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Research paper

Trehalose–hydroxyethylcellulose microspheres containing vancomycin for topical drug delivery

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Abstract

A new formulation, in which vancomycin is entrapped into trehalose and hydroxyethylcellulose (Natrosol®) spherical matrices, is described. Microspheres were produced by the solvent evaporation method. The entrapped drug was fully recovered following microspheres dissolution. Differential scanning calorimetry analyses proved that Natrosol maintains trehalose in its amorphous form. The stabilizing effects of trehalose on vancomycin were evaluated even after long storage and heating of microspheres. Calorimetric data indicated no decomposition of the entrapped drug. In vitro drug release, already performed by using a general two-compartment linear time-invariant open model, suggests that the new delivery system is suitable for topical application on extensive and purulent or burn wounds, when the skin is heavily damaged and the barrier disrupted. The system activation is determined by osmotic phenomena. The prepared new delivery system seems to have characteristics suitable for topical applications on extensive and purulent wounds. The system is able to take away serous exudates from wounds, thus letting the matrix to swell and form a viscous gel-like dispersion that, in turn, enables drug diffusion.

Keywords: Vancomycin; Trehalose; Natrosol; Microsphere; Solvent evaporation method; Differential scanning calorimetry; Drug release

1. Introduction

Wound contamination by virulent microflora is one of the major problems in treatment of patients with extensive skin barrier disruption. The proliferation of mixed bacterial flora is characteristic of patients exhibiting decubitus ulcers, burns or diabetic foot, with serous exudates formation. To prevent septic complications, like e.g. those produced by polychemoresistant *Enterococci*, *Staphylococcus aureus* and *pyogenes*, therapy protocols require systemic administration of high doses of broad-spectrum antibiotics. Unfortunately, intravenous (i.v.) administration of antibiotics often transforms wounds into sites of multiresistant virulent bacteria, with consequent appearance of unwanted side effects [1].

Statistical analysis of antibiotic assay indicated the well-known antibiotic vancomycin as one of the most useful drugs for treatment of several wound infections [2]. Vancomycin, a glycoprotein derived from *Streptomyces orientalis*, is active against most Gram-positive *cocci*, *Neisseria* and *Clostridia*. This antibiotic resulted to be the drug of choice in treatment of methicillin-resistant staphylococcal infections caused by *Staphylococcus epidermidis*, and in treatment of colites caused by *Clostridium difficile* [3]. Usually, vancomycin is administered by slow intravenous infusion since it is poorly absorbed from gastrointestinal tract and i.v. bolus may increase the risk of hypersensitivity reactions.

Systemic administration of vancomycin may be associated with several adverse effects such as anaphylactoid reactions, syndrome of flushing of the upper body, hypotension, angioedema, pruritus, thrombophlebitis, nephrotoxicity and ototoxicity [4,5]. These side effects could be lowered by topical applications; it must be considered, however, that the skin barrier properties may affect the amount of penetrating drug. Moreover, when the skin is seriously damaged and no barrier opposes to drug transfer into biological tissues, appropriate choice of formulation components may regulate drug penetration. Release profile optimization can be achieved by conferring suitable physical and chemical properties to the dosage form.

Topical conventional pharmaceutical dosage forms, such as lipophilic ointments, oil-in-water creams and oil-type

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preparations, are not recommended for applications on injured skin. Notwithstanding the fact that vancomycin has good water-solubility characteristics, the administration of simple aqueous sterile solutions would involve in situ fluctuations of drug concentration. This implies the presence of 'peaks and valleys', or often, subtherapeutic drug levels thus requiring high applications frequency [1]. In addition, vancomycin shows marked instability in aqueous solutions at 37°C, with consequent reduction of drug effectiveness [6]. In view of the above reasons, the administration of vancomycin is nowadays performed by intravenous infusions of extemporaneously prepared solutions [4].

