryoprotectants for the investigated formulations are demonstrated in Fig. 4(a, b),

respectively. In general, the addition of cryoprotectants to the nebulizing solutions decreased the surface tension and increased the viscosity of the solutions compared to 0.9% (w/v) NaCl. By increasing the Na-polyphosphate concentration from 20 to 100 mmol/l only a 0.04 mPa s increase of the viscosity and a small decrease of the surface tension could be observed. Increasing the concentration of $CaCl_2 \times 6H_2O$ from 1 to 10 mmol/l resulted in a 0.05 mPa s increase of the viscosity, while a 1.15 mN/m reduction of the surface tension has been observed. When $MgSO_4 \times 7 H_2O$ was used, no significant increase of the viscosity occurred while the surface tension was decreased by 1.51 mN/m. It is suggested that this small decrease of the viscosity has a minor influence on the droplet size whereas the decrease in the surface tension could explain the decrease in the droplet sizes in the case of the $CaCl_2 \times 6 H_2O$. However, other factors as the temperature, the concentration effect and the different principles of droplet formation of the used nebulizers could also play a role with respect to the droplet size.

3.2.3. Effect of nebulization time on aviscumine activity

Aiming to determine the effect of the nebulization time on the stability of aviscumine and the factors affecting it, the aviscumine activity has been determined after 5, 10, 15 and 20 min nebulization time. Fig. 5 presents (as an example) the effect of the nebulization time on the nebulized aviscumine for the formulation with 10 mmol/l $CaCl_2 \times 6H_2O$. A decrease of approx. 11% of the activity of aviscumine could be observed after the first 5 min using the PariBoy® jet nebulizer. After that, no further significant decrease in the activity was determined. Opposing to that a continuous decrease in the activity of aviscumine could be observed using the Systam® ultrasonic nebulizer (approx. 37% during 20 min nebulization time) while a sharp decrease in the activity within the first 5 min (approx. 26%) followed by fluctuating values with a final increase was determined using the Multisonic® ultrasonic nebulizer. To explain these observations, the changes in the concentration of CF as an internal standard over the observed nebulization time had to be taken into account as well (Fig. 6). Generally, the decreasing activity of aviscumine during the nebulization process could be attributed to the longer exposure to stress conditions such as heat and ultrasonic waves in the ultrasonic nebulizers or shear forces due to the multiple recycling of large droplets in the jet nebulizer. According to previous studies and other work, it has been observed that the protein degradation is typically lower in jet nebulizers as compared to ultrasonic nebulizers [24,32]. However, the steady state which has been reached in the PariBoy® jet nebulizer could be explained by the increasing protein concentration (8%) during the last 15 min of nebulization which opposes the destabilizing effects of the nebulization process itself. On the other hand, the sharp decrease in the activity within the first 5 min in the Multisonic® nebulizer followed by a fluctuation could be attributed to the sharp increase in the temperature in the medicament reservoir (11°C during the first 3 min) followed by an increasing drug concentration during the last 15 min, which reaches 15%. Opposing to that, the continuously decreasing activity in the Systam® nebulizer is due to the higher destabilizing conditions compared to the Multisonic® nebulizer due to

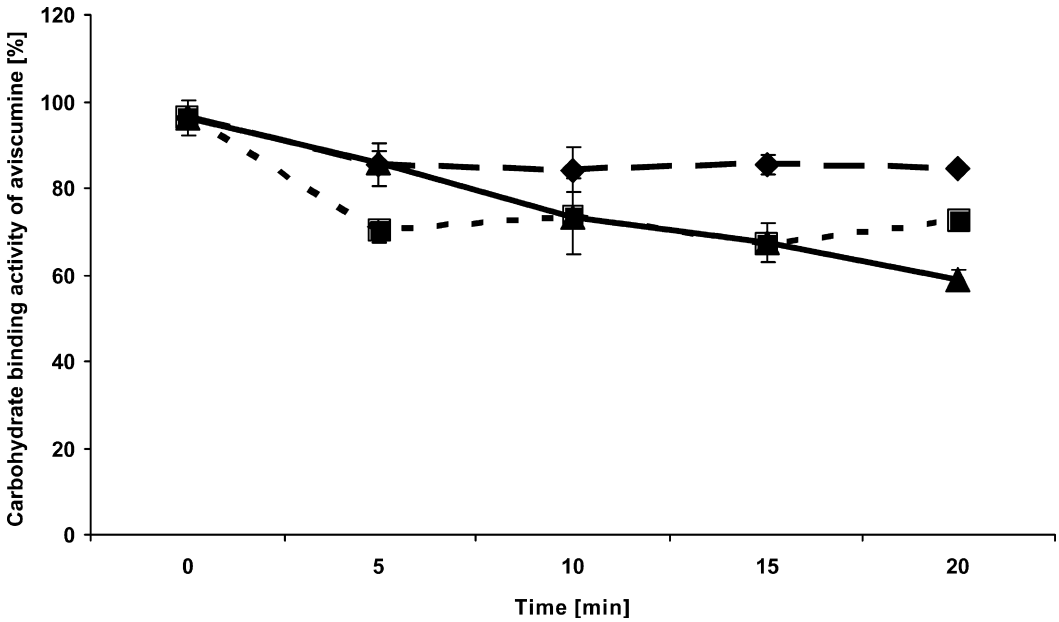

Fig. 5. Influence of nebulization time on the carbohydrate binding activity of aviscumine during a time range of 20 min, for the formulation: 10 mmol/l $CaCl_2 \times 6H_2O$, 100 mmol/l Tricine, 0.03% (w/v) OMEGA and 80 mmol/l NaCl in PariBoy® Jet (◆), Multisonic® (■) and Systam® (▲) ultrasonic nebulizer (error bars = SD).

