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International Journal of Pharmaceutics 270 (2004) 83–91
international journal of pharmaceutics
www.elsevier.com/locate/ijpharm

In vivo study of different ointments for drug delivery into oral mucosa by EPR oximetry

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Received 30 April 2003; accepted 10 October 2003

Abstract

The aim of the present study was to determine the rate of transport and long-term effect of a drug applied to the oral mucosa in different ointments. Three ointments with bioadhesive properties: Orabase, Carbopol 935P, and polymethyl methacrylate (PMM) and the ointment Miglyol without such properties were used. Benzyl nicotinate (BN) was used as an active ingredient that causes hyperemia. The kinetics of drug action was measured by electron paramagnetic resonance (EPR) oximetry in vivo using the paramagnetic probe (Lithium phthalocyanine) implanted beneath the epithelium of the buccal mucosa in rats. EPR spectra line-width was proportional to local changes of partial pressure of oxygen (pO_2) in tissue and was monitored for 90 min after the application of ointments mixed with BN. The greatest increase in pO_2 and the highest efficiency of drug action was observed after the application of 2% BN in PMM ($P < 0.01$). Additionally in PMM the drug effect increased linearly with BN concentration up to 3%, at higher concentrations (3.5 and 4% BN) no further effect was observed. The results demonstrated that the greatest and the longest effect caused by a hyperemic drug in PMM. By increasing the concentration of the drug in PMM higher pO_2 in the oral mucosa can be established but only until the saturation is reached.

Keywords: Drug delivery; Hyperemic drug; Mucoadhesive ointments; In vivo EPR oximetry; Rat

1. Introduction

One of the characteristics of the oral mucosa is its selective permeability which can be used for local or systemic drug delivery (Hicks, 1973; Squier and Johnson, 1975; Galey et al., 1976; Pimlott and Addy, 1985; Barsuhn et al., 1988; Harris and Robinson, 1992; Sveinsson and Holbrook, 1993). Long-term adhesion of drugs to the oral mucosa is normally prevented by the continuous salivary flow and the mechanical movements of the tongue. Consequently, the major part of a successful application of local drug delivery systems in the oral cavity is a proper selection of vehicles. Therefore, ointments acting as vehicle for local drug delivery to the oral mucosa need to have excellent mucoadhesive properties (Bremecker et al., 1984). They are composed mainly as a hydrogel suspension in a hydrophobic base and primarily adhere to the mucus layer and swell to form gel after contact with aqueous media (Anlar et al., 1993). However, it is possible for them to over-hydrate to form a slippery mucilage which

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doi:10.1016/j.ijpharm.2003.10.030

may limit their use (Smart et al., 1984; Smart, 1991). Additionally the interaction between various types of bioadhesives and epithelial cells has direct influence on the permeability of mucosal epithelia (Lehr, 1994).

Several mucoadhesive ointments have already been studied in vitro (Bremecker et al., 1984; Smart et al., 1984; Smart, 1991; Anlar et al., 1993; Petelin et al., 1998). However, in vitro conditions could differ appreciably from that in vivo, which are much more relevant for clinical circumstances. The method by which it would be possible to follow the role of different ointments on a drug action after topical application to the oral mucosa in vivo is electron paramagnetic resonance (EPR) oximetry. The method made it possible to follow the local changes of partial pressure of oxygen in tissue (pO_2) in vivo after topical application of a vasodilator mixed with an ointment, using an implantable paramagnetic probe (Kržič et al., 2001).

EPR oximetry is based on the fact that molecular oxygen is paramagnetic and causes the fast relaxation of the surrounding paramagnetic probes by Heisenberg exchange interaction. As a consequence the line-width of the paramagnetic probe is broadened. For this purpose a paramagnetic probe sensitive to oxygen has to be implanted into the tissue and from the line-width of its EPR spectra the response of the organism to the application of the hyperemic drug can be followed continuously over a longer period of time (Swartz and Clarkson, 1998; Gallez et al., 1999; Ilangovan et al., 2002). Recently, a similar study has been performed on the skin and it showed that the type of ointment significantly influences the time when the drug starts to act and the duration of its action (Kržič et al., 2001).

In our previous in vitro study the washing out properties and transport of spin labeled molecules into oral mucosa from Orabase, Carbopol and polymethyl methacrylate (PMM) was investigated by the EPR reduction kinetic imaging method (Petelin et al., 1998). It was shown that with PMM the reduction of paramagnetic probe was the fastest, indicating the best contact with the tissue. In the following study the same three mucoadhesive ointments: Orabase, Carbopol and PMM were tested in vivo on rat oral mucosa and the results compared with Miglyol ointments without adhesive properties using EPR oximetry method.