An appropriate delivery system is, therefore, needed for obtaining the desired release rates and bioavailability.

In the present study we report on the development of a microparticulate drug delivery system suitable for topical application on extensive and purulent wounds.

1.1. System design

Vancomycin was entrapped into a mixture of trehalose and hydroxyethylcellulose (Natrosol®) spherical matrices. Microspheres were produced by the solvent evaporation method. Trehalose was chosen on the basis of its characteristics of hydrophilic biocompatible sugar, useful to prevent degradation of biomaterials [7,8].

In the last decade large interest has been devoted to the study of physical and chemical properties of trehalose (α-D-glucopyranosyl-α-D-glucopyranoside), a non-reducing disaccharide of glucose, found in organisms (such as some desert plants) able to survive in extreme drought and high temperatures conditions, without suffering any irreversible damage. Indeed, following re-hydration they resume their normal lifecycle. Preservation against adverse conditions like extremely high or low temperature has also been observed for isolated enzymes, membranes or artificial systems, such as liposomes, embedded in solid trehalose matrices (trehalose coated) [8].

It has been suggested that trehalose can hydrogen-bond to biostructures (like polar headgroups of membranes or OH group of proteins) thus replacing water. This direct interaction results in maintenance of dry proteins and membranes in a physical state similar to that seen in the presence of excess of water [9].

Moreover, trehalose forms glasses in the dry state (vitrification) [10], thus bringing about several advantages. Indeed, due to the glass high viscosity, diffusive processes are hindered, chemical reactions are slowed down to negligible rates and degradative processes are prevented [11,12]. In particular, it has been reported that, for carbon monoxy myoglobin embedded in a trehalose matrix, the non-harmonic motions, arising from thermal fluctuation of a protein among conformational substates [13], are strongly hindered [14,15]. Moreover, the hydrogen atoms mean square displacements and density of states resulted in being the ones of a perfectly harmonic solid even at high temperature [16].

Natrosol® was chosen on the basis of its characteristics of amorphous, biocompatible, non-toxic, hydrophilic, swellable polymer. In contact with aqueous media the polymer starts swelling, forms hydrophilic and viscous gel-like dispersions, and gradually becomes permeable, thus determining drug diffusion.

In a previous work [1] we described the development of a new antibiotic delivery system for application on burn wounds. The system was constituted of a reservoir compartment containing the drug molecularly dispersed in a Natrosol gel. The gel was then applied on one side of a dressing membrane (Veloderm®) carrying a cultured keratinocyte layer fixed on the other side. The application of the system, by putting the keratinocyte layer side on the wound, results in withdrawal of serous exudates from lesions, which in turn leaves the treated area dry. Lesions' healing, in burn patients, was observed 8 days after the application.

We report here preliminary results on a similar solid system, consisting of trehalose–Natrosol spherical matrices containing vancomycin, which could be more efficiently applied on extensive and purulent wounds. The system is designed to release vancomycin with pre-programmed rate characteristics maintaining the glycopeptide antibiotic stable in the long term.

2. Materials and methods

Vancomycin hydrochloride (Eli Lilly, Indianapolis, IN) and Veloderm (formerly distributed in Italy by BIO's, Castelfidardo, and more recently by Remedia Italia, Rome) were kindly supplied by A.R.N.A.S. 'Ospedale Civico e Benfratelli – G. Di Cristina – Maurizio Ascoli', Palermo, Italy. Trehalose (dihydrate) was purchased from Hayashibara Shoij (Hayashibara Shoij Inc., Okayama, Japan). Hydroxyethylcellulose (Natrosol) and liquid paraffin were purchased from Galeno (Florence, Italy); SPAN® 60 was purchased from Fluka (Buchs, Switzerland). All chemicals and solvents were used without further purification.

