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Enhanced permeability of the urinary bladder wall: the role of polymer charge

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The urothelium is usually impermeable to substances present in the urine. In the current work the possibility of using different absorption enhancers in the development of intravesical drug delivery systems was explored. To establish the role of the polymer charge on its ability to improve bladder wall permeability, cationic poly-L-arginine, anionic NaCMC and alginate as well as nonionic HPC and HPMC were tested. The permeability experiments were performed on isolated pig urinary bladders. We established that the charge of the polymer affects its ability to enhance the permeability of the urinary bladder wall, but to a limited extent. Positively charged polymers were the most promising absorption enhancers for the urinary bladder wall. In order to significantly enhance the permeability of the bladder wall, higher concentrations of poly-L-arginine were needed compared to chitosan. Moreover, chitosan reached the plateau of its absorption enhancement effect after 60 min, while poly-L-arginine increased the permeability continuously over 90 min. In contrast to polycarbophil, two other anionic polymers, NaCMC and alginate, did not significantly enhance the permeation of pipemidic acid into the tissue. Interactions between the polymers and the drug might prevail over the potential effect of NaCMC and alginate on tissue permeability. Furthermore, for the nonionic polymers HPMC and HPC an insignificant influence on bladder wall permeability was determined. Therefore, the selection of absorption enhancers for intravesical drug delivery systems is limited and cannot be done only on the basis of polymer charge.

1. Introduction

Urothelium lies on the top of the *lamina propria* and covers the luminal surface of the urinary bladder wall. It is a transitional epithelium composed of several cell layers whose differentiation increases closer to the luminal surface. The number of the cell layers depends on distension of the bladder (Jost et al. 1989; Lewis 2000). Urothelium belongs to tight epithelia, and is usually impermeable to substances present in the urine with the exception of actively transported substances (Lewis 2000; Ward et al. 2000). Besides the urothelium, the thin layer of negatively charged glycosaminoglycans on the surface of the urothelium acts as an important diffusion barrier (Lilly and Parsons 1990; Parsons et al. 1990). Moreover, 70–90% of the apical membrane surface of superficial urothelial cells is composed of specific proteins called uroplakins. They are organised in membrane plaques that are much thicker than normal membrane regions and act as another permeability barrier of the urinary bladder wall (Hu et al. 2002; Lewis 2000).

In the cases of severe urinary bladder infection or superficial bladder cancer, intravesical instillation of an appropriate drug can be beneficial. A local treatment lowers the systemic toxicity of the applied drug and increases its concentration at the site of action. Chitosan and polycarbophil have already been revealed to significantly increase the permeability of the urinary bladder wall (Grabnar et al. 2003; Kerec et al. 2005), and when applied together with the drug they could improve the effectiveness of intravesical therapy.

In the scope of the present work, some polymers other than chitosan and polycarbophil were tested for their ability to increase the permeability of the urinary bladder wall. Namely, the possibility of using different absorption enhancers facilitates the development of drug delivery systems. Cationic poly-L-arginine, anionic NaCMC and alginate, and nonionic HPC and HPMC were chosen. Some of these polymers have already been tested for their absorption enhancement activity on other tissues or cell cultures (Clausen and Bernkop-Schnürch 2001; Di Colo et al. 2007; Hosoya et al. 1998; Ohtake et al. 2002; Uchida et al. 1996); among these some have been tested even on urinary bladders (Tzan et al. 1993), while for the others literature data regarding their absorption enhancement is not available. Establishing the role of the polymer charge on its ability to enhance bladder wall permeability was then attempted. Permeability experiments were performed on isolated pig urinary bladders.

