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# Physicochemical compatibility of nebulizable drug mixtures containing dornase alfa and ipratropium and/or albuterol

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Patients suffering from cystic fibrosis (CF) often need to inhale multiple doses of different nebulizable drugs per day. Patients attempt to shorten the time consuming administration procedure by mixing drug solutions/suspensions for simultaneous inhalation. The objective of this experimental study was to determine whether mixtures of Pulmozyme® inhalation solution with Atrovent® or Sultanol® are physicochemically compatible. Drug combinations were prepared in accordance with the product information and clinical practice by mixing the content of one respule Pulmozyme® with 2 mL Atrovent® LS and 0.5 mL Sultanol® Inhalationslösung (inhalation solution) or with one respule of either Atrovent® 500 μg/2 mL Fertiginhalat (unit dose formulation) or Sultanol® forte Fertiginhalat. Test solutions were stored at room temperature and exposed to light. Dornase alfa activity was determined by a kinetic colorimetric DNase activity assay. Ipratropium bromide and albuterol concentrations were investigated by a stability-indicating HPLC assay with ultraviolet detection. Physical compatibility was determined by visual inspection and measurements of pH and osmolality. Ipratropium bromide and albuterol concentrations were not affected by mixing the drug products. Dornase alfa activity is affected by benzalkonium chloride, used as excipient in Atrovent® LS and Sultanol® Inhalationslösung, and disodium edetate used as an excipient in Atrovent® LS. Patients should be advised not to mix Pulmozyme® with Atrovent® LS and/or Sultanol® Inhalationslösung, because of the incompatibility reaction. Mixtures of Pulmozyme® with Atrovent® 500 μg/2 mL Fertiginhalat or Sultanol® forte Fertiginhalat can be designated as compatible for a limited period of time.

## 1. Introduction

For patients suffering from airway diseases inhalation of aerosolized medications is a mainstay of therapy. Especially for patients with poor inhalation pattern, e.g. small children and patients suffering from cystic fibrosis (CF), nebulization is the preferred way of administration. Nebulizers convert drug solution/suspension by ultrasound or a jet stream of compressed air into an aerosol. Aerosolized droplets or particles should be 1 to 5 μm diameter in size, to ensure that the droplets reach bronchioles (Boe et al. 2001).

Chronic pulmonary infections are the major cause of morbidity and mortality in patients suffering from cystic fibrosis (CF). Both short and long-term studies indicate that dornase alfa (recombinant human deoxyribonuclease) therapy improves pulmonary function in selected patients with CF. Dornase alfa is to be administered by oral inhalation via nebulization. The usual dosage is 2.5 mg (2.5 mL of undiluted solution) once or twice daily. Generally multiple drug inhalation therapies are indicated in CF patients. Antibiotics, i.e. tobramycin or colistin, bronchodilators, i.e. albuterol and ipratropium, and corticoidsteroids, i.e. budesonide or fluticasone are used as adjunctive therapy. Patients often need to inhale multiple doses of different drugs per day. Each nebulization procedure takes about 15 minutes. Thus patients tend to mix drug solutions or suspensions for simultaneous nebulization. In order to help patients making the best use of their inhalation drugs, knowledge of the compatibility of drug solutions and suspensions for oral inhalation is a prerequisite. However the available data are limited (Kamin et al. 2006).

The official prescribing information and the relevant monografs in the AHFS Drug Information book (Hoffmann-La Roche 2004; McEvoy 2006) of Pulmozyme® (brand of dornase alfa) state that the inhalation solution should not be diluted or mixed with other drug solutions in nebulizers. Known data prove the compatibility and stability of ipratropium and albuterol inhalation solutions mixed together (Nagtegaal et al. 1997; Jacobson and Peterson 1995). To our knowledge compatibility information about mixtures of dornase alfa nebulizer solution with ipratropium and/or albuterol nebulizer solutions is not yet available. The objective of this study was to determine whether mixtures of Pulmozyme® inhalation solution with ipratropium and or albuterol containing brands (Atrovent®

**Table 1: Enzymatic activity of Dornase alfa in mixtures of Pulmozyme® with ipratropium bromide containing brands (Atrovent® LS, Atrovent® 500 µg/2 mL Fertiginhalat) and/or albuterol containing brands (Sultanol® Inhalationslösung, Sultanol® forte Fertiginhalat) or excipient solutions (0.01% benzalkonium chloride solution, 0.05% disodium edetate solution)**

| Test solution | Enzymatic acitivity of dornase alfa ± rel. SD (%) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Nominal (%) | Actual (%) | | | | | | |
| | | Initially after mixing | After 1–2 h | After 7–8 h | After 20–21 h | After 23–24 h | After 25–29 h | After 29–31 h |
| Control solution | | | | | | | | |
| Pulmozyme®[a] | 100 | nd | nd | 97.08 ± 3.4 | 96.83 ± 8.1 | 87.33 ± 7.5 | 95.00 ± 5.1 | nd |
| Test solution | | | | | | | | |
| Pulmozyme® + Altrovent® LS + Sultanol® Inhalationslösung[a] | 100 | 62.5 ± 44.5 | 49.17 ± 8.6 | 22.25 ± 7.9 | nd | 16.75 ± 35.3 | 3.83 ± 97.9 | 3.22 ± 39.2 |
| Pulmozyme® + Altrovent® 500 µg/2 mL Fertiginhalat[a] | 100 | 90.33 ±9.7 | 82.08 ± 1.5 | nd | nd | 84.75 ± 9.2 | 79.17 ± 7.9 | nd |
| Pulmozyme® + Sultanol® forte Fertiginhalat[b] | 100 | 91.00 ± 5.0 | 77.50 ± 6.7 | nd | 74.42 ± 10.7 | nd | 67.83 ± 35.1 | nd |
| Pulmozyme® + 0.05% Disodium edetate Solution | 100 | 96.33 ± 7.7 | 69.42 ± 6.3 | nd | 80.75 ± 1.4 | nd | 73.11 ± 10.0 | nd |