2.1. Preparation of vancomycin microspheres

Natrosol (0.1 g) was added to an aqueous solution (5.5 ml) containing vancomycin hydrochloride (0.1 g) and trehalose (dihydrate) (0.8 g). The mixture was sonicated in a Branson 1200 ultrasound bath until a viscous, transparent and homogeneous gel was formed. Warm liquid paraffin (50 g) was then poured into the aqueous gel and mechanically stirred at a constant speed, using a Polimix RW 20 stirrer equipped with a digital KCH-TRON spin counter (Kinematica, Switzerland) and a four-blade stainless-steel impeller of ~4.8 cm diameter.

The addition of a surfactant was required to form a stable emulsion. SPAN 60 (HLB 4.7) was the most suitable surfactant to produce discrete and free-flowing microspheres. The optimum concentration was 0.66% (w/w) of the total formulation.

Reproducible, spherical particles were obtained when the stirring speed was kept at 1900 rev./min. Lower stirring led to losses of material that adhered to the beaker walls and to the impeller.

The temperature was kept at 50°C using an Heidolph MR 3001 K hot stage and an Heidolph EKT 3000 thermostatic probe. It was found that lower temperatures led to aggregate formation; on the other hand, vancomycin degradation phenomena increased when the temperature exceeded 50°C.

The stirring lasted about 6 h until complete water evaporation. The aqueous droplets dispersed in the external oily phase resulted then to be transformed into solid spheres. Petroleum ether (40–60°C, 50 ml) was added to the mixture, under stirring, for rapid separation of spheres. Spheres were then collected by filtration and washed with petroleum ether. Washing was repeated three more times (25 ml × 3) to remove traces of adhering oily material and surfactant residues. Microspheres were then air-dried at room temperature for 24 h, and stored as free-flowing material.

2.2. Microsphere characterization

2.2.1. Morphological characteristics

All batches of microspheres were observed under optical microscope with transmitted light (magnification 500×) to evaluate morphological characteristics, shape, size and homogeneity. Microspheres appeared uniformly round, smooth and globular, without aggregates. No drug crystals, mixed with microspheres, were detected (Fig. 1). Scanning electron microscopy analysis was performed on carbon coated specimens by a Leica Stereoscan 440. The exposure to the electron beam, during focusing, altered the original geometry of microspheres. The shape was fully lost following 10 min of exposure.

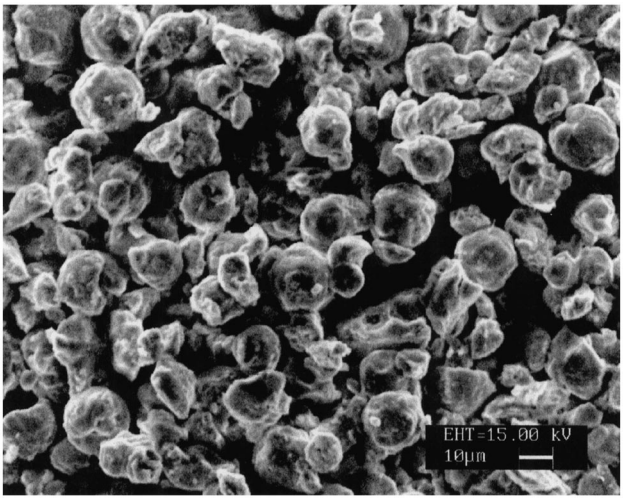

Fig. 1. Morphological appearance of a batch of trehalose–Natrosol microspheres containing vancomycin. Scanning electron microscopy analysis was performed on carbon coated specimens by a Leica Stereoscan 440. Note that the exposure to the electron beam, during focusing, on impact, altered the original geometry of microspheres. The shape was fully lost following 10 min of exposure (data not shown). Bar: 10 μm.