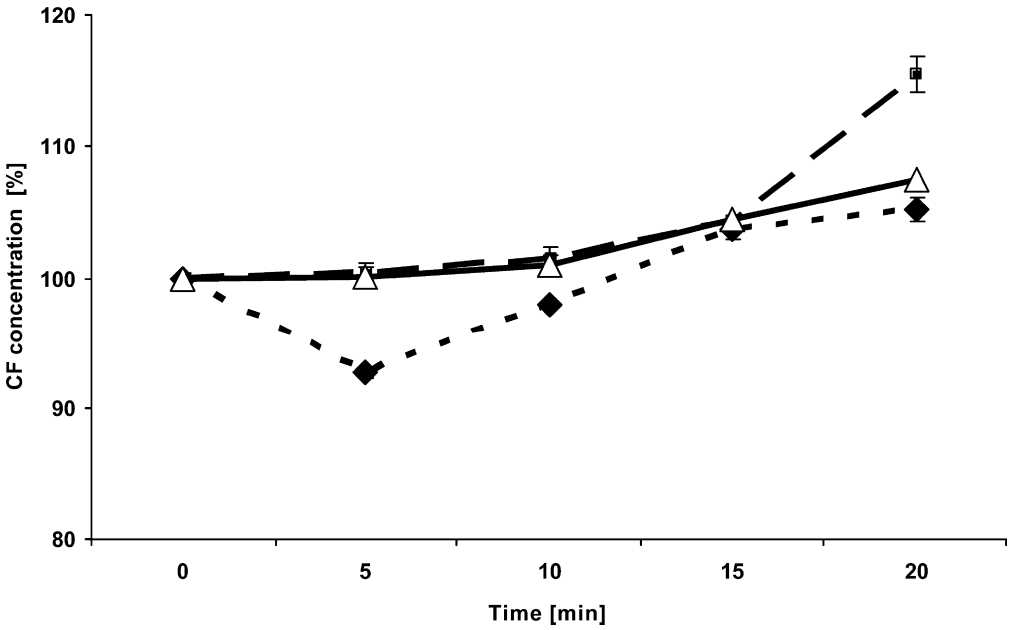

Fig. 6. Increase of carboxyfluorescene sodium concentration in the reservoir solution during nebulization for the formulation: 10 mmol/l $CaCl_2 \times 6H_2O$, 100 mmol/l Tricine, 0.03% (w/v) OMEGA and 80 mmol/l NaCl with PariBoy® Jet (◆), Multisonic® (■) and Systam® (▲) ultrasonic nebulizer.

the higher frequency of vibration (1.7 MHz in the case of the Multisonic® compared to 2.4 MHz in the Systam® nebulizer). Also, the small increase in the drug solution concentration of approximately 5% after 20 min nebulization time was not high enough to oppose the frequency effect. Despite the higher frequency of vibration in the Systam® nebulizer, the increase in the concentration was lower than in the case of Multisonic® nebulizer (15% increase). This observation is attributed to the direct contact of the piezoelectric crystal with the drug solution in the Multisonic® reservoir which results in a higher temperature in the reservoir and so higher solvent evaporation.

4. Conclusions

It has been shown that aviscumine is inactivated by air-jet as well as by ultrasonic nebulization. However, the ultrasonic nebulization destabilized aviscumine to a higher extent than the air-jet nebulization. In this study it was shown, that the addition of cryoprotectants to the reconstitution medium could stabilize the lyophilized aviscumine after reconstitution as well as during nebulization. The effect of increasing salt concentrations on the stability and on the aerosol performance of aviscumine has also been studied and a correlation between concentration and its stabilizing effect could be demonstrated. By the addition of 10 mmol/l $CaCl_2 \times 6H_2O$ to the reconstitution medium, a 16% increase in the aviscumine activity after nebulization with PariBoy® air-jet nebulizer has been achieved. This may be attributed to its capability to stabilize the disulfide bond between the A- and B-chain of aviscumine. Opposing to that no stabilizing effect has been observed when the ultrasonic nebulizers were utilized.

The effect of cryoprotectants on the droplet formation has also been investigated. It was observed that the droplet size tends to decrease with increasing salt concentration. To explain this phenomenon, the effect of the excipients on the surface tension as well as the viscosity and their influence on the droplet size has been analyzed but no clear correlation has been found and further research should be done in this area. In conclusion, the formulation requirements for ultrasonic nebulization may be quite different from those found to be suitable for air-jet nebulization and a clear distinction must be made between the two modes of delivery in this respect.

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