2. Investigations, results and discussion

In the cases of severe urinary bladder diseases, local treatment with intravesical instillation of a suitable drug could

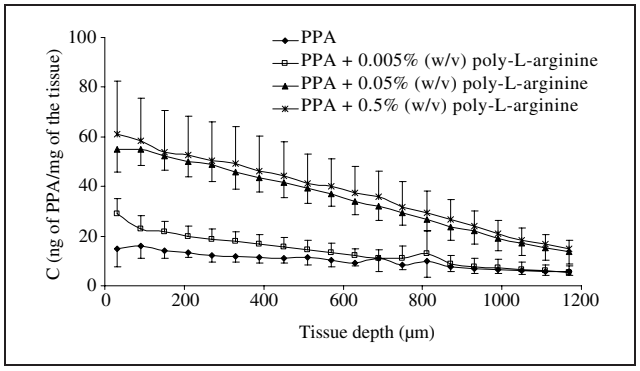

Fig. 1: The amounts of pipemidic acid (PPA) that permeated into the urinary bladder wall in the 1st series of the experiments as a function of the tissue depth (mean ± S.D., n = 6). The tissue was exposed for 1 hour to different concentrations of poly-L-arginine

be favourable. However, the urothelium is impermeable to substances present in urine (Lewis 2000), and it has the highest recorded transepithelial resistance of all epithelia measured to date (Giannantoni et al. 2006). Therefore, substances that increase bladder wall permeability could improve the effectiveness of intravesical therapy. In our previous work (Grabnar et al. 2003; Kerec et al. 2002; Kos et al. 2006) it was revealed that cationic chitosan as well as anionic polycarbophil increased bladder wall permeability to a significant extent. The aim of the present study was to evaluate the ability of some other polymers to enhance the permeation of the model drug pipemidic acid into the urinary bladder wall. Establishing the importance of the polymer charge for its absorption enhancement activity was then attempted. Positively charged poly-L-arginine, negatively charged NaCMC and alginate, and nonionic HPC and HPMC were studied.

Due to the positively charged guanidine group on the side chain of L-arginine, poly-L-arginine is a cationic polyamino acid with a high density of positive surface charges. The effect of poly-L-arginine on the permeability of the urinary bladder wall is shown in Fig. 1. At 0.05% and 0.005% (w/v) concentration of poly-L-arginine, permeation of the model drug pipemidic acid into the urinary bladder wall increased significantly. With greater tissue depth the effect of the polymer diminished. At 0.005% (w/v) concentration the maximal effect of poly-L-arginine on tissue permeability seems to be approached, and a further increase of the polymer concentration did not additionally enhance the permeability. For this reason 0.005% (w/v) concentration of the polymer was chosen to study the time dependence of poly-L-arginine's effect on permeation of the model drug into the bladder wall. Within 90 min the influence of poly-L-arginine on the tissue amounts of pipemidic acid gradually increased (Fig. 2). In our previous work (Kos et al. 2006) it was ascertained that the amounts of the model drug moxifloxacin that permeated into the bladder wall in 15, 30, 60 or 90 min from the solution of moxifloxacin alone did not differ significantly. As moxifloxacin and pipemidic acid have similar physical-chemical properties, we can conclude that increased amounts of pipemidic acid determined in this series of the experiments are due to the effect of poly-L-arginine. The ability of poly-L-arginine to enhance the permeability of the urinary bladder wall is in accordance with Tzan et al. (1993) who ascertained that poly-L-arginine increased transepithelial conductance of rabbit urinary bladders. Moreover, poly-L-arginine was intensively studied as an absorption enhancer for nasal drug delivery, and it increased the intranasal absorption of model drugs without nasal membrane damage (Miyamoto et al. 2001; Natsume et al. 1999; Ohtake et al. 2003).