2.2.2. Particle size analysis

Separation of microspheres into various fractions, according to their size, was carried out using an Endecotts Octagon 200 test sieve shaker (Endecotts Ltd., UK) and standard mesh wire sieves (Endecotts). Three standard stainless-steel sieves, in the range 20–56 μm, were arranged in the order of decreasing aperture size. Drug-loaded microspheres (1 g) were placed on the upper sieve. The sieves were mounted on the mechanical shaker operating for a time interval suitable for complete separation (~15 min). About 90% of the microspheres were < 20 μm. Results are reported as mean of ten batches. Microspheres, prepared at the same drug-loading, stirring speed, surfactant amount and temperature, were found to have reproducible mean size.

2.2.3. Evaluation of drug content and encapsulation efficiency

The active ingredient incorporated into the microspheres was measured by spectrophotometric quantitative determination (UV/Vis Shimadzu model 1601 instrument). Randomly selected microspheres aliquots (20 ± 1 mg) of each batch were transferred into 100-ml flasks and completed to volume by water. The microparticles were dissolved by sonification, thus releasing the incorporated drug. The amount of vancomycin was then measured at $\lambda =$ 280.5 nm using the appropriate blank and calibration curve ($E_{1\%} = 0.0436$ in water). The average drug content into microspheres resulted in 9.99% (w/w), giving an encapsulation efficiency of about 100%.

2.2.4. In vitro release tests

The discharged vancomycin from microspheres was measured using flow-through type Franz diffusion cells equipped with: (i) a formulation chamber i.e. the source of diffusing drug (cell cap); (ii) a receptor chamber, which simulates the biological medium, surrounded by a water jacket to maintain constant temperature (cell body); (iii) a membrane allocation site, which separates the donor from receptor compartment; and (iv) a side arm, which allows samples withdrawal (sample port).

Drug transfer was tested using Veloderm®, a freely permeable membrane 0.1 mm thick, commonly used in wound dressing. Veloderm (also called Bio-Skin) is a natural micro-porous procollagen containing glucosamine and *N*-acetyl galactosamine; its fibers, as evidenced by scanning electron microscopy analysis, show regular structure with inter-fibrillar spaces large enough to allow transfer of large molecules from one side to the other side of the membrane [17,18].

Aliquots of microspheres (0.2 ± 0.01 g) were placed in the cell cap. The receptor chamber was filled with 45 ml of isotonic phosphate buffer solution (pH 7.4), used as a simulated receptor phase. The temperature was kept at 37 ± 0.5°C by using Polimix EH2 thermostatic bath (Kinematica, Switzerland). The exposed surface area was 10.7 cm^2. The receptor solution was stirred using a magnetic

follower rotating at 600 rev./min (stirrer RECO® S5, Kinematica, Switzerland) which largely increased the mixing efficiency and reduced the tendency to form a stagnant boundary layer next to the membrane surface; the primary convective flow under the drug-releasing surface, was also minimized. At regular intervals (15 or 30 min), samples (1 ml) were removed from the center of the receptor chamber through the sample port. To avoid saturation phenomena and maintain the sink conditions, the withdrawn volume was replaced by fresh buffer solution. Drug permeation was monitored by analysis of the cumulative amount of antibiotic reaching the receptor phase. Vancomycin was detected by UV spectrophotometric analysis at $\lambda = 280.5$ nm using the appropriate blank and calibration curve ($E_{1\%} = 0.0436$ in phosphate buffer solution).

Release measurements were performed on ten different batches. Reproducibility was within 2.1% of the mean.

2.2.5. Thermal analysis

Calorimetric measurements were carried out with a Perkin–Elmer DSC7 (power compensation) differential scanning calorimeter connected to a computer via a TAC7/DX thermal analysis instrument controller. The thermal unit was thermostated by means of an external thermocryostat in which the coolant was kept at −30°C; a nitrogen flux was used as a purging gas for the furnace. Calibration was performed using indium and zinc at the same scan rates used in the experiments. DSC scans were run on samples weighing between 5 and 8 mg and sealed in pierced aluminum Perkin–Elmer DSC pans. The temperature range investigated was 40–230°C with a heating rate of 10 K min^{-1}. An empty aluminum pan was used as a reference.