Expressed as percentage [%] ± rel. SD [%] of nominal activity, when stored under ambient light conditions at room temperature
[a] Enzymatic activity expressed as mean (n = 3) of the mean of 4 determinations of one sample of 3 test solutions
b Enzymatic activity expressed as mean (n = 5, *n = 2) of the mean of 4 determinations of one sample of 5 or 2 test solutions
nd = not defined

or Sultanol®) are physicochemically compatible. The results can be used to inform patients and health care personnel, if mixing of dornase alfa and ipratropium and albuterol formulations in nebulizer cups and simultaneous inhalation is feasible. The data presented here have in part been published previously in abstract form (Schwabe et al. 2005).

## 2. Investigations and results

Drug combinations were prepared in accordance with the drug product information and clinical practice by mixing the content of one respule Pulmozyme® with 2 mL Atrovent® LS and 0.5 mL Sultanol® Inhalationslösung (multiple dose containing brands) or with one respule Atrovent® 500 µg/2 mL Fertiginhalat (unit dose containing brand) or with one respule Sultanol® forte Fertiginhalat (unit dose containing brand). Each 2.5 mL respule Pulmozyme® contains 2500 units Dornase alfa dissolved in sodium chloride solution (Hoffmann-La Roche 2004). Calcium chloride is added to ensure stability and activity of Dornase alfa (Shire 1996).
Atrovent® LS is marketed as a multiple unit container preserved with benzalkonium chloride (0.1 mg/mL) (Boehringer Ingelheim Pharma 2005). Additional excipients are disodium edetate and hydrochloric acid 3.6% to adjust pH value. The unit dose form Atrovent® 500 µg/2 mL Fertiginhalat is free from benzalkonium chloride and disodium edetate (Boehringer Ingelheim Pharma 2005). From the different commercially available albuterol nebulizer solutions, we used Sultanol® for our studies. The multiple unit container Sultanol® Inhalationslösung also contains benzalkonium chloride as a preservative, which is not the case for the ready to use respules Sultanol® forte Fertiginhalat (GlaxoSmithKline 2004).
Test solutions were stored at room temperature and exposed to light. Dornase alfa activity and ipratropium bromide as well as albuterol concentrations were determined by a kinetic colorimetric DNase activity assay or a stability-indicating HPLC assay with ultraviolet detection, respectively. Physical compatibility was determined by visual inspection and measurements of pH and osmolality.

According to the conventional definition of compatibility, mixtures of inhalation solutions can be designated as physicochemically compatible, when stability (decomposition 10% or less) of each active ingredient and unchanged pH values, osmolality and physical appearance are proven over the entire test period (24 h or less). Corresponding to this definition, all mixtures of Pulmozyme® with Atrovent® LS and Sultanol® Inhalationslösung or with Atrovent® 500 µg/2 mL Fertiginhalat or with Sultanol® forte Fertiginhalat

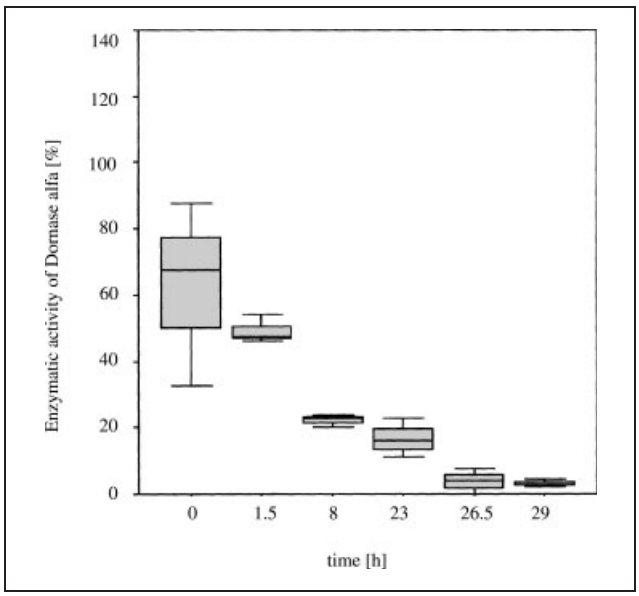


Fig. 1: Enzymatic activity of dornase alfa in mixtures of Pulmozyme® with Atrovent® LS plus Sultanol® Inhalationslösung expressed as percentage [%] of nominal activity stored under ambient light conditions at room temperature. The line in the box indicates the median, the upper and the lower end of the box show the upper and the lower quartile. The ends of the whiskers correspond to the minimum and the maximum (not more than 1.5 times of the inner quartile range). Outliers are shown as circles (1.5 to 3 times of the inner quartile range), extreme outliers are indicated with crosses (more than 3 times of the inner quartile range)

