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EPR study of mucoadhesive ointments for delivery of liposomes into the oral mucosa

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Abstract

Local drug delivery to the oral cavity tissues has been used for treatment of periodontal disease, aphthosus stomatitis, lichen planus, bacterial and fungal infections. The liposome stability in different mucoadhesive ointments, their transport into dog's oral mucosa and gingiva and washing out of ointments from tissue surface was investigated by electron paramagnetic resonance (EPR). Liposomes were composed of soya lecithin, cholesterol and lipoaminosalt (55:35:10 w/w) and encapsulated with spin labeled water soluble spin probe ASL (*N*-1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl-*N*-dimethyl-*N*-hydroxyethylammonium iodide). Polymethyl methacrylate, Carbopol 934P and Orabase as the bioadhesive ointments were used. The stability of liposomes did not change significantly after mixing with polymethyl methacrylate, but decreases in Carbopol and even more in Orabase. Washing out experiment shows that all three ointments adhere well to oral mucosa and gingiva. After 10 min of washing, approximately 30% of hydrogels were washed out. The transport experiment showed that liposomes limit the transport of hydrophilic substance to the superficial layer of epithelium. In oral mucosa, as opposed to gingiva, hydrogels enhance the transport as compared to solution of ASL or liposomes. Among the examined ointments, polymethyl methacrylate proved to be the most appropriate for local application of liposome entrapped drug to oral mucosa or gingiva.

Keywords: Mucoadhesive ointments; Orabase; Carbopol 934P; Polymethyl methacrylate; EPR; Liposome

1. Introduction

Local delivery of drugs to tissues of the oral cavity has numerous applications such as treatment of periodontal disease, aphthosus stomatitis,

Abbreviations: ASL, *N*-1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl-*N*-dimethyl-*N*-hydroxyethylammonium iodide; EPR, electron paramagnetic resonance.

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lichen planus, bacterial and fungal infections. In order to overcome the continuous flow of saliva which prevents long term adhesion of the drug to oral mucosa, several different ointments with mucoadhesive properties were investigated. With respect to mucoadhesive properties of different ointments Bremecker et al. (1984) explored the possibilities to use acrylic polymers based on methacrylic acid and methyl ester. During clinical studies on the skin and mucosa these ointments did not cause any local irritation or systemic side effects. The greatest stability of the mucosa adhesive binding in an in vitro study was assessed for the two polyacrylic acids containing formulations Carbopol 934P and EX55 (Smart, 1991).

Topical treatment of ulcerative inflammatory diseases is associated with several general disadvantages, like high permeability of the oral mucosa (Squier and Johnson, 1975; Harris and Robinson, 1992) for drugs, which could cause uncontrolled release of drug into the blood circulation and some unwanted side effects in organism. In order to localize the effect of drugs the use of liposomal formulations with encapsulated drug has been investigated (Harsayi et al., 1986). The treatment with the liposomal formulations resulted in an increase of local and a decrease of systemic drug concentration (Mezei and Gulasekharam, 1982; Harsayi et al., 1986). For the application to the tissues in oral cavity two mucoadhesive ointments with liposomes were compared, Orabase and polymethyl methacrylate (Sveinsson and Holbrook, 1993). No difference in drug release was found for these mucoadhesive ointments, however longer washing out time was observed for Orabase.

In order to develop the best formulation for local drug delivery to oral mucosa three different mucoadhesive ointments: Orabase, Carbopol 934P and polymethyl methacrylate, were investigated as possible vehicles for liposome delivery. The aim of our EPR study was to determine the level of multilamellar liposomes disintegration in these three mucoadhesive ointments, the transport of liposome-entrapped molecules from the ointments into the oral soft tissues and their washing out time from oral mucosa or gingiva.

2. Materials and methods

2.1. Preparation of liposomes and mucoadhesive ointments

2.1.1. Liposomes

Multilamellar liposomes with mean diameter of 600 nm were prepared in PBS (phosphate buffered saline, pH 7.4, 320 mosM) from hydrogenated soya lecithin (Emulmetic 320, Lucas Mayer GmBH) cholesterol and lipoaminosalt (disodium salt of *N*-stearilglutamic acid) (55:35:10 w/w) (47 mg/ml total weight of membrane components) by thin-film hydration method (Lasic, 1993). Spin probe ASL (*N*-1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl-*N*-dimethyl-*N*-hydroxyethyl-ammonium iodide) was incorporated into the liposomes during hydration (10 mM, final concentration). ASL is readily soluble in water and due to its charge does not penetrate the liposomal membrane easily. Uncaptured spin probe was removed by overnight dialysis at 4°C.