3. Results and discussion

Kinetics of release from microparticulate systems is largely affected by the system geometry and particle size, which determines the effective surface for drug release. Microparticulate systems for external use are frequently described as dusting materials, which should be dispensed as extremely small-sized particles to enhance effectiveness and avoid aggravation of abraded skin [19]. Moreover, the administered dose of drug being subdivided into small units can be spread over a large area, thus decreasing the high, local drug concentration [20].

Attempts to prepare drug-loaded microspheres of suitable particle size using trehalose as sole component of matrix failed; indeed, the obtained spheres, observed by optical microscopy, showed gradual geometrical transformation during storage. Modification was complete in a few hours. This phenomenon was attributed to conversion of amorphous trehalose into the dihydrate crystalline form, due to interactions with environmental moisture [21]. When Natrosol was added as a further matrix component, particles remained spherical even following long storage. We ascribed this behavior to Natrosol–trehalose interactions that avoid trehalose crystallization. This was confirmed by differential scanning calorimetry (DSC) techniques.

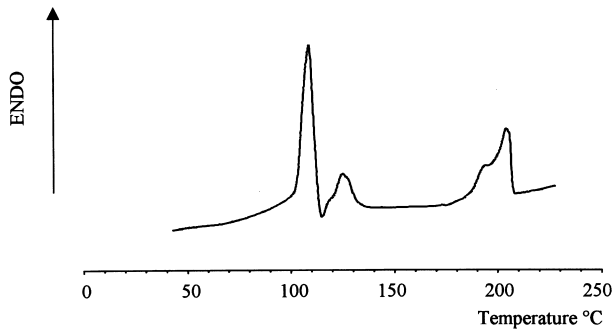

Fig. 2. DSC profile of trehalose dihydrate (10 K min^{-1}; 40–230°C).

Fig. 2 shows the thermogram of trehalose dihydrate. In agreement with previously reported data [22], our results showed the existence of five forms.

(i) One dihydrate crystalline form (T_h) whose existence was suggested by the sharp endotherm, centered at about 110°C, indicative of water release from crystals.
(ii) One anhydrous crystalline form (T_γ) whose existence was suggested by the exothermic peak, ranging from 110 to 120°C, next to the right of the water loss, ascribed [22] to molecular rearrangement. This form (T_γ) 'melts' at a temperature slightly above its formation (120–130°C), thus suggesting it to be a rather unstable form.
(iii) One 'amorphous form' stable up to about 180°C.
(iv) Two anhydrous crystalline forms (T_α and T_β), whose melting is evidenced by a double peak, appearing above 200°C and indicating the 'final' melting [22].

Fig. 3 shows the thermogram of microparticles containing trehalose dihydrate as sole component of the matrix. These microparticles were prepared by using the above described microencapsulation process. The thermogram (performed after 24 h storage at room temperature and moisture) shows behavior analogous to that in Fig. 1.

Fig. 4a–c report the thermograms of Natrosol alone, trehalose/Natrosol physical mixture (ratio 9:1 w/w), and treha-

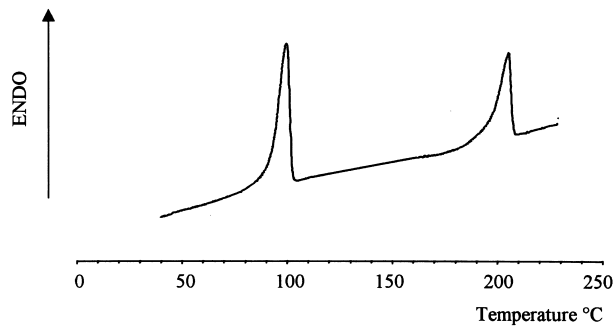

Fig. 3. DSC profile of trehalose dihydrate microparticles (10 K min^{-1}; 40–230°C).