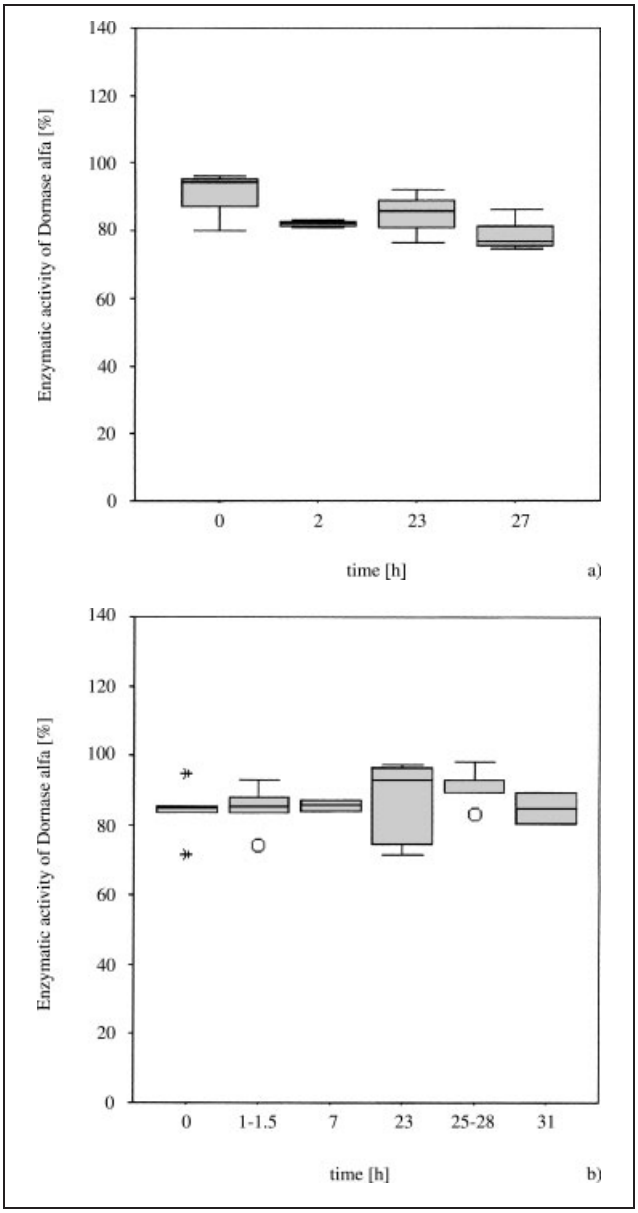


Fig. 2: Enzymatic activity of dornase alfa in mixtures of Pulmozyme® with a) Atrovent® 500 µg/2 mL Fertiginhalat and b) Sultanol®, forte Fertiginhalt expressed as percentage [%] of nominal activity stored under ambient light conditions at room temperature.
[1] See Fig. 1

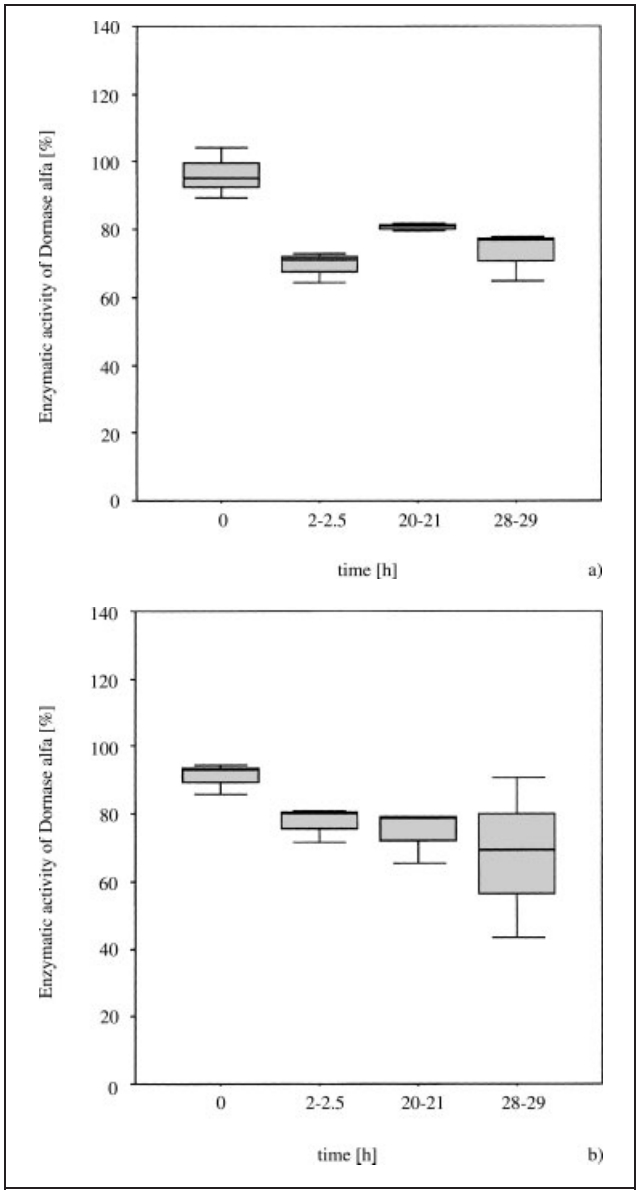


Fig. 3: Enzymatic activity of dornase alfa in mixtures of Pulmozyme® with a) 0.01% benzalkonium chloride and b) 0.05% disodium edetate solution expressed as percentage [%] of nominal activity stored under ambient light conditions at room temperature.
[1] See Fig. 1

tested are designated as incompatible. The mixtures of Pulmozyme® with the unit dose forms of Atrovent® or Sultanol® are merely to be designated as incompatible, because of particle appearance, but proved to be chemically stable.