The obtained results with poly-L-arginine were compared with another positively charged polymer, chitosan, whose effect on permeability of the urinary bladder wall was evaluated previously (Kos et al. 2006). As different model drugs (pipemidic acid and moxifloxacin) were used in the permeability experiments with poly-L-arginine and chitosan, a relative comparison of the enhancement effect was made. Therefore, the amounts of the drug that permeated into the tissue in the presence of chitosan or poly-L-arginine were relatively compared to the amount of the same drug that permeated into the tissue in the absence of the polymer. The results are shown in Figs. 3 and 4. For both cationic polymers the permeability of the urinary bladder wall for the model drugs increased with increasing polymer concentration (Fig. 3). The enhancement ability of chitosan was slightly higher compared to that of poly-L-arginine. In one hour even 0.0005% (w/v) dispersion of chitosan significantly enhanced the permeability of the bladder wall, while poly-L-arginine in the same concentration was not yet effective. For both polymers the absorption enhancement activity was not significantly different when 0.005 and 0.05% (w/v) concentrations of the polymer were compared. Within 90 min 0.005% (w/v) poly-L-arginine increased the bladder wall permeability more even when compared to the dispersion of chitosan of the same concentration (Fig. 4). There was still a significant difference in the amounts of the drug that permeated into

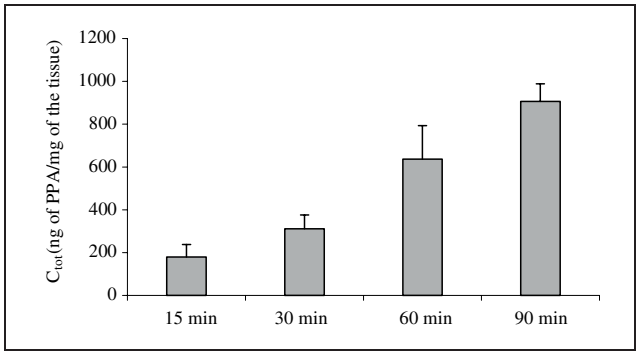

Fig. 2: The cumulative amounts of pipemidic acid (PPA) that permeated into the urinary bladder wall when the tissue was exposed for different time periods to a solution of pipemidic acid with 0.005% (w/v) poly-L-arginine (mean ± S.D., n = 6)

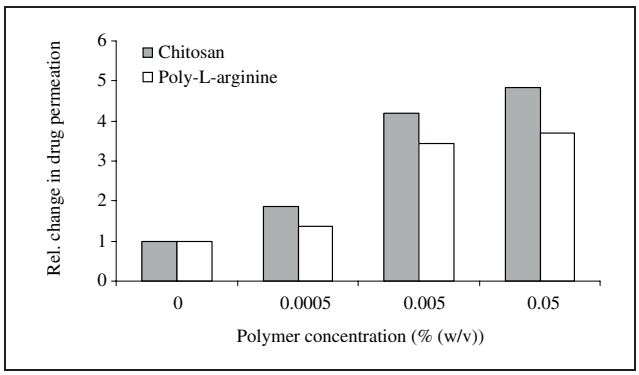

Fig. 3: The relative change in the permeability of the urinary bladder wall for quinolones (pipemidic acid, moxifloxacin) in the presence of different concentrations of chitosan or poly-L-arginine (mean, n = 6–14). The amount of the drug that permeated into the tissue in the presence of the polymer was compared to the amount of the drug determined in the tissue after exposure to the drug alone. The tissue was exposed to the tested solutions for 1 hour

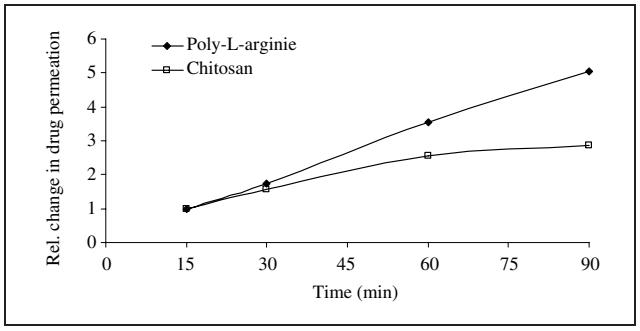

Fig. 4: The relative change in the drug permeation into the urinary bladder wall, when the tissue was exposed for different time periods to the solution of pipemidic acid with 0.005% (w/v) poly-L-arginine or to the solution of moxifloxacin with 0.005% (w/v) chitosan (mean, n = 6). The amount of the drug that permeated into the tissue in a particular time period was compared to the amount of the same drug permeated in 15 minutes