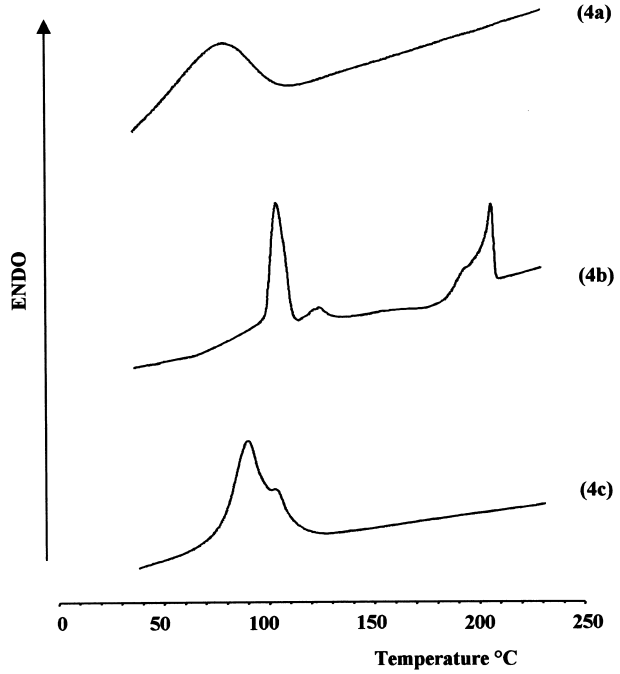

Fig. 4. DSC profiles of Natrosol (a), trehalose/Natrosol physical mixture (9:1 w/w) (b) and trehalose/Natrosol microspheres (9:1 w/w) (c) (10 K min^{-1}; 40–230°C).

lose/Natrosol microspheres (ratio 9:1 w/w), respectively. Natrosol alone shows one broad signal, in the temperature range 80–90°C, typical of amorphous cellulose derivative polymers; trehalose/Natrosol physical mixture exhibits several peaks corresponding to the combination of signals observed in the thermograms of the two sole substances; trehalose/Natrosol microspheres show a double peak at 90 and 105°C. We ascribe the first peak to water depletion from amorphous trehalose and the other to the loss of residual water entrapped into the cellulose polymer network during microencapsulation process. No peak indicative of transitions between different trehalose forms is observed.

The stabilizing effects of trehalose on vancomycin were evaluated by DSC analysis. The thermogram of vancomycin (Fig. 5) shows only a broad endotherm centered at 90°C due to drug decomposition by melting.

Fig. 6 shows the thermograms of the physical mixtures of

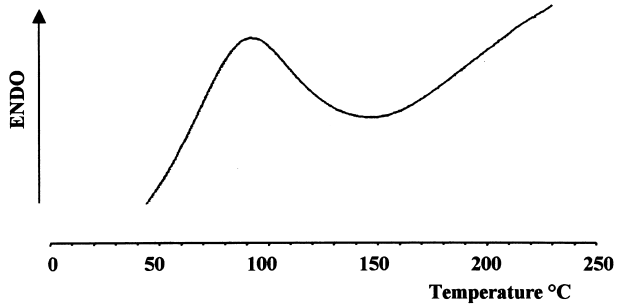

Fig. 5. DSC profile of vancomycin; (10 K min^{-1}; 40–230°C).