Dornase alfa activity in undiluted Pulmozyme® control solutions did not change after 28–29 h of storage (see Table 1). Triple mixtures of Pulmozyme® with Atrovent® LS and Sultanol® Inhalationslösung showed reduced enzymatic activity immediately after mixing (see Table 1 and Fig. 1). Loss of activity increased correlating with storage time, and nearly complete inactivation of the enzyme was observed after 29 h of storage. In mixtures of Pulmozyme® with Atrovent® 500 µg/2 mL Fertiginhalat loss of dornase alfa activity was hardly measurable. In mixtures of Pulmozyme® with Sultanol® forte Fertiginhalat no loss of enzymatic activity could be detected with the assay used (see Table 1 and Figs. 2a, b). The suggestion that benzalkonium chloride or disodium edetate are responsible for the DNAse inactivation, was confirmed by the test results of the corresponding mixtures (see Table 1 and Figs. 3a, b).

Ipratropium bromide and albuterol concentrations were found to be stable over a period of 5 h in each inhalation mixture tested as well as in the Atrovent® LS/Sultanol® Inhalationslösung control solution (see Table 2). No additional peaks of degradation products were detectable in HPLC analysis (see Figs. 4 and 5). The concentrations retained 100% of the initial ipratropium bromide or albuterol concentrations during storage of the mixtures in glass containers at room temperature. Measured variations of the concentrations fell within the range of the relative standard deviation of the method.

Results of pH and osmolality measurements are shown in Table 3. Each of the nebulizable drug products (Pulmozyme®, Atrovent® 500 µg/2 mL Fertiginhalat and Sultanol® forte Fertiginhalat) and their mixtures exhibited a pH in the range of 4.0 to 5.5, which corresponds

**Table 2: Concentrations of ipratropium bromide and albuterol in mixtures of Pulmozyme® with Atrovent® 500 µg/2 mL Fertiginhalat or Sultanol® forte Fertiginhalat stored under ambient light conditions at room temperature**

| | Ipratropium bromide | | | | Albuterol | | | |
|---|---|---|---|---|---|---|---|---|
| | Concentration (µg/ml)* ± rel. SD (%) | | | (%) initial concentration remaining ± rel. SD (%)** | Concentration (µg/ml)* ± rel. SD (%) | | | (%) initial concentration remaining ± rel. SD (%)** |
| | nominal | actual | after 5 h storage | after 5 h storage | nominal | actual | after 5 h storage | after 5 h storage |
| *Control solution* Atrovent® LS + Sultanol® diluted with saline | 11.11 | 11.16 ± 0.21 | 11.16 ± 0.49 | 100.06 ± 0.29 | 55.56 | 55.78 ± 0.37 | 55.86 ± 0.13 | 100.14 ± 0.24 |
| *Test solution* Pulmozyme® + Atrovent® 500 µg/2 mL Fl | 11.11 | 11.11 ± 0.80 | 11.02 ± 1.29 | 99.15 ± 0.8 | n.a. | n.a. | n.a. | n.a. |
| Pulmozyme® + Sultanol® forte Fl | n.a. | n.a. | n.a. | n.a. | 50.00 | 54.66 ± 2.40 | 55.67 ± 1.71 | 101.88 ± 2.7 |

n.a. = not appropriate; Fl = Fertiginhalat
* Concentrations expressed as mean (n = 9) ± relative SD (%) of triplicate determinations of 3 test solutions
** Drug concentrations in samples taken at time 0 were designated as 100%

to the specific limits set for nebulizer solutions in the Ph. Eur. 2005 (pH 3 to 8.5). The pH of the mixtures did not change over the storage period. Each of the nebulizer solutions and the mixtures were isotonic and exhibited no significant changes after nearly 6 h of storage.

Visible changes were detectable in each mixture compounded. In mixtures of Pulmozyme® with Atrovent® LS plus Sultanol® Inhalationslösung visible changes were detectable immediately after mixing. Particles increased in number and size depending on storage time.

