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Activity loss on room temperature storage of Survanta®, a bovine lung extract based surfactant

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Abstract

The rapid loss in Survanta®, a bovine lung extract based surfactant, activity upon storage at 30°C is investigated. During 3 months, the minimum surface tension as measured by both Wilhelmy plate and pulsating bubble methods rose by 8–9 mN/m. Additionally, a decrease in the in situ rat lung bioactivity of approximately 40% was observed. Disaturated phosphatidylcholine levels decreased while free fatty acid levels increased under the same storage conditions. Thus, the presence of both lysolecithins and additional free fatty acids is believed to result in the loss in activity.

Keywords: Survanta®; Lung surfactant; Wilhelmy surface balance; Pulsating bubble surfactometer; Dipalmitoylphosphatidylcholine; Lysolecithin

1. Introduction

Survanta® is a bovine lung extract based surfactant which is currently marketed for the treatment of surfactant deficient, neonatal respiratory distress syndrome. Survanta® is a complex mixture of lipids, mostly DPPC, and apoproteins (SP-B and SP-C). Lung surfactants, such as Survanta®, reduce the surface tension at the air–alveolar lining interface, thus facilitating breathing.

Animal models have traditionally been used in the evaluation of lung surfactant mixtures (Ikegami et al., 1979; Tanaka et al., 1983a,b; Bermel et al., 1984; Notter et al., 1985). In vitro techniques such as the Wilhelmy surface balance (WSB) and pulsating bubble surfactometer (PBS) are also commonly used to demonstrate the surface activity of lung surfactant mixtures at the air–water interface (Notter, 1984; Enhorning, 1977; Yu and Possmayer, 1986; Sarin et al., 1990). Preliminary in vitro surface tension and in situ rat lung bioactivity measurements suggested a rapid loss in Survanta® activity at room temperature storage (30°C). This loss in activity was further investigated in the current study. Chemical degra-

Abbreviations: RLA = Rat lung activity; WSB = Wilhelmy surface balance; PBS = Pulsating bubble surfactometer; %TLC = Percent of total lung capacity; DST = Dynamic surface tension; γ_{wsb} = WSB surface tension at 40% of total area (mN/m); γ_{pbs} = PBS surface tension (mN/m); DPPC = 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine; DPPE = 1,2-Dipalmitoyl-sn-glycero-3-phosphoethanolamine.

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dation of the major lipid component (disaturated phosphatidylcholines) and subsequent formation of degradation products (free fatty acids) were also investigated. A relationship between the chemical degradation and loss in surface activity is discussed.

2. Methods

Vials of Survanta® (lot 53-141AN) were stored protected from light at 5°C until the initiation of the surface activity/bioactivity experiment. The initial data points ($t = 0$) were considered to be at the time the assays were conducted prior to storing the remaining vials at 30°C, protected from light for 78 days. The 30°C storage temperature represents the high end of the room temperature range. Sample vials were tested at predetermined time intervals over the course of the study to demonstrate the loss in activity. The following measurements were made on two samples per interval:

1. Rat lung activity,
2. Wilhelmy surface balance-dynamic surface tension,
3. Pulsating bubble surfactometer. All of the above tests were performed on each sample vial in order to pair the results. The time to conduct the assays was minimized to maintain a common time interval per sample per assay, typically 2 days apart.