2.1.2. Mucoadhesive ointments

As the bioadhesive ointments three different hydrogels were used: Orabase, Carbopol 934P (B.F. Goodrich, Cleveland, OH) and polymethyl methacrylate (Sigma-Aldrich, Germany). Mucoadhesive ointment should be sufficiently hydrophobic to permit intimate contact with and adhesion to the oral mucosa and gingiva surface and should include a lipophilic component to prevent the ointment from being washed away. Orabase (sodium carboxymethylcellulose, pectin and gelatin combination in a polyethylene–paraffin base) was delivered from the drug store. Carbopol 934P (polyacrylate–calcium carbonate–liquid paraffin) powder was mixed with water (0.1:10 w/w) and NaOH (10% water solution) was added to keep pH value in the range between 4–6.5. Carbopol suspension was freshly prepared before use. Polymethyl methacrylate (neutralized co-polymer of methacrylic acid and methyl methacrylate) was prepared as described by Sveinsson and Holbrook (1993). The hydrogels were diluted with PBS before use in the weight ratio 1:10. Liposomes were mixed with tested ointments in the volume ratio 1:4.

2.2. EPR measurements

2.2.1. Stability of liposomes in ointments

Immediately after mixing of liposomes with tested ointments, 50 μl of the formulation was mixed with 50 μl of 0.1 mol/l sodium ascorbate (Asc) water solution, or in control experiments with 50 μl of PBS, the EPR spectra intensity was measured. The procedure was repeated at different time intervals after preparation of the liposomal formulation. Asc is a reducing agent which reduces nitroxides to the EPR non-measurable hydroxylamines. Due to its charge, Asc does not penetrate the membrane of intact liposome easily. Asc thus reduces only those spin probes which are in the external volume, i.e. those released from liposomes due to membrane disintegration. The reduction is reflected in decreased intensity of EPR spectra. EPR spectra intensity does not change after addition of Asc as long as the liposome formulation is stable. After disintegration the intensity is proportional to the amount of ASL in intact liposomes.

2.2.2. Washing out of the formulations

Slices of a Beagle dog's buccal mucosa and gingiva tissue ($10 \times 5 \times 0.9$ mm) were used not later than 24 h after biopsy. Tissue specimens were kept at 4°C in humid atmosphere until used. To the tissue surface 50 μl of liposomal formulation was applied. At 2-min intervals, tissues were washed with 50 μl of artificial plasma Haemaccel® solution (Behring Institut, Germany) and the samples of washed out mucoadhesive ointments were collected and put in glass capillaries for EPR measurements. EPR spectra intensity was measured from the amplitude of the first peak of the spectra, as the line-shape does not change during the procedure. EPR spectra intensity of the collected solution is directly proportional to the amount of hydrogel which was removed from the tissues after washing.

2.2.3. Transport of liposome-entrapped spin probe into mucosa or gingiva tissue

Approximately 1 mm thick, 2 mm wide and 10 mm long tissue slice was placed into the tissue cell with the stratified squamous epithelium (the narrow surface) mounted in contact with a cotton thread (0.3 mm thick) soaked with liposome formulation. Transport of liposome-entrapped spin probe into mucosa or gingiva was measured simultaneously by one dimensional EPR imaging (1D-EPRI) and EPR kinetic imaging methods (Gabrijelčič et al., 1994) on a Varian E-9 X-band EPR spectrometer.

2.2.3.1. 1D-EPRI method. A magnetic field gradient of 0.3 T/m was applied in the direction perpendicular to the narrow surface of the sample and parallel to the direction of magnetic field. From the EPR spectra line-shape variation with time it is possible to follow continuously the evolution of concentration profiles of ASL in tissue. The variations in the line-shape of 1D-EPRI spectra was measured empirically by asymmetry parameter l, as described elsewhere (Gabrijelčič et al., 1994). The parameter l describes the asymmetry of the 1D-EPRI spectrum, and depends strongly on the transport of spin probe into the tissue. Its alteration with time ($\Delta l = l_{25\,\mathrm{min}} - l_{5\,\mathrm{min}}$) is a measure of the transport. Only the transport into the layers more than 100 μm deep can be observed by this method.