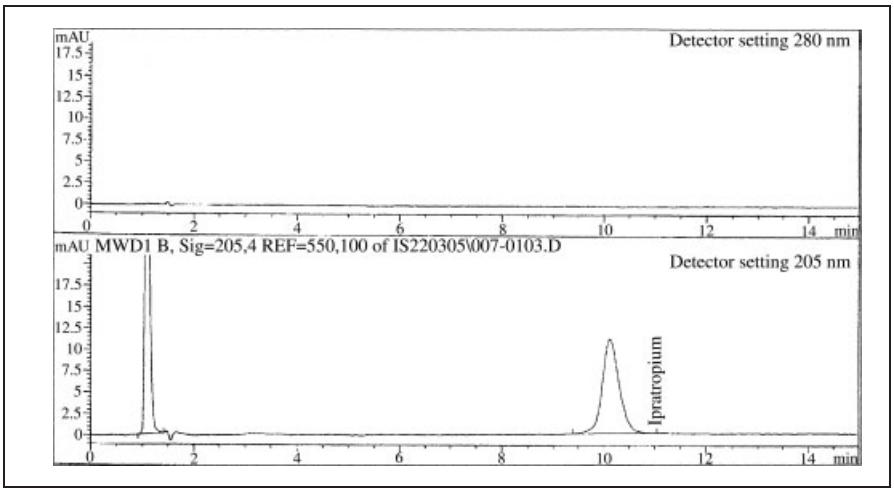


Fig. 4:
Typical HPLC-chromatogram of the mixture of Pulmozyme® with Atrovent® 500 µg/2 mL Fertiginhalat after 5 h storage under ambient light conditions at room temperature. The peak at retention time 1 min derives from excipients in the nebulizable drugs

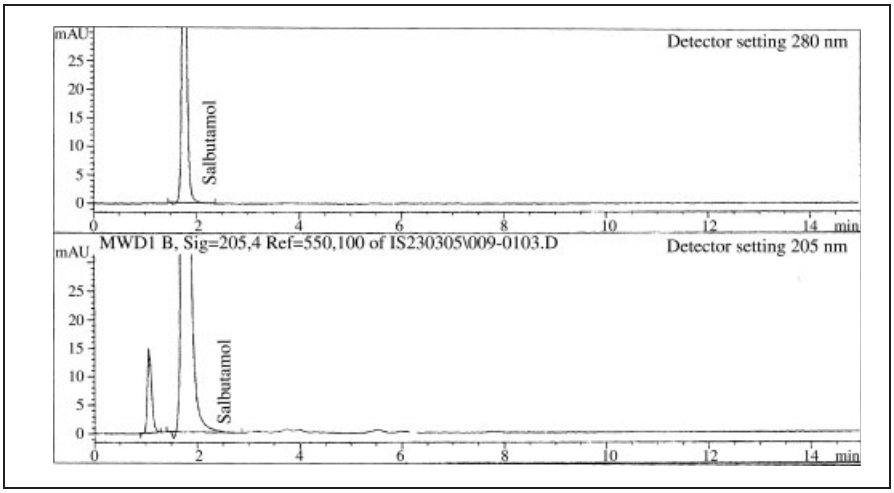


Fig. 5:
Typical HPLC-chromatogram of the mixture of Pulmozyme® with Sultanol® forte Fertiginhalat after 5 h storage under ambient light conditions at room temperature. The peak at retention time 1 min derives from excipients in the nebulizable drugs

**Table 3: Osmolality and pH values of Pulmozyme®, Atrovent® 500 µg/2 mL Fertiginhalat and Sultanol® forte Fertiginhalat unmixed and mixed nebulizable drug products, stored under ambient light conditions at room temperature**

| Nebulizer solution | pH | Mixture stored | | Osmolality (osmol/kg) + rel. SD (%) | Mixture stored | |
|---|---|---|---|---|---|---|
| | | 0.5–1 h | 5.5–6.5 h | | 0.5–1 h | 5.5–6.5 h |
| Pulmozyme® | 5.5 | n.a. | n.a. | $0.276 \pm 0.27$ (n = 8) | n.a. | n.a. |
| Atrovent® 500 µg/2 mL Fertiginhalat | 4.0 | n.a. | n.a. | $0.272 \pm 0.46$ (n = 8) | n.a. | n.a. |
| Sultanol® forte Fertiginhalat | 5.0 | n.a. | n.a. | $0.287 \pm 0.45$ (n = 8) | n.a. | n.a. |
| Pulmozyme® + Atrovent® 500 µg/2 mL Fertiginhalat | n.a. | 5.0 | 5.0 | 0.274# | $0.275 \pm 1.66$ (n = 5) | $0.278 \pm 0.47$ (n = 5) |
| Pulmozyme® + Sultanol® forte Fertiginhalat | n.a. | 5.2 | 5.2 | 0.282# | $0.287 \pm 0.55$ (n = 5) | $0.290 \pm 0.52$ (n = 5) |