the tissue after a 60 and 90 min exposure to the dispersion of poly-L-arginine, while the effect of chitosan approached its plateau after 60 min. On nasal epithelium the absorption enhancement effect of poly-L-arginine was comparable or stronger to chitosan of the same concentration, dependent on poly-L-arginine molecular weight (Natsume et al. 1999).

Cationic polymers are obviously capable of increasing the permeability of the bladder wall. Their positive charge is important for interactions with a negatively charged urothelial surface. In nasal epithelium poly-L-arginine increased the paracellular transport of hydrophilic macromolecules through disorganisation of the proteins of tight and adherens junctions (Ohtake et al. 2003). A similar mechanism of absorption enhancement, studied mostly on Caco-2 cells, was also proposed for chitosan (Smith et al. 2004). However, in our previous studies (Kerec et al. 2005; Kos et al. 2006) it was revealed that in concentrations that significantly enhanced permeability of the urinary bladder wall, chitosan triggered necrosis of superficial urothelial cells and desquamation of the urothelium. The revealed mechanism is not so unusual for the urinary bladder, as urothelial cells normally respond to bacterial infection with desquamation and consecutive excretion of infected superficial urothelial cells with attached bacteria (Mulvey et al. 1998). Due to different time-dependent effects of chitosan and poly-L-arginine on bladder wall permeability, it is possible that poly-L-arginine also triggers urothelial desquamation, but the rate at which urothelial cells desquamate is different. On the other hand, a different mechanism of absorption enhancement as determined for chitosan cannot be completely excluded.

Furthermore, we aimed to determine the effect of negatively charged NaCMC and alginate on permeability of the urinary bladder wall. In our previous studies (Grabnar et al. 2003; Kerec et al. 2002) anionic polymer polycarbophil significantly increased the permeation of pipemidic acid into the bladder wall. Moreover, Clausen and Bernkop-Schnürch (2001) established that 1% (w/v) NaCMC significantly increased the transport of sodium fluorescein across the intestinal mucosa of guinea pigs. Therefore, increased permeability of the urinary bladder wall in the presence of NaCMC and alginate was expected. However, neither of the polymers significantly enhanced the permeation of pipemidic acid into the tissue in any of the concentrations tested (Fig. 5). Besides not being effective as permeability enhancers of the urinary bladder wall, the reason for insignificant results obtained with NaCMC and