2.1. Rat lung activity

The rat lung activity (RLA) was determined in a similar manner to that described by Bermel et al. (1984). Briefly, the intact lung is excised from the rat. Following a vacuum degassing step, the lung is placed in a thermostated (37°C) chamber suspended by a tracheal cannula. Volume–pressure (VP) measurements are made over the pressure range 30 to 0 cm H_2O using a constant rate of withdrawal of 2.0 ml/min. After the initial VP curve is generated, the lung is lavaged with normal saline to deplete the natural surfactant. A second VP curve is generated (surfactant deficient curve) in the same manner as above. The lung is then instilled with 2.5 ml of Survanta® and a third VP curve is generated. The total area between the initial and surfactant deficient curves is compared to the total area between the Survanta® and surfactant deficient curves from 0 to 30 cm H_2O pressure giving the percent total lung capacity (%TLC) restored. In addition, the percent of lung capacity between the initial and surfactant deficient curves is compared to the percent of lung capacity between the Survanta® and surfactant deficient curves at 5 and 10 cm H_2O pressure. The parameters, percent total lung capacity restored at 5 cm H_2O pressure (%TLC 5 cm H_2O pressure) and percent total lung capacity restored at 10 cm H_2O pressure (%TLC 10 cm H_2O pressure), respectively, are generated. Lung compliance is best assessed at low pressures where surfactant deficient lungs collapse. The %TLC 5 cm H_2O pressure parameter gives an estimate of lung compliance under conditions of low pressure. The %TLC 10 cm H_2O pressure parameter was also chosen to assess lung compliance since it is the approximate pressure at which the surfactant deficient lung collapses. Also, it is the approximate pressure at which the surfactant deficient and freshly excised lung curves exhibit the greatest difference.

2.2. Wilhelmy surface balance

Dynamic surface tension diagrams of Survanta® films spread at the air/water interface were generated as described previously with a modified Wilhelmy surface balance (Sarin et al., 1990). The instrument consisted of a Teflon trough with a movable Teflon ribbon which defines the surface area of the interface. The surface tension was measured directly with a roughened platinum plate suspended from a Cahn 2000 electrobalance. The entire apparatus was contained within an incubation chamber which maintained the 0.9% saline subphase solution at 37 ± 1°C. The instrument was calibrated with 950 ml of the normal saline subphase to give a surface tension of 70.4 mN/m prior to the initiation of each experiment. A surfactant film was formed by evenly depositing portions of a 27 μl aliquot of sample (equivalent to 675 μg of phospholipids) across the subphase

surface. The film was allowed to equilibrate (3 min) prior to initiation of the compression/expansion cycles. The film was compressed and expanded between 445 cm^2 (100% area) and 178 cm^2 (40% area) at a rate of 3 min per cycle for a minimum of seven cycles. Surface tension (γ_{wsb}) values were based upon the mean of the highest and lowest values generated from cycles two through seven, at 40% of total area.

2.3. Pulsating bubble surfactometer

The pulsating bubble surfactometer (PBS) surface tension data were collected with an Electronetics Corporation surfactometer using a method similar to that described by Enhorning (1977). A total phospholipid concentration of 1 mg/ml was used. Minimum bubble radius surface tension readings were taken in triplicate every minute for 10 min. The surface tension data were plotted as a function of time to determine when a steady state surface tension was achieved. All data points collected after achieving steady state were averaged to give a steady state surface tension value (γ_{pbs}).

2.4. Chemical stability

The chemical stability of five Survanta® lots (35-942AR, 35-943AR, 42-590AR, 43-671AR and 43-672AR) at 30°C in thermostated ovens was determined. The Survanta® vials were protected from light during the course of the study. The disaturated phosphatidylcholines were assayed using a previously reported HPLC method (Scarim et al., 1989). The free fatty acid content was determined using a validated HPLC assay as follows. An aliquot of Survanta® was mixed with a stearyl alcohol internal standard solution and sodium chloride. The sample was extracted with mobile phase hexanes (99.2% v/v, Fisher HPLC Grade), isopropyl alcohol (0.74% v/v, Fisher HPLC grade) and formic acid (0.06% v/v, 88% Fisher A.C.S. certified grade), mixed with silica gel and filtered through a 0.45 μm Zetapor™ membrane. The organic layers from three extractions were collected and diluted to a final volume (25 ml) with mobile phase. An aliquot of the diluted extract was evaporated at 40°C in a thermostated bath. The residue was dissolved in a chloroform (Fisher, HPLC grade) and acetic acid (Fisher Reagent grade) eluent. The sample was then passed through a Sep-Pak® silica cartridge, evaporated to dryness at 40°C under a stream of nitrogen and reconstituted with HPLC mobile phase. The sample free fatty acid content was determined via an HPLC assay utilizing a silica guard column (Alltech), a 30 g × 3.9 mm i.d., microparticulate silica, 10 μm analytical column (μ-Porosil, Waters Associates) with refractive index detection. A 20 μl injection volume was used with a 2.0 ml/min flow rate. The columns and detector were maintained at 35°C. Palmitic acid was used as a standard.