n.a. = not appropriate; # calculated

## 3. Discussion

Compounding of test solutions was selected to observe normal inhalation practice. In order to determine the stability of the active ingredients of the inhalation mixtures we used two different methods. Quantitative analysis of dornase alfa activity was done with a kinetic colorimetric activity assay. This biochemical assay is not as accurate as a chemical assay, but this method was the only available one. Hydrolysis of DNA, caused by dornase alfa, results in production of free methyl green and change of absorbance at 600 nm is determined in the colorimetric activity assay. For each assay run a non-linear calibration curve had to be performed. Calibration failures resulted in varying determination time intervals for the test solutions. The DNase activity measured for an identical sample decreases during the assay course probably due to the instability of the DNA-methyl green substrate solution. For example within the same assay for an unmixed sample with a nominal dornase alfa activity of 100% results varied between 99% at the beginning and 81% at the end of the run. Therefore we analyzed each sample once followed by a 3-fold repetition of the analysis in the identical sample order. The mean of 4 results for one sample was used for further interpretation. Results outside the calibration curve ("greater/lower than test range", "sign") were ignored. While determining the precision of the chosen method this proceeding resulted in a mean dornase alfa activity of $89\% \pm 8.3\%$ (n = 15) for nominal 100% and $78\% \pm 9.3\%$ for nominal 85% activity (n = 39). In part the relative standard deviation of 8.3% may also be caused by the 4000 fold dilution in several steps during sample preparation. Due to the relative standard deviation of 8.3% a 10% loss of enzymatic activity cannot be determined. As shown by Lichtinghagen the kinetic method can differentiate between 100% and 85% (i.e. 15% loss) of enzyme activity (Lichtinghagen 2006). Because of the limited precision of the method, we chose a study period of two days in order to get information about the inactivation course. In clinical practice a test period longer than a few hours is not of relevance.

The test results confirm the importance of simulating clinical practice as close as possible, when performing compatibility studies. The different drug products used contained different excipients which caused different compatibility results. Dornase alfa activity was more compromised in mixtures of Pulmozyme® with Atrovent® LS plus Sultanol® Inhalationslösung than in mixtures of Pulmozyme® with Atrovent® 500 µg/2 mL Fertiginhalat or Sultanol® forte Fertiginhalat. As shown by additional experiments the enhanced decrease in activity is most probably caused by benzalkonium chloride and disodium edetate, which are used as excipients in the multiple dosage forms of Atrovent® or Sultanol®. The mode of action of the excipients is prone to alter the integrity of proteins and thereby to inactivate the proteinaceous drug substance. Benzalkonium chloride, which acts as a cationic surfactant, does not belong to the series of preservatives recommended to use in multiple dose drug formulation of biopharmaceuticals and is most probably incompatible with a large number of recombinant proteins (Bontempo 1997).

Mixtures of Pulmozyme® with the unit dose forms of Atrovent® or Sultanol® could probably be designated as compatible for a clinically relevant period of time. To ensure this designation a more precise assay for determination of dornase alfa activity is required. In addition the nebulization properties of the proposed inhalation mixture are to be studied before a final recommendation encouraging simultaneous nebulization can be made (EMEA 2006). The process of aerosolization might also influence compatibility of different drug solutions (physical and mechanical forces, temperature drop during nebulization) (Clay et al. 1983). Moreover simultaneous nebulization of nebulizable medications can affect drug delivery of the components by e.g. altering the aerosolized particle size distribution or the total mass output and inhaled mass (Clay et al. 1983; McKenzie et al. 2002; Smaldone et al. 2000).

Mixing drug products generally decreases concentrations of active ingredients and excipients. By this the bronchoconstrictive effects of excipients like benzalkonium chloride are diminished to the patients' advantage (Beasley et al. 1988). On the other hand decreased concentrations of preservatives may lead to reduced microbiological stability of the mixtures. Therefore dilution of the multiple dosage forms should only be prepared directly before nebulization and surplus quantities should not be stored.

## 4. Experimental

### *4.1. Materials*

Pulmozyme® nebulizer solution 2.500 units/2.5 mL: Roche, Germany, lot: L00130, L00121, L00115 and L00147, Atrovent® LS: Boehringer Ingelheim Pharma GmbH & Co. KG, Germany, lot: 432137A, 433418A, 433238A and 433812A, Atrovent® 500 µg/2 mL Fertiginhalat: Boehringer Ingelheim Pharma GmbH & Co. KG, Germany, lot: 4387825, Sultanol® Inhalationslösung: GlaxoSmithKline GmbH & Co. KG, Germany, lot: C100234, C109486, C112951 and C153571, Sultanol® forte Fertiginhalat: GlaxoSmithKline GmbH & Co. KG, Germany, lot: 4C007, sodium chloride injection solution 0.9%, preservative free: Braun, Germany, lot: 4411C12 and 4191C12.

Ipratropium bromide monohydrate, Boehringer Ingelheim GmbH. Albuterol sulfate, catalog number 701154, Fagron GmbH & Co. KG. Benzalkonium chloride, catalog number 700174-0002, Synopharm, Germany.

Disodium edetate, catalog number 8418, Merck, Germany. DNA, catalog number 223646, Roche, Germany. Methyl green, catalog number M884, Sigma, Germany. Hepes, catalog number H-3375, Sigma, Germany. Acetat-NaOH, catalog number A1045, Applichem, Germany. $CaCl_2$, catalog number 2381, Merck, Germany. $MgCl_2 \times 6\ H_2O$, catalog number 5833, Merck, Germany. BSA (Bovines serum albumin), Albumin Fraktion V, catalog number 735078, Roche, Germany. Thiomersal, catalog number T-8784, Sigma, Germany. Tween® 20, catalog number P-1379, Sigma, Germany. $H_2O_2$ 35%: catalog number 1.08600.1000, Merck, Germany. NaOH 1 mol/l: catalog number 1.09137.1000, Merck, Germany. HCl 1 mol/l: catalog number 1.09057.1000, Merck, Germany. Water HPLC gradient grade: catalog number 4218, Mallinckrodt J. T. Baker, Germany. Acetonitrile: catalog number 9128, Promochem, Germany. Phosphoric acid 85%: catalog number 1.00573.1000, Merck, Germany. Methanol HPLC grade: catalog number M/4056/17, Fisher Scientific, Germany. KOH: catalog number 1.05021.0250, Merck, Germany. Triethylamine: catalog number A3845, 0025, Applichem, Germany. Spezialindikator pH 4.0–7.0: catalog number 109542, VWR International GmbH, Germany. Acilit® pH 0–6.0: catalog number 109531, VWR International GmbH, Germany. Universalindikator pH 0–14: catalog number 109535, VWR International GmbH, Germany.
Plastic containers: catalog number 55.468, Sarstedt, Germany. Glass containers: catalog number Lenz 3.0214.13, VWR International GmbH, Germany