2. Materials and methods

2.1. Animals and implantation of the paramagnetic probe

Adult female Wistar rats, weighting 200–250 g and 7–9 weeks old were purchased from the Laboratory of Pathophysiology Faculty of Medicine in Ljubljana, Slovenia. Protocol was approved by the Veterinary administration of the Republic of Slovenia (No. 323-02-76/01). Rats were anaesthetised by intraperitoneal injection of a mixture containing xylazine hydrochloride, 10 mg/kg (Rompun, Bayer, Leverkusen, Germany) and ketamine hydrochloride, 75 mg/kg (Ketanest 50, Parke-Davis, Berlin, Germany). The paramagnetic probe Lithium phthalocyanine (LiPc) (a generous gift from EPR centre for viable tissues, Dartmouth college of Medicine, Hanover, New Hampshire, USA) was implanted beneath the buccal mucosa epithelium using an injection needle 23 Gauge (Microlance, Becton Dickinson, Fraga, Spain). The needle top was filled with approximately 0.1 mm^3 of amorphous powder of LiPc and placed approximately 1 mm below the mucosa surface. Measurements started 48 h after the implantation of the paramagnetic probe in order to equilibrate the probe with the surrounding tissue and to overcome the initial stress after the implantation of the probe into the tissue. Also we wanted to avoid possible permeation of benzyl nicotinate (BN) through the wound caused by the implantation of LiPc.

2.2. Mucoadhesive ointments with benzyl nicotinate

Three different hydrogels with bioadhesive properties were used: Orabase (sodium carboxymethylcellulose, pectin and gelatin combination in a polyethylene–paraffin base), Carbopol 934P (polyacrylate–calcium carbonate–liquid paraffin), and PMM (neutralized co-polymer of methacrylic acid and methyl methacrylate). In addition, Miglyol 812 (Hüls, Witten, Germany) a mixture of triglicerides with medium length chains without such properties was administered. Orabase was delivered from the drug store. Carbopol 934P suspension (B.F. Goodrich, Cleveland, Ohio, USA) and PMM (Sigma–Aldrich, Steinheim, Germany) were prepared as described elsewhere (Sveinsson and Holbrook, 1993; Petelin et al., 1998).

The hyperemic drug BN (Lek; Ljubljana, Slovenia) was added to each ointment to achieve 2% concentration (vol.%). BN increases local blood flow indirectly through releasing nicotinic acid followed by prostaglandin D2 formation (Wilkin et al., 1985; Morrow et al., 1992). The increase of blood flow leads to the elevation of tissue pO_2, which can be measured by EPR oximetry.

2.3. EPR measurements

The prepared solution (0.05 ml) was applied by a syringe (Plastipak, Becton Dickinson, Madrid, Spain) to the surface of the buccal mucosa over the site where LiPc had been implanted. The surface coil of an extended loop resonator (11 mm diameter) was placed over the implanted area and the EPR spectra were recorded on Varian E-9 EPR spectrometer with a custom made low frequency microwave bridge (designed by Dr. T. Walczak, Dartmouth college of Medicine, Hanover, N.H., USA), operating at 1.1 GHz. Spectra were recorded at the following conditions: magnetic field density 44–45 mT, modulation amplitude 2.5×10^{-3} mT, and microwave power 20 mW.

The EPR spectra line-width (Fig. 1), which is proportional to local pO_2 changes in the tissue was converted into mucosal pO_2 according to the calibration curve for the LiPc (Šentjurc et al., 2001).

Each chosen ointment was tested in five rats. In order to obtain average pO_2 in the buccal mucosa five EPR spectra were recorded before the application of the prepared solution. The local pO_2 changes after the application of the ointments with BN was measured for 90 min in 2–5 min intervals. In the control group of rats the ointments without hyperemic drug were administered. In PMM ointment the concentration dependence of BN action was also measured. In addition to the 2% also higher concentrations of BN: 2.5, 3, 3.5, and 4% (vol.%) were measured. The time, when BN starts to act (lag time), the maximal pO_2 (ΔpO_{2max}), the time when pO_{2max} was reached and area under the curve (AUC), which represents the efficiency of drug action were evaluated for each ointment and different BN concentrations.