2.2.3.2. Nitroxide reduction kinetic imaging. The rate of reduction of spin probe molecules which had been released after destruction of liposomes was measured. The released spin probes are reduced by different oxy-redoxy systems in tissue to EPR non-visible hydroxylamines (Kveder et al., 1988). Therefore, the kinetics of nitroxide reduction can be determined by measuring the intensity decrease of the EPR spectra with time for the whole sample (thread and mucosa or gingiva). The reduction kinetics and evolution of the concentration profiles of ASL molecules in mucosa or gingiva were calculated using a model which included the diffusion of intact liposomes and of released spin probes into the tissue, the decay rate of the liposomes, the reduction rates of ASL as well as the differences between epithelium and lamina propria (Gabrijelčič et al., 1994). The parameters varied as long as the best fit to the experimental kinetic curve was obtained. The determined transport parameters were used to calcu-

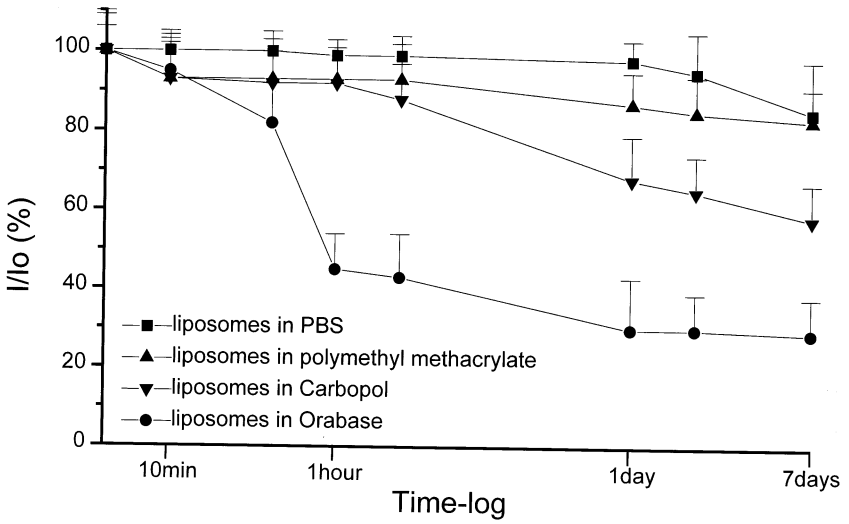

Fig. 1. The stability of liposomes in PBS solution and in different mucoadhesive ointments. Time is represented in logarithmic scale due to measurements which were performed in different time intervals (5, 10, 30 min, 1, 2 h, and 1, 2–7 days). I and I_o are EPR spectra intensities in presence and absence of 10 mM ascorbate, respectively.

late the concentration distribution profiles ($C(x, t)$) of liposome-entrapped substance in oral mucosa and gingiva.

3. Results

3.1. Stability of liposomes

The amount of intact liposomes after mixing with hydrogels is presented in Fig. 1. Liposomes were the most stable in PBS solution. Stability did not change significantly after mixing with polymethyl methacrylate but changed appreciably in Carbopol and even more in Orabase. In the first hour, 50% of liposomes in Orabase was disintegrated, but only 10% in the two other hydrogels. Twenty four hours after mixing of liposomes with different hydrogels, 30% of liposomes remained undamaged in Orabase, 68% in Carbopol, and 87% in neutralized polymethyl methacrylate.

3.2. Washing out of liposomal formulations from tissue surfaces

Washing out of liposomal formulations from tissue surfaces was recorded at 2-min intervals. From the EPR spectra intensity measurements of washed out solution it was evident that after 10 min about 30% of hydrogels was washed out from mucosa or gingiva. The largest amount of Carbopol and polymethyl methacrylate was washed out in first 2 min after application of the formulation to the oral mucosa or gingiva (Fig. 2A and B). After that period, only a very slow washing out was observed indicating good mucoadhesive properties of these two hydrogels. Washing out of Orabase from both tissue surfaces was slower but constant. So, the amount of Orabase washed out from the tissues after 10 min was about the same as for the other two hydrogels.

3.3. Transport of liposome-entrapped spin probe into oral mucosa or gingiva

3.3.1. 1D-EPRI method

No significant change in the asymmetry parameter l was observed by 1D-EPRI ($\Delta l \approx 0$). No significant penetration of spin labeled substances into the tissue layers deeper than 100 μm was observed.