### 4.2. Sample preparation

All tests were performed with the commercially available nebulizer solutions Pulmozyme®, Atrovent® LS, Atrovent® 500 µg/2 mL Fertiginhalat, Sultanol® Inhalationslösung and Sultanol® forte Fertiginhalat. Mixtures were prepared by mixing 2.5 mL of Pulmozyme® (withdrawn from a 2.5 mL respule containing 2500 units Dornase alfa) with 2.0 mL of Atrovent® LS (withdrawn from a 20 mL multiple unit container containing 261 µg/mL ipratropium bromide × 1 $H_2O$ equivalent to 250 µg ipratropium bromide) and 0.5 mL of Sultanol® Inhalationslösung (withdrawn from a 10 mL multiple unit container containing 6 mg/mL albuterol sulfate equivalent to 5 mg/mL albuterol), or with 2.0 mL of Atrovent® 500 µg/2 mL Fertiginhalat (withdrawn from a 2 mL ampoule containing 261 µg/mL ipratropium bromide × 1 $H_2O$ equivalent to 250 µg ipratropium bromide) or with 2.5 mL of Sultanol® forte Fertiginhalat (withdrawn from a 2.5 mL ampoule containing 1.2 mg/mL albuterol sulfate equivalent to 1 mg/mL albuterol) in polysterene or glass containers. For each assay three test solutions (except for Pulmozyme® plus Sultanol Fertiginhalat 5 test solutions) were prepared, gently mixed and stored at room temperature under ambient light conditions (mixed daylight and normal laboratory fluorescent light).
Determination of dornase alfa activity was performed on test solutions stored in polystyrene-containers. Samples containing nominal 25 units dornase alfa (i.e. 100% activity) were withdrawn from each test solution immediately after mixing, and after 1–2, 7–8, 23, 25–28 and 29–31 h of storage. Samples from test solutions of Pulmozyme® with Atrovent® 500 µg/2 mL Fertiginhalat were obtained immediately after mixing, and after 2, 23 or 27 h of storage. Samples were diluted with buffer C (25 mM Hepes, 4 mM $CaCl_2$, 4 mM $MgCl_2$, 0.1% BSA, 0.01% thiomersal, 0.05% Tween® 20, pH 7.5) in order to fit the calibration curve. Therefore samples were first diluted to a volume of 10 mL and an aliquot of 500 µL was further diluted 1 : 10. Each dilution step was completed by vortexing.
Dornase alfa activity was also determined in triplicate admixtures of Pulmozyme® with 0.01% benzalkonium chloride solution or with 0.05% disodium edetate solution. 500 µL aliquots of undiluted Pulmozyme® inhalation solution were added to 400 µL 0.01% benzalkonium chloride solution or 0.05% disodium edetate solution (each one dissolved in 0.9% NaCl solution) in polystyrene-containers. Samples containing nominal 25 units dornase alfa (i.e. 100% activity) were withdrawn from each test solution immediately after mixing and after 2, 20–21 and 28–29 h of storage and further diluted. Each sample prepared was assayed in quadruplicate immediately.
Determination of ipratopium bromide and albuterol concentrations was performed on test solutions stored in glass containers. Test solutions were prepared in triplicate and 1 mL samples were withdrawn immediately after mixing and after 5 h of storage. Samples were diluted in glass containers to a nominal volume of 10 mL by adding mobile phase (s. Table 1) and were assayed in triplicate.
Samples of pure Pulmozyme® inhalation solution or 2.0 mL Atrovent® LS plus 0.5 mL Sultanol® Inhalationslösung diluted with 2.0 mL 0.9% NaCl solution were assayed as control solutions.

