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ORIGINAL ARTICLE

Pulmonary disposition of vancomycin nebulized as lipid vesicles in rats

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Formulation of antibiotics as inhalable products is proposed to improve their therapeutic index when intended for the treatment of pulmonary infections; as vancomycin shows reduced values of lung partition coefficient, pulmonary administration might be an interesting alternative to conventional administration routes. An experimental study has been performed to compare the pulmonary disposition of vancomycin after inhalation of the drug formulated as a solution and as lipid vesicles (conventional liposomes or liposomes modified with chitosan). Vancomycin concentrations were determined in bronchoalveolar fluid, pulmonary tissue and blood samples from 27 Wistar rats distributed in three groups subjected to nebulisation of the drug formulated as a solution, conventional liposomes or chitosomes. Statistically significant differences between the mean drug concentrations in bronchoalveolar lavage (BALF) and lung tissue were found upon comparing the solution to lipid vesicles ($116.95 \mu g ml^{-1} \pm 62.13$ versus $68.34 \mu g ml^{-1} \pm 28.90$ for liposomes and $65.36 \pm 22.11 \mu g g^{-1}$ for chitosomes in BALF; $222.74 \pm 37.15 \mu g g^{-1}$ versus $357.17 \pm 65.37 \mu g g^{-1}$ for liposomes and $378.83 \pm 85.87 \mu g g^{-1}$ for chitosomes in pulmonary tissue). The amount of available drug estimated by mass balance reached the highest values for chitosomes followed by liposomes ($24289.66 \pm 4795.48 \mu g$ and $20207.91 \pm 5318.29 \mu g$, respectively) and the lowest for the solution ($18971.64 \pm 4765.38 \mu g$). The drug transport and tissue uptake processes showed to be dependent on the nebulized formulation, being facilitated by the lipid vesicles that improved drug passage from the airway space to the pulmonary tissue and systemic circulation.
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INTRODUCTION

Pulmonary delivery is currently a promising administration route to improve local effects of drugs used for treatment of respiratory diseases and this is also proposed as an alternative route for biotechnological drugs intended for systemic effects.

Among the nanotechnology-based drug delivery systems assayed for these purposes, liposomes have a relevant role and offer a feasible way of delivering drugs to the lung owing to their attractive biological properties. These spherical, self-closed, biocompatible structures allow entrapment of either hydrophilic or hydrophobic drugs and show different size, charge and surface characteristics according to the formulation procedure applied.

Several strategies based on the pulmonary administration of different types of lipid vesicles have been assayed, and important achievements related to lung targeting of antinfective and anti-asthmatic as well as the systemic delivery of biotechnological drugs have been reported in the recent literature. Amphotericin B, rifampicin, isoniazid, ciprofloxacin or gentamicin have been formulated as liposomes in order to improve their therapeutic index by increasing the lung/plasma concentration ratios. Chitosan or specific ligands, such as mannan and pullulan, added to the surface of amphotericin-loaded vesicles provide sustained drug levels for longer times.[1,2] Liposomes of isoniazide formulated with dipalmitoyl-phosphatidylcholine[3] have been proposed with the dual aim of providing tuberculosis patients with a pulmonary formulation and also with the phospholipid material that is lacking as pulmonary surfactant in these patients. The use of anionic compounds or specific ligands such as maleylated bovine serum albumin or O-steroyl amilopectine[4] has been proposed to formulate liposomes aimed at pulmonary delivery and the macrophage targeting of rifampicin, as these approaches lead to higher and more sustained drug concentrations in the lung tissue. Aerosolization of pro-liposomes is another promising strategy for the pulmonary administration of this drug.[5] The formulation of ciprofloxacin[6] and gentamicin[7] as inhalable liposomes is also considered a good approach to improve their therapeutic index when intended for the treatment of pulmonary infections. Regarding non-antiinfective drugs, budesonide[8] or vasoactive intestinal peptide included in sustained-release formulations have been proposed as a 'dispersable drug depot' that produces longer-lasting effects than those observed with the

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corresponding free drug.[9] Many attempts have been made to achieve good selectivity in the targeting of tumor cells by preparing specialized carrier agents that are profitable for anticancer therapy. Among these, in the context of antitumor therapy, liposomes are the colloidal particles that to date have received the most attention.[10–12] The relevance of research work on pulmonary delivery is not restricted to local effects but also for the systemic delivery of proteins and peptide drugs. Studies carried out with liposomal formulations of insulin[13,14] or heparin[15–17] reveal that this strategy seems to increase the serum half-life of these drugs.