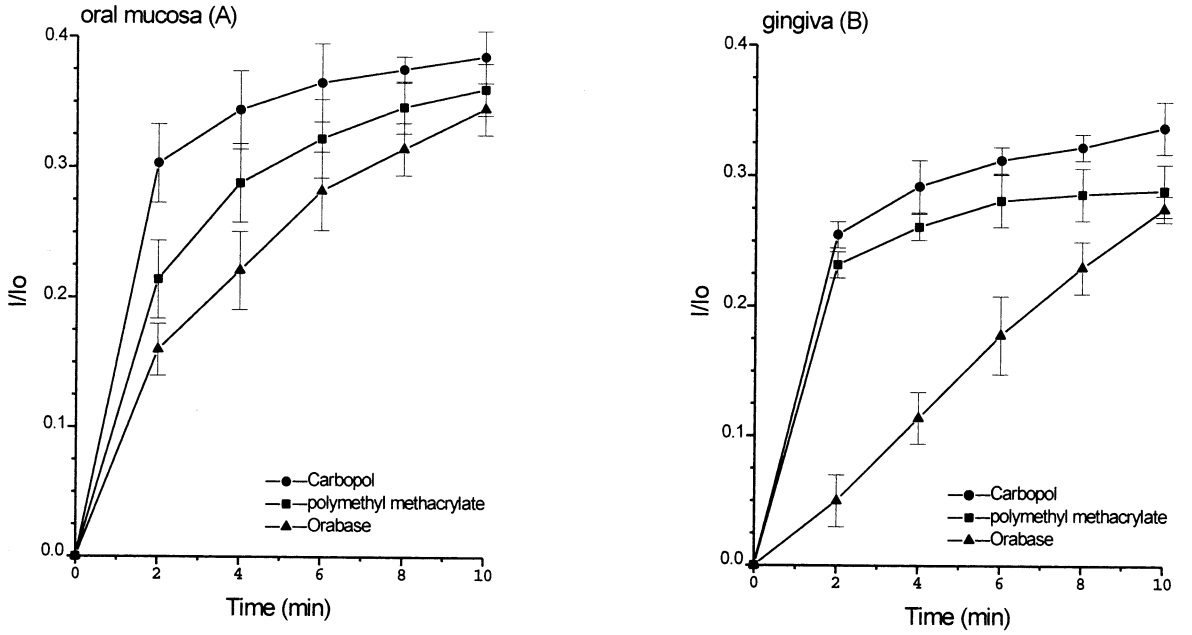

Fig. 2. Total amount of liposomes which were washed out from oral mucosa (A) or gingiva (B). I_o means EPR spectra intensity of ASL entrapped in liposomes applied to the tissue (I is the sum of intensities I_i) measured in subsequent washed out solutions $I = \Sigma I_i$.

3.3.2. Kinetics of nitroxide reduction

The rate of spin probe reduction in oral mucosa or gingiva was measured up to 27 min (Fig. 3A–D). It was interesting to note that the reduction of free ASL was much faster when applied to oral mucosa in mucoadhesive ointments as when applied in the solution (Fig. 3A). With polymethyl methacrylate, the reduction was the fastest indicating the best contact with the tissue and consequently the best penetration into the region where reduction occurred. Similar trend was also observed with liposomes but the differences are not significant (Fig. 3C). However, on gingiva the opposite trend was observed (Fig. 3B and D). Unexpected kinetics was observed when liposomes were applied to both tissues in Orabase. It can be explained with fast disintegration of liposomes in Orabase (Fig. 3C and D). ASL was released from liposomes before it came in contact with tissue[1]. In Fig. 3, the curves represent the best fits of reduction kinetics to the experimental data. The parameters by which the best fits were evaluated are presented in Table 1.

4. Discussion

The oral mucosa consists of an outermost layer of stratified squamous epithelium, below which lies a basement membrane, lamina propria and submucosa. The thickness of the oral epithelium varies for different tissues: for the buccal mucosa it is between 500 and 800 μm, while the hard and soft palates, floor of the mouth and gingiva measures 100–200 μm (Harris and Robinson, 1992). The composition of the epithelium also varies with its location in the oral cavity. Thus the epithelium of the gingiva and hard palate are keratinized but soft palate and subgingival and buccal regions on the other hand, are not keratinized (Squier and Johnson, 1975;

[1] EPR spectra amplitude increase observed for Orabase was a consequence of fast liposome disintegration in this hydrogel and release of ASL from liposomes. Due to the dilution of ASL solution the interaction between ASL molecules was diminished, the lines became narrower and the amplitude increases, if the intensity of the spectra ($I \approx b \times \Delta H^2$, b = amplitude of one spectral absorption line and ΔH = peak to peak line width) remains the same.