2.3.1. Effect of body temperature on local pO_2

The effect of body temperature changes on local pO_2 was determined. Animals were warmed with a heater until body temperature reached 38.5 °C, afterwards their spontaneous cooling to 33.5 °C was measured rectally. EPR spectra were recorded at 0.5 °C intervals.

In the temperature range where the lowest influence of body temperature on mucosal pO_2 was observed the measurements were repeated at 0.1 °C intervals for 90 min.

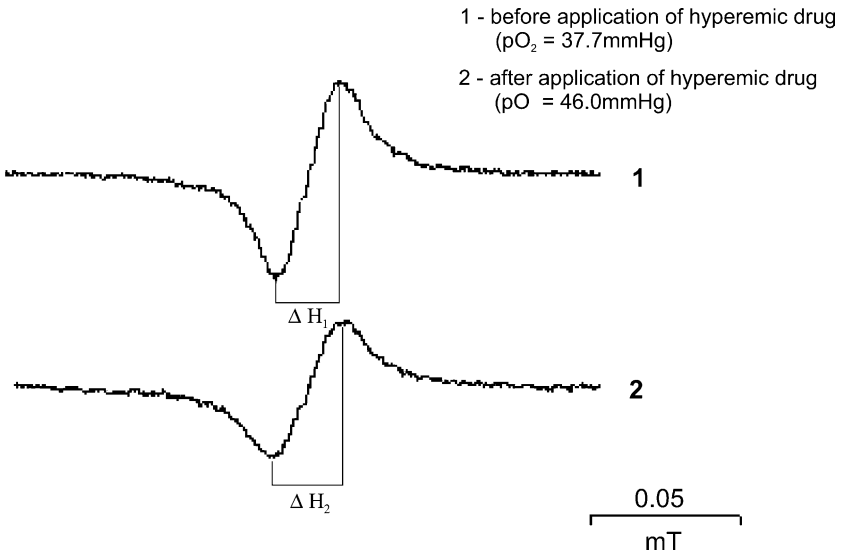

Fig. 1. EPR spectra of the paramagnetic probe Lithium phthalocianine (LiPc) implanted into the mucosa tissue. The line-width changes of the recorded spectra after the application of the hyperemic drug to the mucosa surface was measured. ΔH represents EPR spectra line-width.

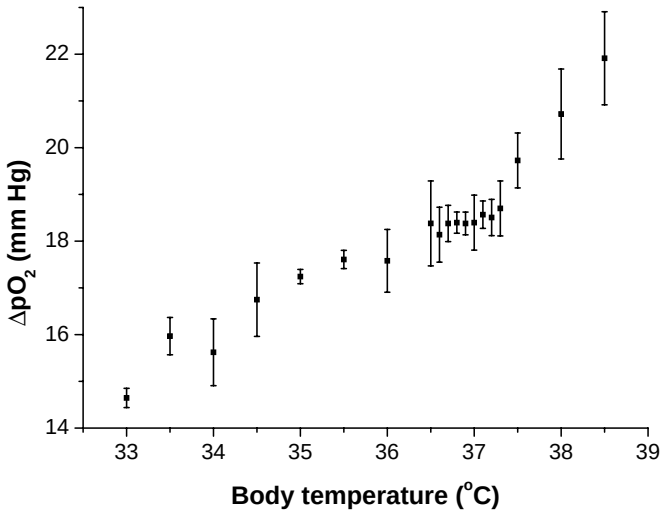

Fig. 2. Dependence of partial pressure of oxygen (pO_2) on body temperature in rat oral mucosa. Body temperature was recorded rectally on an anaesthetised rat. Minimal changes in pO_2 were observed between 36.6 and 37.2 °C.

2.4. Histological examination

The position of the paramagnetic probe and tissue reaction around it were assessed histologically. Animals were sacrificed by administration of T61 (Hoechst Roussel Veterinär GmbH, Wiesbaden, Germany). Samples of buccal mucosa (8 mm × 8 mm × 4 mm) with the paramagnetic probe in the middle were excised and fixed in 10% paraformaldehyde, dehydrated in ethanol and embedded in paraffin. Tissue reaction on hematoxilin–eosin stained specimens at 4 and 7 days after the placement of LiPc were compared with normal tissue by light microscopy.

2.5. Statistical analysis

Data were analysed using ANOVA followed by Tukey honesty significant-difference method. A level of $P < 0.05$ was chosen for statistical significance.