### 4.3. Assays

#### 4.3.1. Dornase alfa activity

Enzymatic activity of dornase alfa was determined by a kinetic colorimetric DNase activity assay developed by Lichtinghagen (2006). Degradation of DNA-methyl green substrate by dornase alfa was determined by measuring ΔA/min at 600 nm and 37 °C using the Cobas Mira (Roche Diagnostics) automated analyzer. 90 µL samples of the diluted test solutions were transferred into a sample tube of the analyzer containing 90 µL DNA-methyl green substrate-solution diluted and 100 µL buffer C. The colorimetric substrate-solution was prepared by mixing 10 mL DNA-solution (0.5 g DNA in 250 mL buffer A) with 600 µL methyl green-solution (0.4 g methyl green in 100 mL buffer B), 2396 µL buffer C and 10 µL $H_2O_2$ 35% in order to remove free methyl green. Buffer A consisted of 25 mM Hepes and 1 mM EDTA, pH adjusted to 7.5. Buffer B contained 20 mM sodium acetate, pH adjusted to 4.2. According to Lichtinghagen (2006) the substrate solution was always freshly prepared and preincubated overnight at room temperature. For each assay calibration was performed with dilutions of nominal 0, 20, 40, 60, 80, 100, 120% dornase alfa activity. Quadruplicate determination of control and mixture samples was performed by assaying each sample once, and 3-fold repetition of the analysis in the identical sample order.
Assay precision was determined by analyzing Pulmozyme® solutions on different days. Samples containing nominal 100% or 85% dornase alfa activity yielded a mean dornase alfa activity of 89% ± 8.3% (n = 15) or 78% ± 9.3% (n = 39).
Diluted ipratropium or albuterol nebulizer solutions (Atrovent® LS, Atrovent® 500 µg/2 mL Fertiginhalat, Sultanol® Inhalationslösung and Sultanol® forte Fertiginhalat) as well as diluted benzalkonium chloride or disodium edetate solutions did not exhibit enzymatic activity.
Samples with a nominal dornase alfa activity ≥85% (mean), corresponding to measured dornase alfa activities ≥78% (mean), were defined as compatible with regard to the drug substance dornase alfa.

#### 4.3.2. Ipratropium bromide and albuterol concentrations

Analysis of ipratropium bromide and albuterol concentrations were performed simultaneously using a HPLC method described previously by van den Bemt et al. (1997). The assay conditions are summarized in Table 4. The assays were conducted on an HPLC system consisting of a Hewlett Packard HP series 1050 autosampler, a HP series 1050 on-line degasser, a HP series 1050 pump and a HP series 1050 UV detector MWD. Injection volume was 40 µL and run time was 15 min. Data acquisition and integration were performed with the Hewlett Packard Software HPLC ChemStation (version Rev.A.02.05). Peak areas were used for quantification.
Chromatograms of benzalkonium chloride solution (prepared from reference substance) assayed under the same conditions, showed a peak of benzalkonium chloride (retention time ~7 min) at the detection wavelength 205 nm, which did not interfere with the peaks of ipratropium or albuterol. Dornase alfa was not detectable with this assay.

**Table 4: HPLC conditions for the simultaneous determination of iptratropium and albuterol**

| | Stationary phase | Mobile phase[a] | Detector setting (nm) | Retention time (min) | Flow rate (mL/min) | Mean concentration (mg/mL) ± rel. SD (%) | |
|---|---|---|---|---|---|---|---|
| | | | | | | Intraday (n = 18) | Interday (n = 24) |
| Ipratropium | STIP Lichrocart[b] | Phosphate buffer[c]: acetonitrile 87.5 : 12.5 | 205 | 10 | 1.2 | 11.39 ± 1.48 | 11.29 ± 1.33 |
| Albuterol | STIP Lichrocart[b] | Phosphate buffer[c]: acetonitrile 87.5 : 12.5 | 280 | 1.8 | 1.2 | 56.18 ± 1.67 | 55.36 ± 3.21 |

[a] Each mobile phase was degassed in an ultrasonic bath
[b] STIP Lichrocart column with precolumn, 5 µm particle size, 125 mm × 4 mm inner diameter, MZ Analysentechnik GmbH, Mainz, Germany
[c] The phosphate buffer was prepared by mixing 875 mL water, 241 µL triethylamine and 660 µL 85% $H_3PO_4$. pH value was adjusted to 3.35 with KOH solution 10 mol/L

The assay was validated as stability-indicating by analyzing forced-degraded ipratropium bromide and albuterol solutions. 2 mL Atrovent® LS mixed with 0.5 mL Sultanol® Inhalationslösung and solutions of the reference substances ipratropium bromide × 1 $H_2O$ (224 μg/mL) or albuterol sulfate (1.2 mg/mL) were degraded at 70–75 °C for 3–6 h using NaOH 1 mol/l or HCl 1 mol/L or $H_2O_2$ 35%. The resultant chromatograms indicated that the degradation products were clearly separated from the parent drug peaks.

The linearity of the method was evaluated at eight concentrations injected in triplicate (varying from 14% to 130% of Atrovent® LS and Sultanol® Inhalationslösung). The calibration curve constructed from plots of peak areas versus concentrations of ipratropium bromide or albuterol was linear and the correlation coefficients were 0.9999 and 0.999, respectively.

Assay precisions were determined with Atrovent® LS and Sultanol® Inhalationslösung. Solutions containing 11.11 μg/mL or 55.56 μg/mL ipratropium bromide or albuterol were prepared and analyzed at the same day (intra-day precision) or at eight different days (inter-day precision) in triplicate. Conditions and results are given in Table 4.

Samples with drug concentrations ≥90% (mean) of the initial concentrations taken at time zero were defined as chemically compatible with regard to the drug substance determined.

### *4.4. Physical compatibility*

Physical properties of the mixtures and of the mixture components were determined 0.5–1 h after mixing. Values of pH were measured with pH test strips and osmolality was determined via the freezing depression method with an osmometer (Osmomat –030, Gonotec GmbH, Germany). Test solutions were visually inspected with the unaided eye for any changes over the entire test period. Test solutions with no change in pH, osmolality or visual appearance were defined as compatible and physically stable.

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