Despite the efforts being made in research into pulmonary administration and the development of drug delivery systems through this route, current applications in the clinical setting are still very limited. The lung toxicity of excipients may be one of the main limitations to further advances in dosage forms able to produce efficacious pulmonary delivery. Phospholipids are natural components of the pulmonary surfactant and chitosan has shown a high biocompatibility even with the pulmonary environment;[18] accordingly, formulations based on these compounds seem to be a reasonable proposal for pulmonary delivery systems of clinical usefulness. Building on this hypothesis, the present work addresses the evaluation of lipid vesicles as vehicles for the pulmonary administration of antibiotics used in respiratory infections. Conventional and chitosan-modified liposomes (chitosomes) were studied to determine the pulmonary disposition of vancomycin in laboratory animals after the nebulisation of drug-loaded vesicles. Vacomycin was chosen as the loading drug due to its characteristics of low partition coefficient value in lung together with narrow therapeutic window, both making this drug a good candidate for inhalatory administration. Comparison of results obtained for aerosolized free drug was made in order to determine the influence of the formulation on the kinetic behavior of the antibiotic after pulmonary delivery.

MATERIALS AND METHODS

Reagents

Vancomycin was provided by Combino Pharm Labs (Barcelona, Spain), egg L-α-phosphatidylcholine, lanolin cholesterol, soluble low-molecular weight chitosan, Na_2HPO_4 $2H_2O$ and KH_2PO_4 were purchased from Sigma-Aldrich Quimica SA (Madrid, Spain). Acetonitrile for HPLC and Triton were provided by Merck SA (Madrid, Spain); perchloric acid and acetic acid of analytical grade were from Panreac Quimica SA (Barcelona, Spain). Ultra-pure water was obtained with a MilliQ Millipore device (Millipore Iberica SAU, Madrid, Spain). Methanol HyperSolv Chromanorm of HPLC-gradient grade was provided by VWR International Eurolab SL (Barcelona, Spain), and trichloromethane was from Panreac Quimica SA.

Procedures

Preparation of liposomes. A mixture of phosphatidylcholine and cholesterol (molar ratio 0.7) was used to prepare the vesicles, applying the method of solvent evaporation and lipid hydration.[19] Briefly, the lipid mixture was dissolved in a chloroform-methanol (2:1 v/v) mixture and poured into a glass flask placed in a water bath at 40 °C connected to a rotary evaporator (60 r.p.m. and 50 mBar pressure) for 30 min for solvent evaporation. Following this, the temperature was reduced to 25 °C and a vacuum was maintained for 24 h in order to eliminate residual solvents. Then, the vancomycin solution (5 mg ml^{-1}) and 1 g of glass beads were added to the flask containing the dried lipids and returned to the rotary evaporator at 40 °C (60 r.p.m. for 30 min) for lipid hydration. Next, the suspension was kept at room temperature for 10 min to complete the formation of liposomes and was subsequently subjected to sonication for 15 min. An additional step was performed to obtain chitosomes; 0.1% chitosan (w/v) dissolved in a 1% (w/v) acetic acid solution was added dropwise to 10 ml of the previous lipid vesicle suspension at room temperature with continuous stirring.