3. Results

3.1. Effect of body temperature and anaesthesia on pO_2 in oral mucosa

pO_2 in oral mucosa increased with elevation of body temperature (Fig. 2). Rectally recorded body temperature of an anaesthetised rat was 37.9 ± 0.3 °C. According to our measurements in the temperature range between 37.5 and 38.5 °C mucosal pO_2 is greatly influenced by body temperature. Therefore, during our experiment body temperature of animals was kept at slightly lower temperature, between 36.6 and 37.2 °C where minimal changes in pO_2 were observed.

In addition, anaesthesia affects the tissue pO_2 directly through its effect on the respiratory centre and indirectly due to peripheral vasoconstriction (Liu et al., 1995). It is therefore impossible to avoid certain influence of anaesthesia during measurements in vivo.

Table 1
The effect of 2% benzyl nicotinate (BN) in different ointments on partial pressure of oxygen (pO_2) in rat oral mucosa

Ointment	t_{lag} (min)	ΔpO_{2max} (mmHg)	t_{max} (min)	AUC (mmHg × min)
Miglyol	8 ± 2	4.0 ± 0.2	19 ± 2	89 ± 17
Orabase	20 ± 3	3.1 ± 0.5	20 ± 3	57 ± 18
Carbopol	18 ± 3	4.2 ± 0.6	33 ± 2	71 ± 22
PMM	10 ± 2	6.2 ± 0.3*	43 ± 3*	189 ± 31*

The time, when BN starts to act (lag time, t_{lag}), the largest increase in pO_2 (ΔpO_{2max}), the time when it was reached (t_{max}), and the area under the curve (AUC) after the application of 2% benzyl nicotinate in different ointments to the oral mucosa. The difference between pO_2 after the application of the drug and the basic level of pO_2 before the application of the drug is made.
* $P < 0.01$.