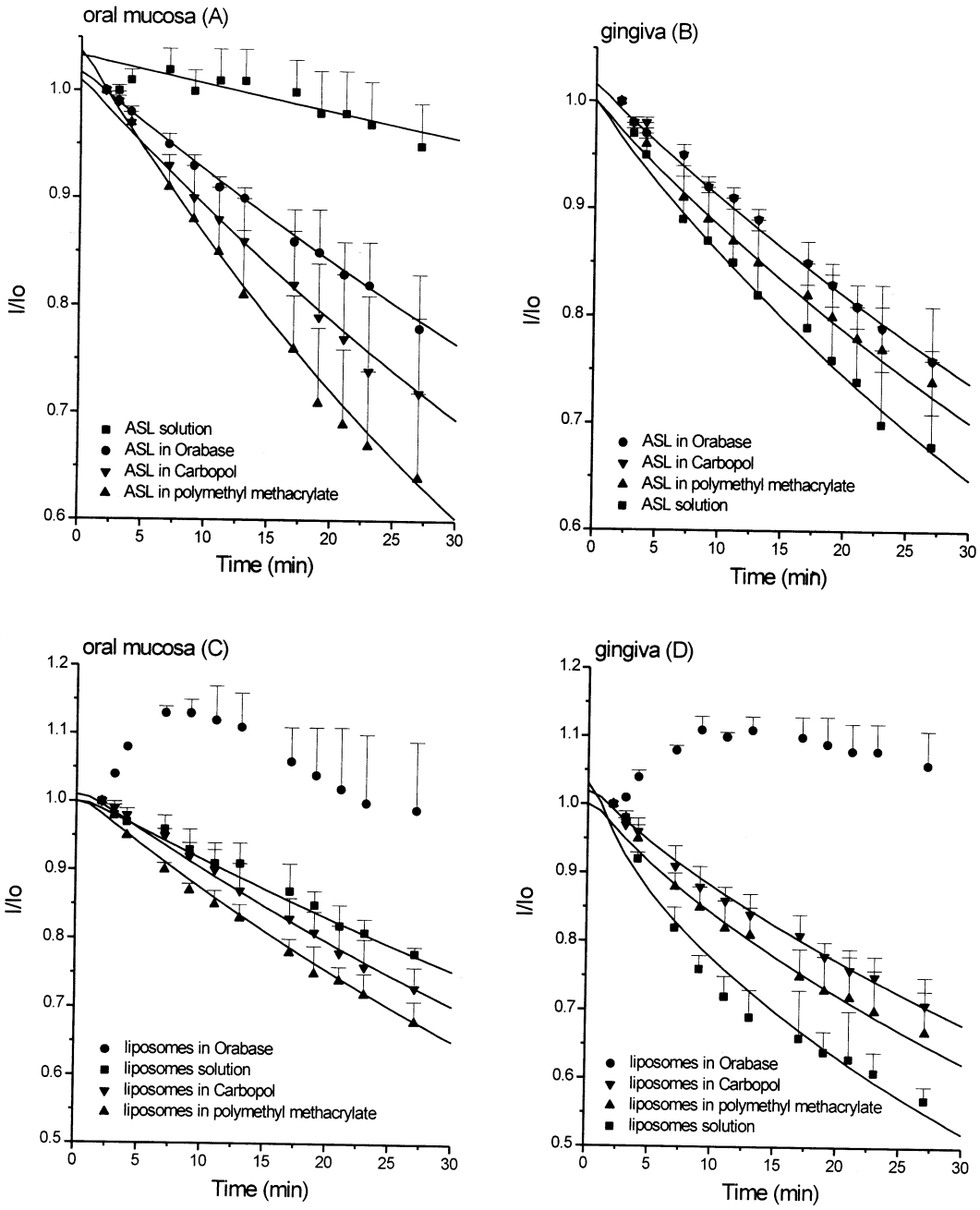

Fig. 3. Kinetics of the EPR spectra amplitude decrease of ASL applied to the surface of the oral mucosa (in solution (A), and in liposomes (C)) or gingiva (in solution (B), and in liposomes (D)) (mean values of five experiments, bars denote the standard deviations). Curves represent the best fit to the experimental data obtained with the parameters presented in Table 1. I_o is the EPR spectra amplitude immediately after application of the formulation to the tissue surface.