Pulmonary administration. Twenty-seven Wistar male rats from Charles River (Barcelona, Spain) with a mean body weight of 247.41 ± 20.40 g were included in the study. Twelve hours prior to the experiments, the animals were isolated in cages and allowed access to tap water *ad libitum*. The housing and experimental treatment of the animals were in accordance with the corresponding Guide from the Institute for Laboratory Animal Research (ILAR 1996). The experiments complied with current Spanish legislation and adhered to the 'Principles of Laboratory Animal Care'. After being weighed, the animals were anesthetized with 80 mg kg^{-1} sodium thiopental (intraperitoneal route) and 1000 IU sodium heparin was injected by the same route to avoid clotting. Then, tracheotomy and tracheal cannulation were performed with the animals on their backs and the cannula was connected to the respiratory equipment, which consists of a nebulizer (Ultrasonic Aerosol Generator 700700-UV system TSE, Technical & Scientific Equipment GmbH, Bad Homburg, Germany) and an artificial ventilator (7025 Rodent Ventilator, Ugo Basile, Comerio VA, Italy). The latter was set at 60 respirations per min and 2 ml of tidal volume was delivered to the rat lungs. All animals were subjected to 20 min of nebulization of the drug formulated as an aqueous dissolution (Group I), a liposome suspension (Group II), or a chitosome suspension (Group III). At the end of this period, samples were collected as follows: blood by direct puncture of the left ventricle, bronchoalveolar fluid by bronchoalveolar lavage (BALF) using 0.3 ml of 0.9% saline solution, and pulmonary tissue by lung excision. The excised tissue was weighed and a 1-g aliquot was separated for processing by homogenization with 50 mM phosphate buffer, pH = 7.4 and 0.1% Triton X-100.

Blood samples were split into two aliquots: one was used to quantify vancomycin concentrations in plasma and the other to determine concentrations in whole blood. In all samples, drug concentrations were determined with an HPLC technique validated and described previously.[20] The actual nebulized volume was determined from the initial and final amounts of the formulation in the nebulizer equipment.

Data analysis. The amount of drug available (D_{av}) was estimated by mass balance according to the disposition model shown in Figure 1 and the following equations:

$$D_{av} = Q_{bal} + Q_L + Q_b + Q_t + Q_{el} \quad (1)$$

$$Q_{bal} = V_{bal} \times C_{bal} \quad (2)$$

$$Q_L = W_L \times C_L \quad (3)$$

$$Q_b = V_b \times C_b \quad (4)$$

$$Q_t = C_p \times R \times BW \quad (5)$$

$$Q_{el} = CLr \times C_p \times 20\,min \quad (6)$$

where, Q_{bal}, Q_L, Q_b, and Q_t represent the amount of drug in the BALF, lung tissue, blood and rat body, respectively; Q_{el} is the estimated amount of drug excreted in urine; V_{bal} is the bronchoalveolar fluid volume; C_{bal} is the bronchoalveolar fluid concentration; W_L is lung weight; C_L is the lung concentration; C_b is the blood concentration; V_b is the blood volume; R is the tissue/plasma partition coefficient; C_p is the plasma concentration; BW is body weight, and CLr is the renal drug clearance.

V_{bal}, C_{bal}, W_L, C_L, C_b and C_p and BW were determined experimentally. The V_b, R and CLr values ($V_b = 60$ ml kg^{-1}; $R = 0.5$ and CLr = 3.2 ml min) were obtained from the literature.[21–23]

Comparison of the results obtained for vancomycin in the three groups was performed by statistical analysis (analysis of variance, ANOVA)[24] using GraphPad Prism, version 4, package (GraphPad Software, San Diego, CA, USA; www.graphpad.com).

RESULTS

Table 1 shows the mean values of the available dose (D_{av}) estimated from equation 1, together with the amounts of vancomycin in