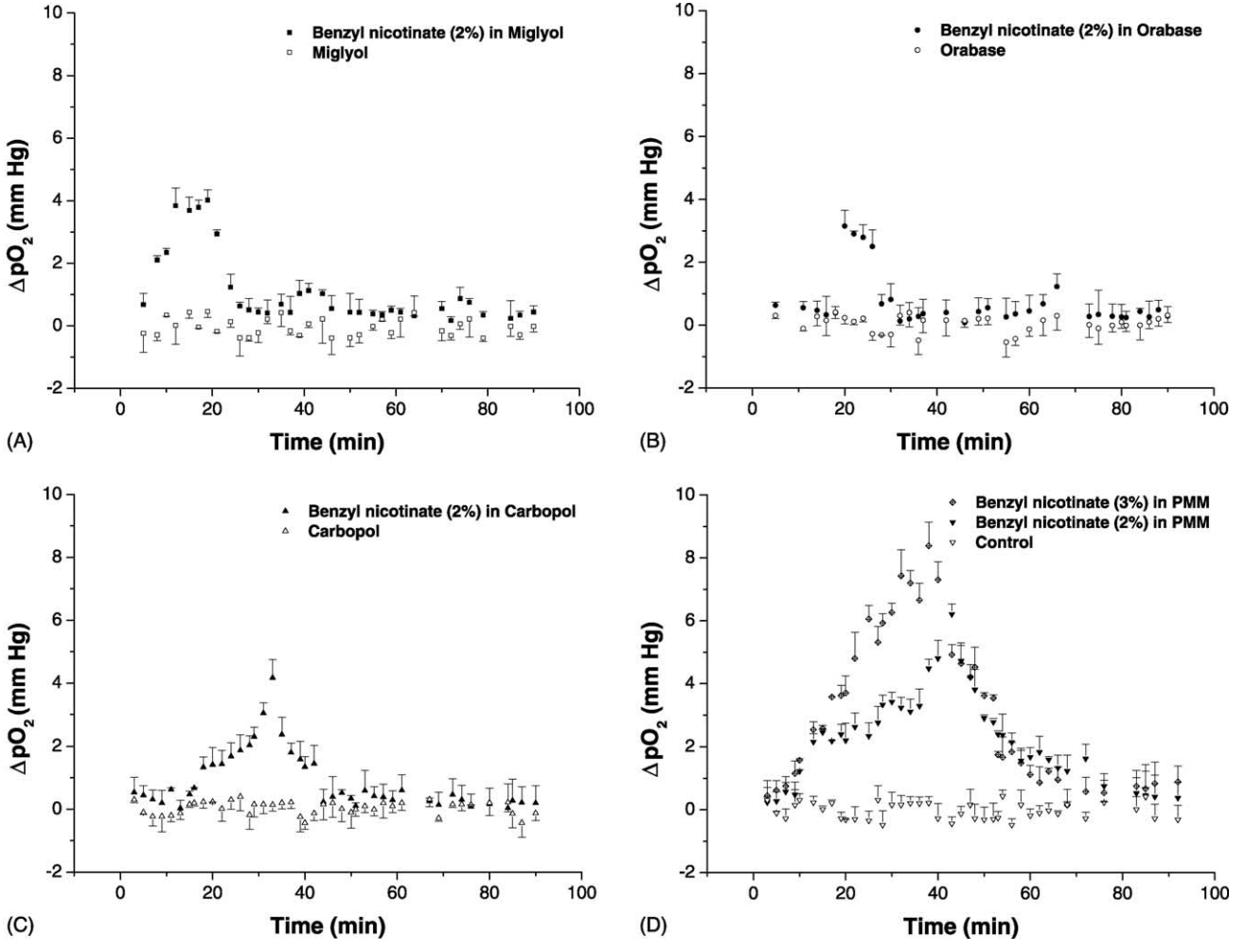

Fig. 3. Changes of local pO_2 (ΔpO_2) in the oral mucosa with time after the application of 2% benzyl nicotinate (BN) in Miglyol (A), Orabase (B), Carbopol (C) and 2 or 3% BN in polymethyl methacrylate (PMM) (D). Each point represents mean value ± S.D. of five measurements.

It was shown as some oscillations of pO_2 changes around the basic value of pO_2. The basic value was obtained as an average of the EPR spectra line-widths taken before the application of the ointment.

3.2. Effect of benzyl-nicotinate release from different ointments

As the basic value of pO_2 varies from rat to rat, probably due to different position of the paramagnetic probe in the mucosa, the basic values were subtracted from each pO_2 obtained after the application of the tested solution. In that way only the differences in pO_2 (ΔpO_2) values after the application of BN were compared for different ointments.

The effect of BN released from different ointments on local pO_2 is shown in Table 1, the lag time when BN starts to act (t_{lag}), the maximal pO_2 (ΔpO_{2max}) the time when maximal pO_2 is reached (t_{max}) and the AUC, which gives information about the effectiveness of the drug action, are presented for the ointments with 2% concentration of BN. The highest pO_2 value and the AUC were obtained with BN in PMM ($P < 0.01$). Also the time, when the maximal pO_2 is reached is the longest for PMM as compared to other ointments used in this study ($P < 0.01$). In control groups where only ointments without BN were applied no significant changes in pO_2 were obtained (Fig. 3). From Fig. 3 it is also well visible that the duration of BN action differs for different ointments and it is the longest for PMM.

Table 2 presents the concentration dependence of BN action on rat oral mucosa in PMM. The drug effect (pO_{2max} and AUC) was increasing with BN concentration up to 3%, at higher concentrations (3.5 and 4% BN) no further effect was observed. On the other hand, the time when the maximal increase in pO_2 is reached was slightly decreasing with increasing concentration of BN.

3.3. Histological examination

Four days after the implantation of the paramagnetic probe into the tissue, slight reaction in the mucosa was observed, which became obvious after 7 days. The cells around and between LiPc particles were mainly fibroblasts (Fig. 4). After that time LiPc seems to become insensitive to pO_2, as no effect of BN was detected 7 days after the implantation of LiPc.

Table 2
The effect of different concentrations of benzyl nicotinate (BN) mixed with polymethyl methacrylate (PMM) on partial pressure of oxygen (pO_2) in rat oral mucosa

Concentration of BN in PMM (%)	t_{lag} (min)	ΔpO_{2max} (mmHg)	t_{max} (min)	AUC (mmHg × min)
2.0	10 ± 3	6.2 ± 0.3[a]	43 ± 2	189 ± 31[a]
2.5	13 ± 2	7.1 ± 0.2	40 ± 3	194 ± 26
3.0	9 ± 3	8.4 ± 0.7[b]	38 ± 4	254 ± 37[b]
3.5	10 ± 3	8.7 ± 0.7[b]	36 ± 3	253 ± 22[b]
4.0	9 ± 2	8.7 ± 0.9[b]	36 ± 3	256 ± 33[b]

The superscript letters (a, b) gives probability $P < 0.01$.
The time, when BN starts to act (lag time, t_{lag}), the largest increase in pO_2 (ΔpO_{2max}), the time when it was reached (t_{max}), and the area under the curve (AUC) for different concentration of benzyl nicotinate in PMM applied to the oral mucosa of rat. The difference between pO_2 after the application of the drug and the basic level of pO_2, before the application of the drug is made.