Table 1
Diffusion coefficients (D, 10^{-7} cm^2/s) for ASL and liposomes of oral mucosa or gingiva epithelium

		Solution	Polymer	Carbopol	Orabase
Oral mucosa	ASL solution	0.1 ± 0.04	0.5 ± 0.2	0.3 ± 0.1	0.2 ± 0.05
	Liposomes	0.2 ± 0.04	0.4 ± 0.1	0.3 ± 0.1	___[a]
Gingiva	ASL solution	0.5 ± 0.2	0.4 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
	Liposomes	3.2 ± 1.3	0.9 ± 0.2	0.7 ± 0.1	___[a]

The reduction rate K of spin probe ASL in oral mucosa was $K = 1.2$ l/mol/s, in gingiva $K = 1.6$ l/mol/s. The concentration of reducing agent in mucosa was $C = 0.06$ mol/l and in gingiva $C = 0.07$ mol/l. Decay rate constant of liposomes K_1 in epithelium of both tissues should be more than 8.3×10^{-3} s in order to obtain good fit. Diffusion coefficient in tread for ASL solution was in the range $250 > D > 1.7 \times 10^{-7}$ cm^2/s and for liposomes $333 > D > 0.8 \times 10^{-7}$ cm^2/s. In the indicated regions, the reduction decay curve is not sensitive to the alterations of the parameters.
[a] Could not be fitted by our model, see the comment in p. 197.

Harris and Robinson, 1992). Non-keratinized oral epithelium is more permeable to water than the keratinized oral epithelium (Kaaber, 1974). Inflammatory infiltrate in connective tissue also increases epithelial permeability (Squier and Johnson, 1975). However, the ulcerated surface, without the epithelial barrier provides an easier entry but also an easier exit of drugs (Harsayi et al., 1986). The permeability of the buccal mucosa is 4–4000 times greater than that of the skin. The permeability of dog's buccal mucosa for water is 2.6×10^{-5} cm/s is similar to the permeability observed in human dermis (Galey et al., 1976).

With respect to much faster reduction of ASL mixed in mucoadhesive ointments as compared to ASL solution (Fig. 3A), when applied to the oral mucosa, we suppose that an intimate contact of hydrogels with mucosa surface is obtained and consequently the best penetration into the regions, where the reduction occurs. This is also reflected in diffusion constants for ASL and liposomes in oral mucosa, which are higher in mucoadhesive ointments than in solution (Table 1). However, in gingiva the diffusion is slightly decreased by hydrogels (Table 1). It is possible that the keratinized layer of gingiva epithelium does not allow such a good contact with hydrogels as mucosal epithelium. It is also interesting to note that diffusion constant of ASL does not change if ASL is applied to the oral mucosa free or entrapped in liposomes, while in gingiva the diffusion does increase when ASL is entrapped in liposomes. Polymethyl methacrylate ensures slightly better penetration than the other two mucoadhesive ointments (Table 1).

Transport parameters presented in Table 1 can be used for the calculation of concentration distribution profiles in oral mucosa or gingiva for any applied hydrophilic liposome-entrapped substance, taking into account that it is not metabolized in contact with the tissue. In such a case in the calculation, the reduction rate constant K for ASL should be taken as zero. Concentration distribution profiles of such hydrophilic substance 27 min after application to the oral mucosa or gingiva are presented in Fig. 4. Better penetration of substance when applied to the oral mucosa in hydrogel instead of in solution could be observed. It can also be seen that liposome preparations chosen do not allow penetration into the deeper layers of the tissue. After 27 min of experiment molecules remain in the superficial layer of oral mucosa or gingiva epithelium, where a permeability barrier is observed in both type of tissues (Squier and Hall, 1984). The best possible explanation for the origin of the permeability barrier of oral epithelium involves the membrane-coating granules which are found in both keratinized and non-keratinized epithelia. They are spherical or oval shape, 100–300 nm in diameter and found in the intermediate cell layers (Harris and Robinson, 1992).