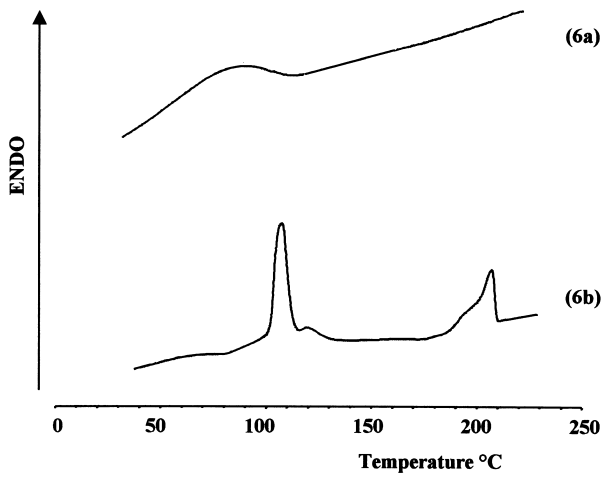

Fig. 6. DSC profiles of vancomycin/Natrosol physical mixture (1:1 w/w) (a) and vancomycin/Natrosol/trehalose physical mixture (1:1:8 w/w) (b) (10 K min^{-1}; 40–230°C).

vancomycin/Natrosol (ratio 1:1 w/w) (Fig. 6a) and vancomycin/Natrosol/trehalose (ratio 1:1:8 w/w) (Fig. 6b). The observed thermograms result as a combination of the signals observed for the corresponding pure substances.

The thermogram of the microspheres of vancomycin and Natrosol (ratio 1:1 w/w) (Fig. 7a) exhibits a single broad signal indicative of drug decomposition. The thermogram of vancomycin trehalose and Natrosol microspheres (ratio 1:1:8 w/w) (Fig. 7b) shows a single, sharp endotherm, attributed to loss of water from the amorphous system.

Integration of the area under the curves in the thermograms suggested that no drug decomposition occurred. Indeed, the area of trehalose/Natrosol microspheres signal ascribed to water loss corresponded to the area observed for vancomycin/trehalose/Natrosol® microspheres. This enabled us to

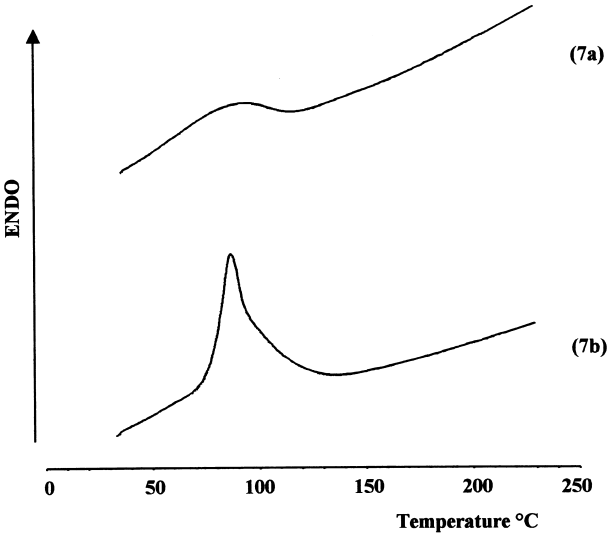

Fig. 7. DSC profiles of vancomycin/Natrosol microspheres (1:1 w/w) (a) and vancomycin/Natrosol/trehalose microspheres (1:1:8 w/w) (b) (10 K min^{-1}; 40–230°C).

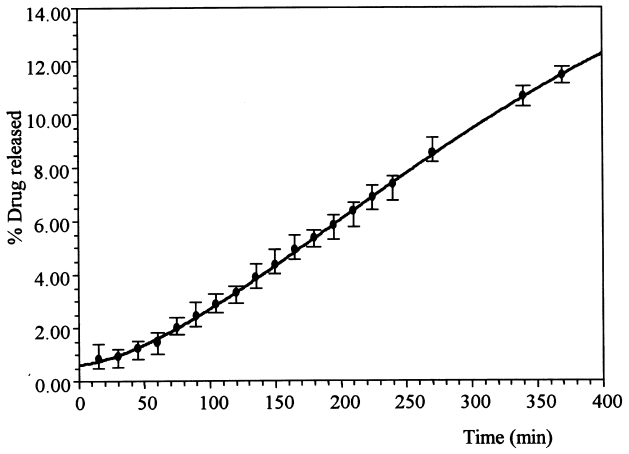

Fig. 8. Release profile of vancomycin from microspheres.

conclude that the entrapped vancomycin, besides being preserved during encapsulation and storage, is also preserved on heating. This conclusion was further supported by the full recovery of entrapped drug following 2 h heating at 90°C of a sample of vancomycin/trehalose/Natrosol microspheres. At variance, analogous heating of pure drug or vancomycin/Natrosol microspheres resulted in sample decomposition.