4. Discussion

In our search for the ointment, which would be the most effective in the transport of the incorporated hyperemic drug into the oral mucosa and would have the best mucoadhesive properties in in vivo conditions we have found that the action of BN is the most effective when applied in PMM (Table 1). Our results showed that the action of 2% hyperemic drug in Miglyol occurred within 10 min after the application of the ointment, and it lasted only for about 15 min (Fig. 3A). This is not surprising as Mygliol is the ointment without mucoadhesive properties. However, it is interesting to note that Orabase behaves very similar to Mygliol (Fig. 3B). The time of action is slightly longer with Carbapol (about 20 min, Fig. 3C) and is significantly longer for BN in PMM (over 40 min) (Fig. 3D). Consequently for PMM the area under the curve was two-fold larger than in other three ointments.

The in vitro washing out study showed that Orabase, Carbopol and PMM adhere well to the oral mucosa surface (Petelin et al., 1998). After 10 min of washing approximately 70% of hydrogels still remained on it. As the duration of BN action in the three ointments is so different in in vivo conditions we can conclude that mucoadhesive properties are not the only properties important for the duration of a drug action. This was already shown in the mentioned in vitro study (Petelin et al., 1998) where we found that PMM ensured better

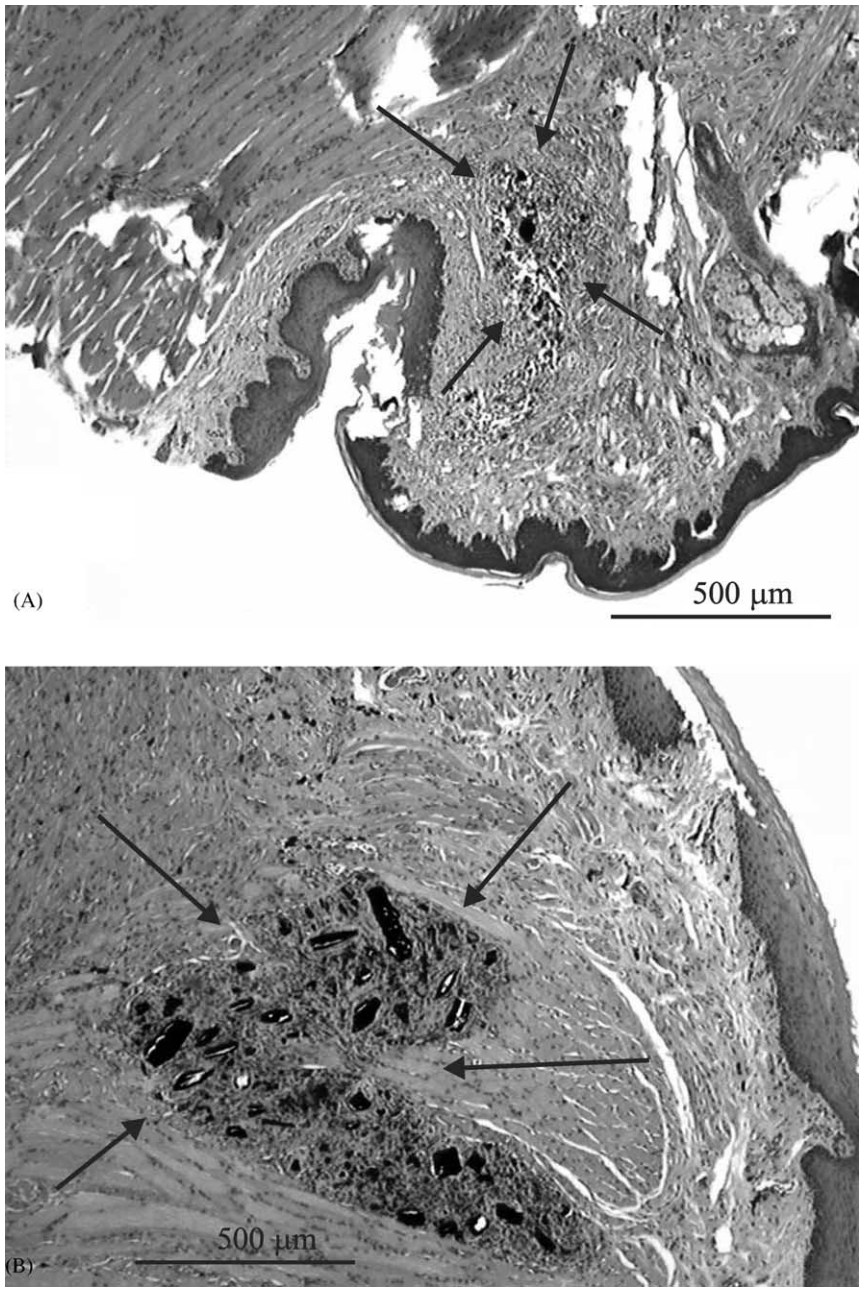

Fig. 4. Representative histological micrographs of the oral mucosa tissue. The oral mucosa 4 days (A), and 7 days (B) after implantation of the paramagnetic probe. Arrows point on cell infiltrate around the paramagnetic probe particles. H&E staining.

permeation of spin labeled molecules into the mucosa tissue than the other two ointments, which agrees well with the results presented here in in vivo conditions. Therefore we suppose that an intimate contact of hydrogels with the mucosa surface influences their transport, which seems to be much better for PMM as for the other two ointments. It is also possible that mucoadhesivity, which is about the same in first 10 min

for all three ointments, becomes superior in PMM after a longer period of time.

Concentration dependence study showed that the local changes in pO_2 can not be detected by EPR oximetry if the concentration of the hyperemic drug is smaller than 1% (Table 2). Statistically significant increase in local pO_2 with increasing concentration of BN was observed up to 3% BN in PMM. Further increase of the hyperemic drug concentration did not produce further increase in pO_2, showing that the saturation had been achieved. This is in accordance with the recent findings in mouse skin (Kržič et al., 2001). Biological effect of higher hyperemic drug concentration depends on the number of active sites or receptors for the final vasodilator prostaglandin D2 and