Studying the influence of different mucoadhesive ointments on washing-out time, we have found that 2 min after application the washing out of Carbopol and polymethyl methacrylate is

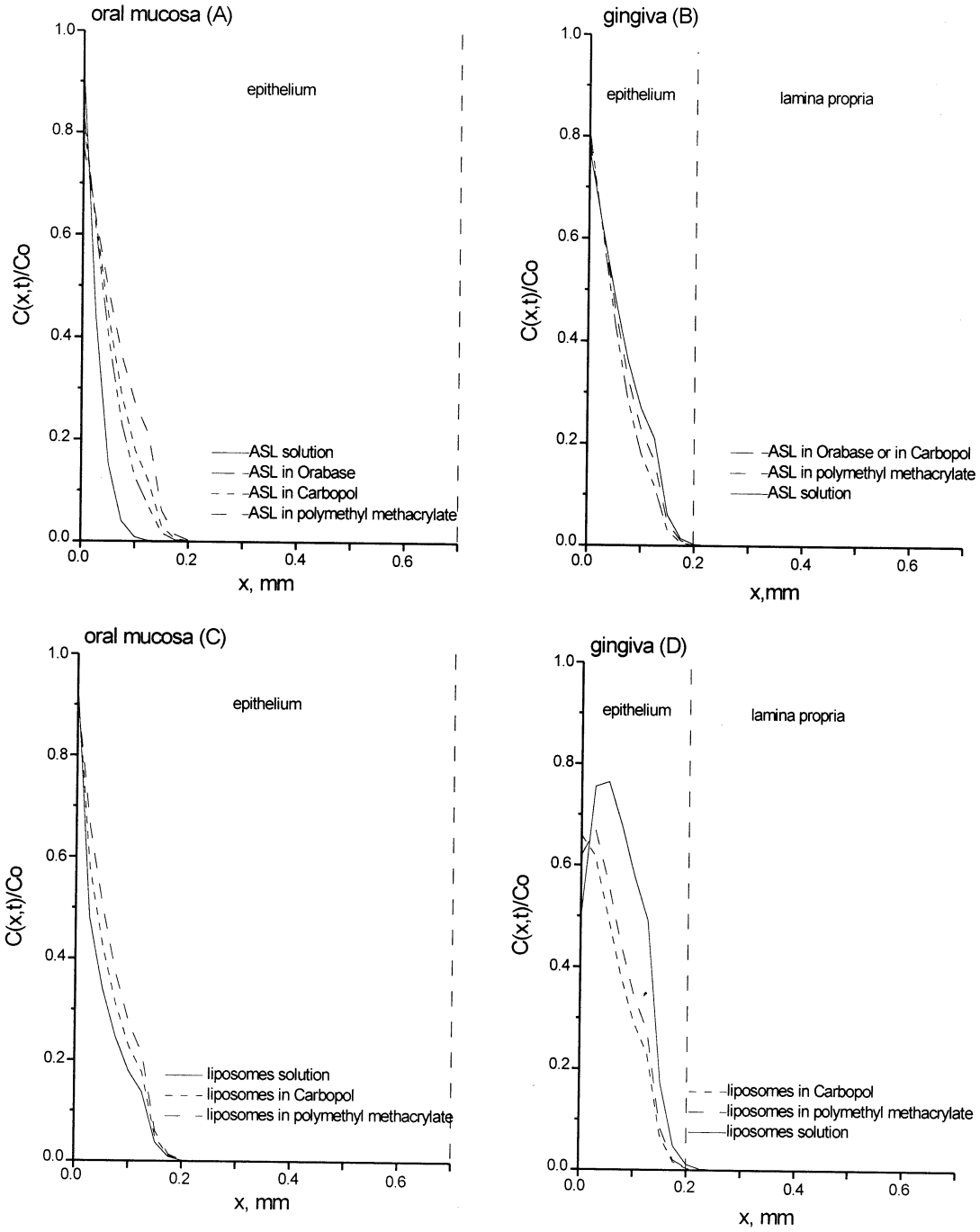

Fig. 4. The concentration distribution profiles ($C(x, t)$) in oral mucosa or gingiva tissues which was calculated for a hydrophilic substance applied to the tissues dissolved in solution (A and B) or entrapped into the liposomes (C and D). In the calculation the parameters presented in Table 1 were used with the exception of reduction rate, which was taken as zero ($K = 0$). Thickness of thread is 0.3 mm, epithelium thickness of oral mucosa is 0.7 mm and epithelium thickness of gingiva is 0.2 mm. It could be used as a model for the liposome-entrapped substance, which does not metabolise in contact with oral mucosa or gingiva.