3. Results and discussion

Storage of Survanta® at 30°C results in degradative effects on surface activity which are reflected in the dynamic surface tension (DST) diagrams shown in Fig. 1. Figs. 1A and 1B illustrate typical DST diagrams of Survanta® at $t = 0$ and 78 days, respectively, which represent the extremes in terms of the surface activity loss in this study. Several points can be made about the change in surface activity of Survanta® from these diagrams. The reproducibility of the tracings is lost (Fig. 1B) which indicates reduced film stability. The collapse plateau of Survanta®, which is the region where a reduction in surface tension can no longer be achieved by further film compression, is diminished from approximately 30% (Fig. 1A) to between 5 and 15% (Fig. 1B). The extent of film compression necessary to reach the collapse plateau reflects the 'efficiency' of the surfactant to reach a minimum surface tension. Thus, the larger collapse plateau demonstrates that the surfactant can minimize surface tension with minor film compression. Finally, γ_{wsb} increases (loss in surface activity) as is seen in Fig. 2. An increase in surface tension of approximately 8–9 mN/m is seen. Similarly, PBS measurements also demonstrate the loss in Survanta® surface activity with an increase of about 8 mN/m, which is consistent with the WSB results. Survanta® activity as measured by the rat lung bioassay is also observed to

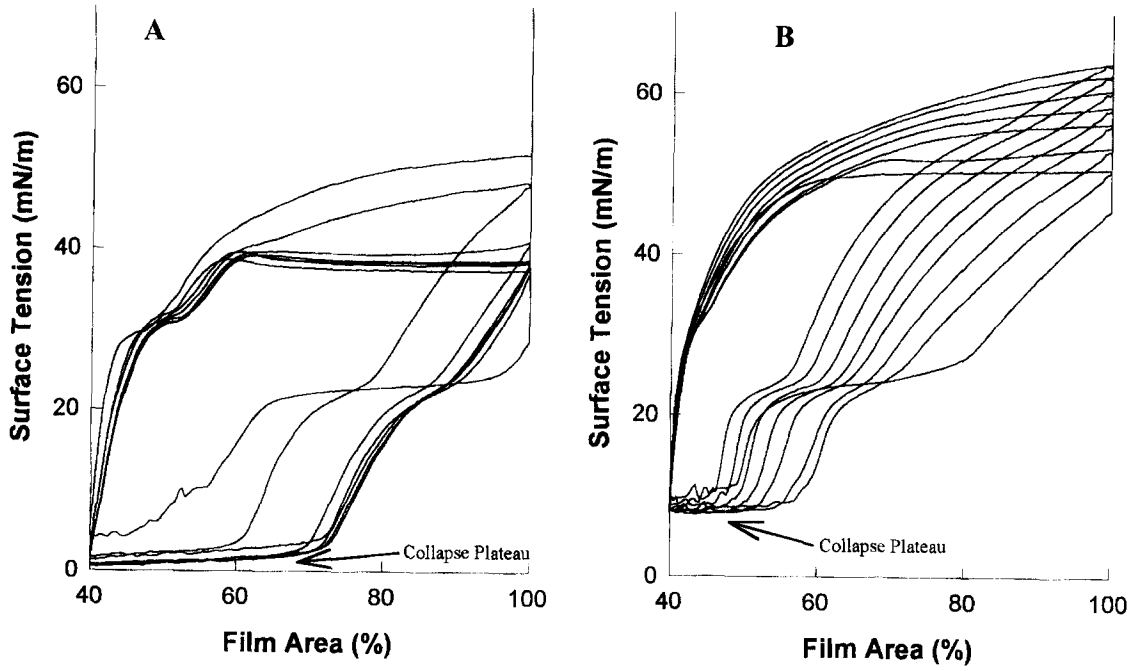

Fig. 1. Wilhelmy surface balance dynamic surface tension diagrams of Survanta® stored at 30°C: (A) $t = 0$ days; (B) $t = 78$ days.