the maximal degree of vasodilation (Kržič et al., 2001). When the maximal vasodilatation is reached further increase in BN concentration could not produce any further effect on the vessels. This assumption can be further confirmed by the fact that comparing the areas under the curves at different concentrations of BN in PMM, where no differences were found after 3% BN concentration. The time when the largest change in pO_2 was reached with higher than 3% concentration of the hyperemic drug was also the same (Table 2).

In the present study we have shown the usefulness of EPR oximetry in vivo for following the time course of the drug action and its effectiveness. By our knowledge this is the first study of this type where the changes in mucosal tissue is followed directly and quantitatively in vivo. It is also possible to follow repeated application of the drug over several days on the same site of action noninvasively. However, from this point of view some disadvantages are documented. It has been already found that some of the paramagnetic probes with optimal spectroscopic properties in vitro may lose or change their responsiveness to oxygen in the tissue (Liu et al., 1995; Swartz and Clarkson, 1998). In present study we found that LiPc became insensitive to pO_2 changes 7 days after its implantation into the tissue. According to the hystologic picture, we suppose that fibroblast's infiltrates might prevent oxygen molecules to interact with particles of LiPc. It was also impossible to measure changes of tissue pO_2 immediately after the implantation of the paramagnetic probe because the hyperemic drug could permeate the oral mucosa through the puncture wound. In addition, certain amount of time is also needed for LiPc particles to equilibrate with the tissue. Measurements were therefore limited to the time between 48 h and 7 days after the placement of paramagnetic probe into the tissue. However, these limitations could be overcome by microencapsulation of such particles into some polymeric materials, which could stabilize their responsiveness to oxygen in vivo (Jiang et al., 2001). The use of silicon permits the diffusion of oxygen inside the implant while the material does not have contact with the biological environment. In that case the pO_2 measurement could be performed over a period of 6 weeks (Gallez et al., 1996).

We conclude that in vivo oximetry is a useful method for measuring the time course of the drug action in the oral mucosa. The present results demonstrated that the greatest and the longest effect was caused by hyperemic drug in PMM. By increasing the concentration of the drug in PMM higher pO_2 in oral mucosa can be established but only until the saturation is reached. Among the ointments tested in in vivo study PMM was found as the vehicle of choice for local drug delivery to the oral mucosa.

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