BALF, lung tissue, blood, and the rest of body at the end of drug nebulisation for all three formulations assayed (solution, liposomes and chitosomes). D_{av} values obtained for lipid vesicles were higher than observed for solution; in particular, chitosomes showed the highest value (24.29 ± 4.79 mg) followed by liposomes (20.21 ± 5.32 mg) and dissolution (18.97 ± 4.76 mg), although the statistical comparison failed to reveal significant differences among the three formulations ($P = 0.13$). On the contrary, the amount of drug remaining at the BALF was higher for solution, while the amounts in the rest of the sampled spaces were lower than the observed for both types of vesicles. As the drug concentration, rather than the amount of drug, is responsible for drug effects, a comparison of the vancomycin concentrations achieved in the different body spaces was performed. Table 2 shows the mean drug concentration values achieved in the BALF, lung tissue and plasma at the end of the nebulization period. Statistical comparison revealed significant differences between the solution and lipid vesicles for concentrations in the BALF and lung tissue ($P = 1.15 \times 10^{-4}$ and $P = 5.80 \times 10^{-3}$, respectively) but no statistically significant differences were detected for plasma ($P = 0.07$).

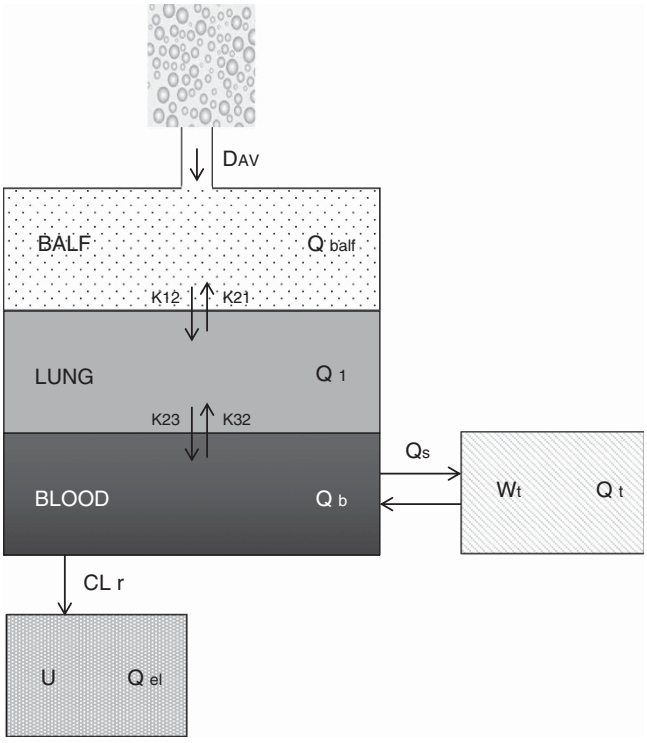

Figure 1 Schematic representation of the physiologically based pharmacokinetic (PBPK) model considered.

Table 2 Vancomycin concentrations after pulmonary delivery

	BALF ($\mu g\,ml^{-1}$)	*Lung tissue* ($\mu g\,g^{-1}$)	*Plasma* ($\mu g\,ml^{-1}$)
Solution	116.95 ± 62.13**	222.74 ± 37.15***	92.98 ± 20.90
Vesicles	66.85 ± 25.01	368.00 ± 74.87	110.65 ± 24.36
Liposomes	68.34 ± 28.90	357.17 ± 65.37	99.34 ± 22.64
Chitosomes	65.36 ± 22.11	378.83 ± 85.87	121.95 ± 21.47

Abbreviation: BALF, bronchoalveolar lavage fluid.
Vesicles: liposomes and chitosomes pulled data.
(***$P<0.001$ and **$P<0.01$).

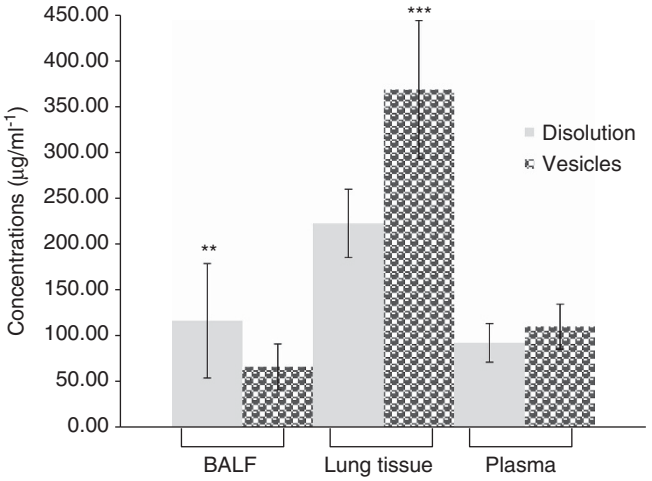

Figure 2 Vancomycin mean concentrations in BALF, lung tissue and plasma samples after drug nebulization as a solution or as lipid vesicles (***$P<0.001$ and **$P<0.01$).