deteriorate with storage at 30°C. This loss is shown in Fig. 3 by the reduction in the percent of total lung capacity restored, %TLC at 5 cm and %TLC at 10 cm water pressure, respectively. A

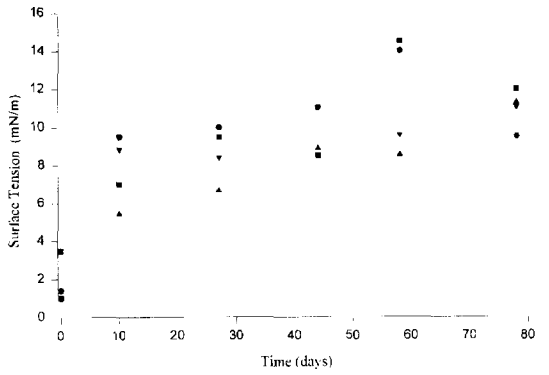

Fig. 2. Time-dependent increase in surface tension obtained from the Wilhelmy surface balance diagrams of Survanta® films compressed to 40% of total surface area, γ_{wsb} ((●) Vial 1, (■) Vial 2) and the pulsating bubble surfactometer steady-state surface tension obtained from Survanta® aliquots containing 1.0 mg/ml phospholipid, γ_{pbs} ((▼) Vial 1, (▲) Vial 2).

40% decrease in bioactivity is observed which is consistent with the loss in surface activity (Fig. 2).

The change in both surface activity (γ_{wsb} and γ_{pbs}) and bioactivity indicates that the Survanta® lipid–protein matrix is altered. This alteration may result from chemical degradation of the lung surfactant components of which the major component is lipoidal in nature. The disaturated phosphatidylcholines which consist mostly of DPPC constitute the majority of the lipids. These lipids can undergo first-(pseudo-first)-order ester hydrolysis to form free fatty acids and lysolecithins (Kensil and Dennis, 1981; Grit et al., 1989, 1993a,b; Grit and Crommelin, 1992, 1993a,b). Fig. 4 shows the loss in the disaturated PC content of five separate Survanta® lots stored at 30°C. The average pseudo-first-order degradation rate constant for the disaturated PC component is 0.041 ± 0.014 month^{-1} at pH 6.3. This value is consistent with predicted hydrolysis rates for natural soybean PC at 30°C and pH 6.5 ($k = 0.08$ month^{-1}) and also for saturated soybean PC at 30°C and pH 7 in the fluid ($k = 0.01$ month^{-1})