more pronounced than that of Orabase. This is in accordance with the results of other authors (Sveinsson and Holbrook, 1993), who found longer washing out time from oral mucosa for two Orabase preparations with and without liposomes than for polymethyl methacrylate preparation with liposomes. Orabase is composed of highly hydrophobic constituents which dissolves slowly, while polymethyl methacrylate when applied to the oral mucosa was retained on it for considerable period of time even after bulk of the ointment eroded. Our observation supported that fact. After washing out time of 10 min from tissue surface, 70% of all mucoadhesive ointments investigated still remained on the tissue. Although all mucoadhesive ointments bound strongly to the tissue there are some differences in washing out behaviors (Fig. 2) which are probably caused by different binding pattern of ointment to the tissue surface. It was found recently (Anclar et al., 1993) that the molecular weight and swelling of mucoadhesive ointments had strong effect on the adhesion force when in contact with oral mucosa. While polymethyl methacrylate and Carbopol washed out for about 30% in the first 2 min; after that almost no washing out was observed. There is slower but constant washing out of liposome-entrapped substance when mixed in Orabase. This could be due to better binding properties of polymethyl methacrylate and Carbopol as compared to Orabase. It can be also caused by very low stability of liposomes in Orabase as compared to the other two hydrogels investigated (Fig. 1).

It is necessary to stress that the great part of successful application of local drug delivery system is a proper selection of vehicle, which is especially critical in oral tissues, due to the flow of saliva and the mechanical stress which prevent any long-term adhesion of ointments on the mucosa surface. The possible limitation of mucoadhesive dosage forms to the oral mucosa may be their interference during drinking and eating (Celebi and Kislal, 1995). When hydrogels adhere to a mucosal epithelium, they primarily adhere to the mucus layer. They swell and form gel at the contact with aqueous media. The rate of water uptake by mucoadhesives depend on the type and number of hydrophilic groups in the polymer structure (Anclar et al., 1993). Lipophilic base of polymer, swells and exhibits its adhesive characteristics only after contact with mucus and saliva (Bremecker et al., 1984). However, it is possible for polymers to over-hydrate to form a slippery mucilage and this may limit their use (Smart et al., 1984; Smart, 1991). The in vitro study of Needleman and Smales (1995) showed that for most bioadhesives, mucin significantly reduced adhesion time. They concluded that the absence of mucus, the control of hydrogel hydration and swelling, and the wetting characteristics are key factors for prolonged adhesion. The results from the in vitro method did not necessarily correlate well with the in vivo data. The in vitro method provided information only on the initial bioadhesion (Bouckaert et al., 1993). There is also increasing evidence that the interaction between various type of bioadhesive polymers and epithelial cells has direct influence on the permeability of mucosal epithelia. Longer bioadhesion could be probably achieved by application of bioadhesive molecules that would bind, instead of to mucus, specifically to epithelial cells as proposed by Lehr (1994).

The lipid soluble compounds, including liposomes have higher permeability into the tissue than the water soluble ones (Siegel, 1984), but liposomes decrease drug diffusion into the neighboring tissues and localize the drug in area of inflammation (Mezei and Gulasekharam, 1982; Harsayi et al., 1986). Bioadhesion could significantly improve oral therapeutics for periodontal disease and mucosal lesions. Clinical trial of Sveinsson and Holbrook (1993) reported about difficult application of liposome-encapsulated corticosteroid in Orabase and Kenalog in Orabase to the mucosa. Several patients suffered a sticky, unpleasant sensation. There were no similar complaints about the polymethyl methacrylate. Another findings of Farshi et al. (1996) was that liposome localize the corticosteroids in the ulcerated area.

Our results show that the liposomes prepared from hydrogenated soya lecithin, cholesterol and lipoaminosalt are a suitable vehicle for transport of hydrophilic substances into the superficial layer of oral epithelium. Among the hydrophilic poly-

mers investigated we found the polymethyl methacrylate to be the most appropriate mucoadhesive ointment for local application in oral cavity since the liposomes are the most stable in this polymer. Its washing out properties and transport characteristics are comparable to Carbopol and better than for Orabase. The penetration of entrapped substance into the oral mucosa or gingiva was also the most enhanced when polymethyl methacrylate was used. From the standpoint of clinical use, the polymethyl methacrylate would be a promising vehicle for treatment of periodontal disease. Due to its proper viscosity there will be possible subgingival application of different liposome-entrapped agents. In the preliminary study using liposome-encapsulated oxygen radicals scavengers for the treatment of experimental periodontitis, we already showed some promising results (Petelin et al., 1998).

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