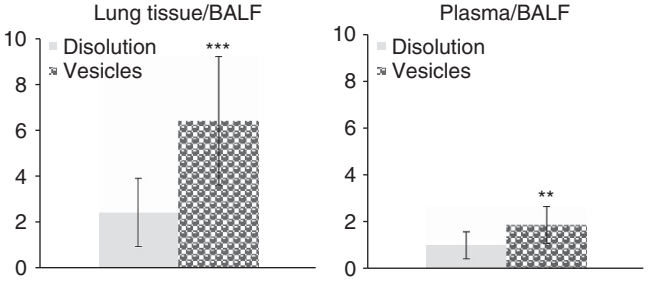

Figure 3 Lung tissue/BALF and plasma/BALF partition coefficient values of vancomycin after nebulization of the solution and lipid vesicles (***$P<0.001$ and **$P<0.01$).

Table 1 Vancomycin amounts in different body spaces after pulmonary delivery

	Q *blood* (μg)	Q *BALF* (μg)	Q *lung tissue* (μg)	Q *rat body* (μg)	Q_{el} (μg)	D_{av} (μg)
Solution	942.57 ± 248.46	35.08 ± 18.64	261.09 ± 54.63	11782.07 ± 3105.80	5950.83 ± 1337.84	18971.64 ± 4765.38
Liposomes	992.28 ± 278.69	20.50 ± 8.67	433.62 ± 98.39	12403.53 ± 3483.61	6357.97 ± 1448.93	20207.91 ± 5318.29
Chitosomes	1182.00 ± 243.04	19.61 ± 6.63	478.18 ± 134.09	14774.96 ± 3037.94	7804.91 ± 1373.78	24289.66 ± 4795.48

Abbreviations: BALF, bronchoalveolar lavage fluid; D_{av}, total available drug; Q_{el}, eliminated drug amount estimated.
Q blood, *Q* BALF, *Q* lung tissue, *Q* rat body: drug amounts in each space at the end of nebulization period.

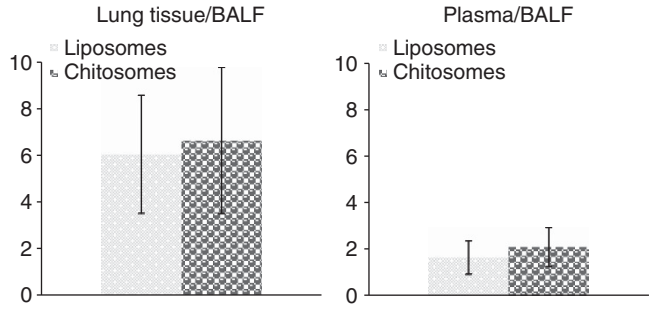

Figure 4 Lung tissue/BALF and plasma/BALF partition coefficient values of vancomycin after nebulization of liposomes and chitosomes.

Figures 2 and 3 illustrate differences in pulmonary drug disposition between the solution and lipid vesicle formulations (data from liposomes and chitosomes were pooled and represented in these figures in order to illustrate and highlight the influence of lipid formulation). As shown in Figure 3, lung tissue/BALF concentration ratio takes a value of 1.9 for drug dissolution and about 5.5 for lipid vesicles; the plasma/BALF ratio also increased from 0.8 to 1.6, the differences being statistically significant in both cases ($P = 5.48 \times 10^{-4}$ and $P = 8.36 \times 10^{-3}$, respectively).