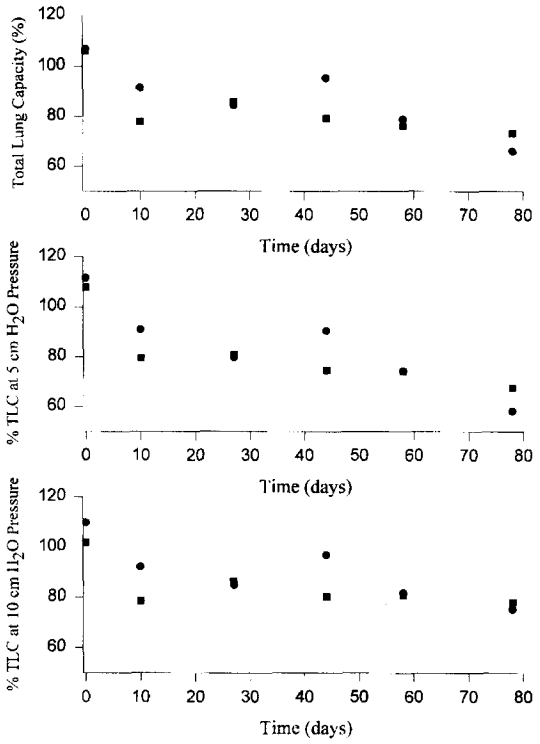

Fig. 3. Reduction in the rat lung activity parameters upon storage of Survanta® at 30°C: Percent of total area restored (total lung capacity (%)), percent of total area restored at 5 cm H_2O pressure and percent of total area restored at 10 cm H_2O pressure: (●) Vial 1, (■) Vial 2.

and gel ($k = 0.02$ month^{-1}) states (Grit et al., 1989, 1993b). These differences may be attributed to the level of chain unsaturation where the greater degree of saturation results in greater stability (Grit et al., 1993b).

An increase in the total free fatty acid content is shown in Fig. 4. The pseudo-first-order rate of free fatty acid formation is 0.15 ± 0.039 month^{-1}. The free fatty acid formation is due to the hydrolysis of all lipids. Therefore, the rate is three- to four-fold greater than the disaturated PC degradation rate. The triglyceride level remained unchanged over the course of the study.

Lysolecithins have been reported to reduce the surface activity of lung surfactant matrices (Holm et al., 1991). Additionally, palmitic acid (a degradation product of DPPC) was also shown to reduce surface activity, although the surface activity appears to be more sensitive to the presence of the lysolecithin.

Klopfenstein et al. (1974) have reported a reduction of both T_m and ΔH of DPPC with the addition of lysolecithin. The amounts of lysolecithin needed were quite high (1:1 lysolecithin:DPPC molar ratio). Blume et al. (1976) confirmed the findings of Klopfenstein et al. (1974). However, lysolecithin at 5% w/w significantly modulated the DPPC:DPPE lipid matrix thermotropism causing the separation of three phases. In this study, the effect was observed at a much lower lysolecithin content than was needed to alter DPPC alone, as reported by others. Such an effect would be expected in the complex lipid matrix of natural lung surfactant.

The increased free fatty acids, such as palmitic acid, could also alter the Survanta® lipid matrix properties which could change the surface activity. Palmitic acid is known to increase the gel-to-liquid phase transition of DPPC even at low concentrations (Mabrey and Sturtevant, 1977; Schullery et al., 1981). Thus, the lipid lamellar phase could be stabilized (opposite the effect of lysolecithin). Films of DPPC on an aqueous subphase have been shown to be stabilized by the addition of as low as 2.5% palmitic acid (Krill et al., 1994). The increased rigidity imparted by the additional palmitic acid on the surfactant lipid matrix could result in poor spreading, resulting in higher γ_{wsb} values. In addition to lipid degrada-

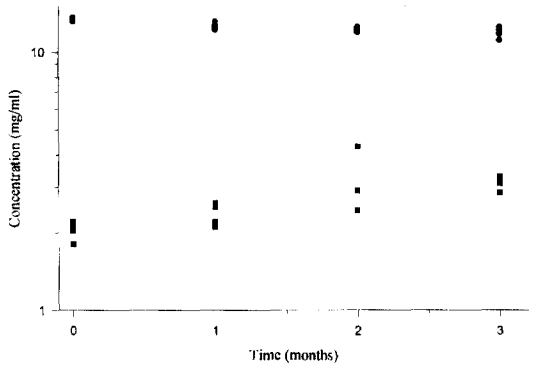

Fig. 4. Survanta® loss in disaturated phosphatidylcholine (●) and increase in free fatty acid (■) content upon storage at 30°C.

tion effects on surface activity, the degradation of the apoproteins (SP-B and SP-C) would also be expected to result in the loss in surfactant activity. Of course, the complexity of the Survanta® lipid–protein matrix precludes drawing any firm conclusions based upon available data. However, the evidence reported in the literature offers some likely explanations of the loss in both surface activity and bioactivity of Survanta®, reported here.

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