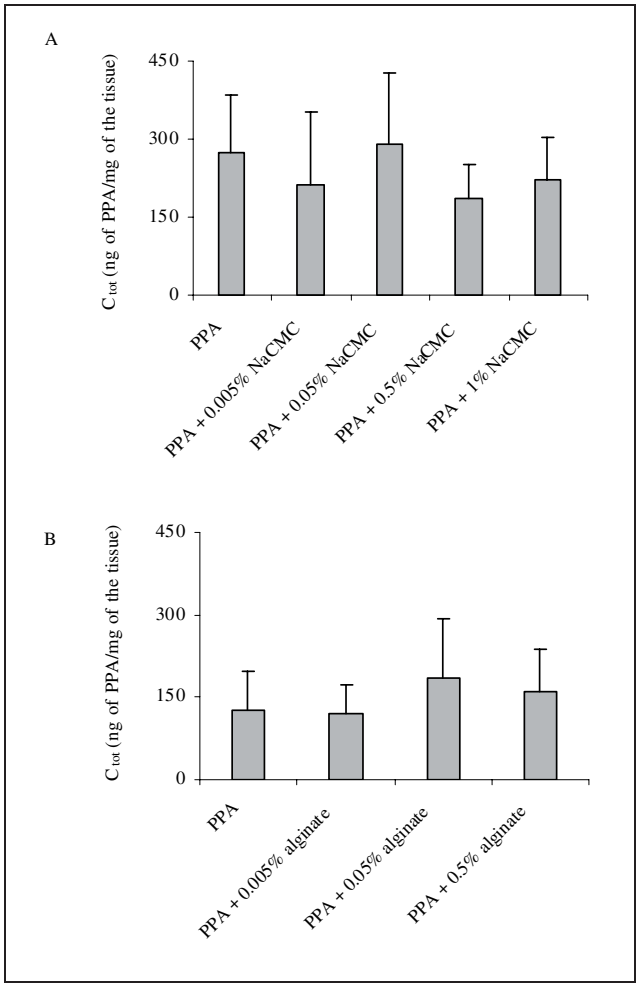

Fig. 5: The cumulative amounts of pipemidic acid (PPA) that permeated into the urinary bladder wall when the tissue was exposed for 60 minutes to a solution of pipemidic acid with different concentrations (% (w/v)) of sodium carboxymethyl cellulose (NaCMC) (Fig. A) or alginate (Fig. B) (mean ± S.D., n = 7–9)

alginate could be in interactions between the polymers and pipemidic acid. Pipemidic acid has pK_{a1} 5.55 (acidic) and pK_{a2} 8.66 (basic) (Izumi and Kitagawa 1989). At pH 4.5, amino groups of pipemidic acid are protonated, and they can form electrostatic interactions with negatively charged NaCMC and alginate. This would hinder the permeation of pipemidic acid into the tissue. Furthermore, the dispersions with higher concentrations of NaCMC or alginate were quite viscous, which could also prevent the diffusion of pipemidic acid into the tissue. Finally, within one hour polymer molecules could partially precipitate and form a thin layer on the surface of the urothelium that additionally hinders the permeation of the drug into the tissue. All the above-mentioned interactions between pipemidic acid and NaCMC or alginate could potentially prevail over the effect of the tested polymers on tissue permeability. However, these interactions with pipemidic acid could also occur in the experiments with 1% (w/v) polycarbophil, which was on the contrary proven as an effective absorption enhancer at pH 4 (Grabnar et al. 2003; Kerec et al. 2002). In the study where 1% (w/v) NaCMC significantly improved the tissue permeability (Clausen and Bernkop-Schnürch 2001), the tested dispersions had pH 7.4, and the experiments were performed on the small intestine, whose luminal surface differs from the urinary bladder.

To eliminate the interactions in the further experiments, pipemidic acid was applied to the tissue separately from