To assess the drug release from microspheres we performed in vitro experiments using a two-compartment linear, time-invariant, open model. A commercially available membrane used in wound dressing (see Section 2) separated the two compartments [1,23]. Measuring vancomycin, which reached simulated plasma, at suitable time intervals, enabled us to follow drug transfer throughout the membrane. Quantitative analysis was performed by UV spectrophotometric method. All experiments lasted 6 h. Fig. 8 shows the drug release profile typical for this kind of formulation. Indeed, a biphasic release mechanism, characterized by a first 'lag phase' followed by a constant slow release, is evident. The lag phase was ascribed to microspheres/water interactions, which determine swelling. This, in turn, allows the system to form a hydrophilic and viscous gel-like dispersion, which becomes progressively permeable, thus enabling drug diffusion.

In order to evaluate the complete liberation time and the shelf life of the system, we fitted the most common models, used in dissolution analysis, to our experimental results (bilog, cube root, exponential, Higuchi, Hill and Weibull [24–26]). Regression analysis verified that the Weibull model was the most appropriate for describing the kinetic of drug release as confirmed by correlation coefficient value (0.999), standard error (0.058) and analysis of residuals (Fig. 9). The χ^2 value was 0.052 while values reached greater than 10 for the other considered models. Geometric extrapolation of the Weibull function enabled us to predict that a single application of the new microparticulate system, containing one dose of vancomycin, would be sufficient for a 48 h of therapy.

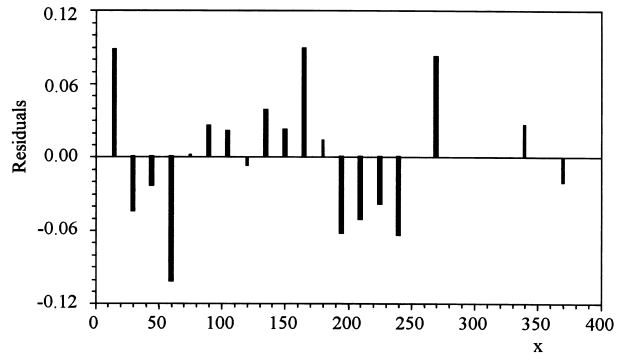

Fig. 9. Distribution of residuals for the Weibull model.

According to the results of in vitro experiments, application on wetted wounds' surfaces will induce osmotic phenomena activating the system. Following the system activation, withdrawal of aqueous media will take the twofold advantage of keeping dry the treated surface and enabling delayed release of vancomycin in situ.

4. Conclusions

Vancomycin-loaded, spherical microparticles were successfully prepared by solvent-evaporation method, using trehalose and Natrosol as matrix support.

Trehalose resulted in being an advantageous component in preventing drug decomposition during microspheres processing and successive storage. Natrosol present in the formulation maintains trehalose in its amorphous form and produces, following interaction with aqueous media, a gel-like viscous dispersion.

The prepared new delivery system seems to have characteristics suitable for topical applications on extensive and purulent or burn wounds, when the skin is extensively damaged and the barrier disrupted. This would limit indiscriminate use of systemic administration of high doses of broad-spectrum antibiotics, thus reducing the incidence of undesirable multiresistance phenomena. The system is able to take away serous exudates from wounds, likely improving healing.

Further investigations are in progress to establish the usefulness of the trehalose-based system to prevent degradation of other unstable antibiotic molecules.

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