Conventional liposomes and chitosomes were also compared with each other and slight but interesting differences were found between both types of vesicles. Both lung tissue/BALF and plasma/BALF ratios were higher for chitosomes compared with liposomes. Figure 4 illustrates these results. Although no statistically significant differences were found for the BALF ($P = 0.81$) and pulmonary tissue concentrations ($P = 0.55$), statistically significant differences were detected for plasma ($P = 0.04$).

DISCUSSION

Data on the available dose estimated from pharmacokinetic model assumed (Figure 1) reveal that a higher amount of drug is able to access the body compartments when nebulized vancomycin is included in lipid vesicles as compared with the solution. Only at the BALF, the remaining drug amount was higher for solution than that observed for both types of vesicles, while in the rest of the sampled spaces these were lower for the solution nebulization. These results indicate that drug transport from the air space to the pulmonary tissue, and subsequently to the systemic blood is facilitated by the lipid formulations. Differences in pulmonary drug disposition between the solution and lipid vesicle formulations are evident in this study (Figures 2 and 3), leading to a pulmonary tissue/BALF concentration ratio of 5.5 when estimated from both lipid formulations (liposomes and chitosomes) versus 1.9 when calculated from solution. This finding means that transport and tissue uptake of aerosolized vancomycin in the respiratory system is dependent on the delivery formulation used, being facilitated by lipid vesicles, which showed an ability to increase the drug passage to deeper lung spaces. Regarding comparison between conventional liposomes and chitosomes, slight but interesting differences were found as both lung tissue/BALF and plasma/BALF ratios were higher for chitosomes compared to liposomes (Figure 4). It seems that the inclusion of chitosan in the vesicles produces a more efficient drug transport from the airways to the body spaces, because the concentrations in pulmonary tissue and plasma as well as the available dose (Dav) were the highest for the chitosomes. As indicated in the Results section, no statistically significant differences were found for the BALF and pulmonary tissue concentrations, but statistically significant differences were detected for plasma. These results are in accordance with those recently reported by Al-Quadi *et al.*,[25] who found a prolonged hypoglycaemic effect of dry powders containing insulin-loaded chitosan nanoparticles administered through the intratracheal route to rats. The mucoadhesive properties of chitosan might minimize particle mucociliary clearance, as well as being responsible for enhancing drug absorption by opening the epithelial tight junctions, facilitating paracellular transport. This has been proposed previously[26] and observed upon incubation with cell lines (Cacu-3 and 16HBE14o) representative of the bronchial epithelium.[27,28] Low toxicity, biodegradability, biocompatibility, mucoadhesivity and permeation enhancement are all desirable characteristics for pulmonary delivery formulations, making chitosan one of the most interesting and promising materials currently under investigation for inhalation of different type of drugs even for gene delivery.[29]

It is interesting to notice that for pulmonary infection treatments, antibiotic delivery by inhalation is proposed for local targeting and that drug access to the systemic circulation may not be desirable in those cases, particularly when the antiinfective agent shows a narrow therapeutic window. Vancomycin nebulization for the treatment of pulmonary infections is an example of this situation, because the drugs showing low lung tissue/plasma partition coefficient but high plasma levels are not desirable due to related toxic effects.[30] Many other antibiotics including aminoglycosides or linezolid show the same peculiarity; passive drug targeting at the respiratory system instead of improved transport to the systemic circulation would be desired for those cases. According to the results of the present study, the nebulization of conventional liposomes would be the best of the three assayed options for pulmonary antibiotic delivery, from both the therapeutic and toxicological points of view, because uptake and permanence in pulmonary spaces instead of systemic access is aimed. Chitosomes would be a better option when systemic effect is pursued and drug solution when BALF is the target.

In summary, the pulmonary disposition of vancomycin after nebulisation is influenced by the type of drug formulation administered. Lipid vesicles improve drug transport from the airways to lung tissue and the systemic circulation, leading to higher available doses as well as higher drug concentrations in pulmonary tissue and plasma. Our results confirm the properties of chitosan as a permeation enhancer in the respiratory system and suggest that, among systems assayed here, conventional liposomes would be the best option for vancomycin nebulization.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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