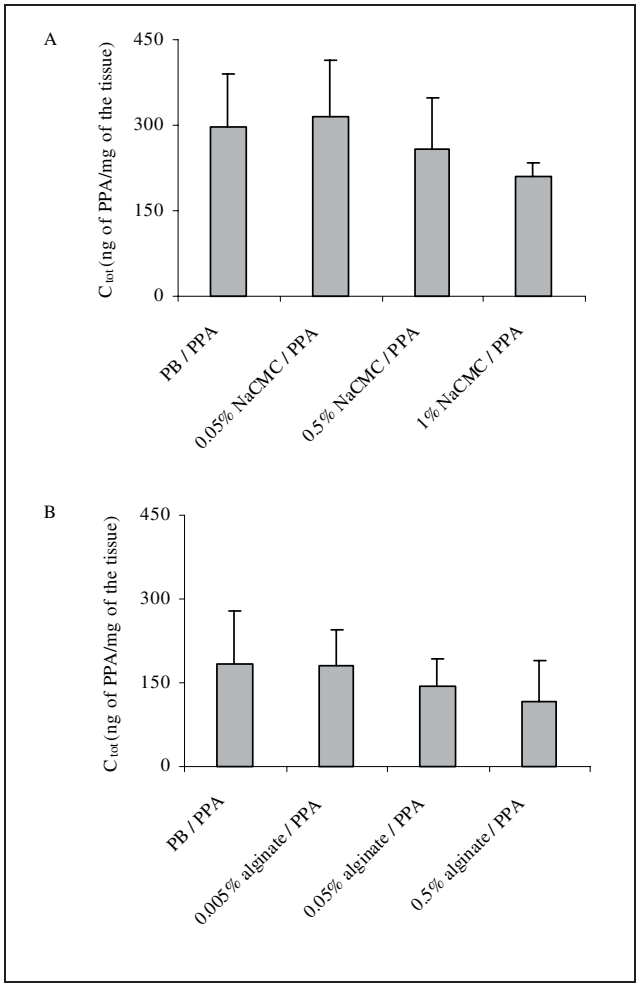

Fig. 6: The cumulative amounts of pipemidic acid (PPA) that permeated into the urinary bladder wall when the tissue was first exposed for 45 minutes to a phosphate buffer (PB) (a control), a dispersion of sodium carboxymethyl cellulose (NaCMC) (Fig. A) or a dispersion of alginate (Fig. B) and then for additional 45 minutes to a solution of pipemidic acid (mean ± S.D., n = 5–6)

NaCMC and alginate. However, preliminary application of the polymers did not significantly enhance the permeation of pipemidic acid into the bladder wall (Fig. 6). It even seems that with increasing concentration of NaCMC and alginate dispersions the permeation of the drug into the tissue decreases. Before applying pipemidic acid the tissue was rinsed with the buffer. Nevertheless, it is possible that due to the mucoadhesiveness and relatively high viscosity of alginate and NaCMC dispersions a certain amount of the polymer still remained on the tissue and hindered the permeation of pipemidic acid. At higher concentrations of the polymers this phenomenon is more likely to occur.

Therefore, anionic polymers do not uniformly influence the permeability of the urinary bladder wall. In contrast to polycarbophil, NaCMC and alginate were not effective as absorption enhancers. Increased permeability in the presence of polycarbophil was explained by the removal of extracellular calcium by binding to carboxylic groups of the polymer, which results in opening of tight junctions and higher paracellular permeability for model drugs (Kerec et al. 2002; Kriwet and Kissel 1996). NaCMC and alginate could affect urinary bladder wall permeability by the same mechanism, but their affinity for calcium binding is too low to determine a significant effect. In any case, interactions between the model drug and the polymers

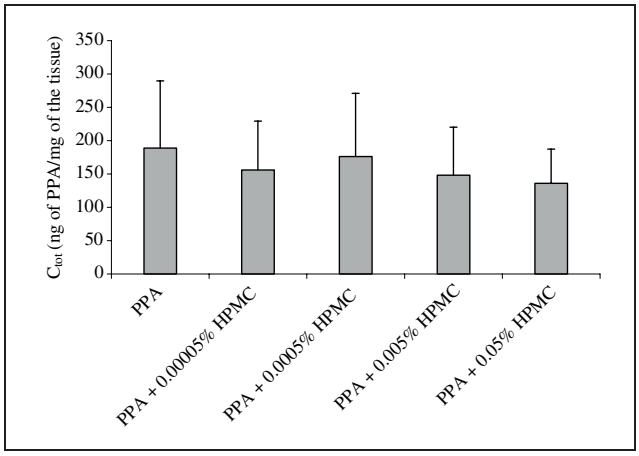

Fig. 7: The cumulative amounts of pipemidic acid (PPA) that permeated into the urinary bladder wall when the tissue was exposed for 60 minutes to a solution of pipemidic acid with different concentrations (% (w/v)) of hydroxypropyl methylcellulose (HPMC) (mean ± S.D., n = 5)

might prevail over the potential effect of NaCMC and alginate on tissue permeability.

In the last part of our study, the absorption enhancement properties of nonionic polymers HPMC and HPC were determined. Firstly, pipemidic acid was applied to the tissue at

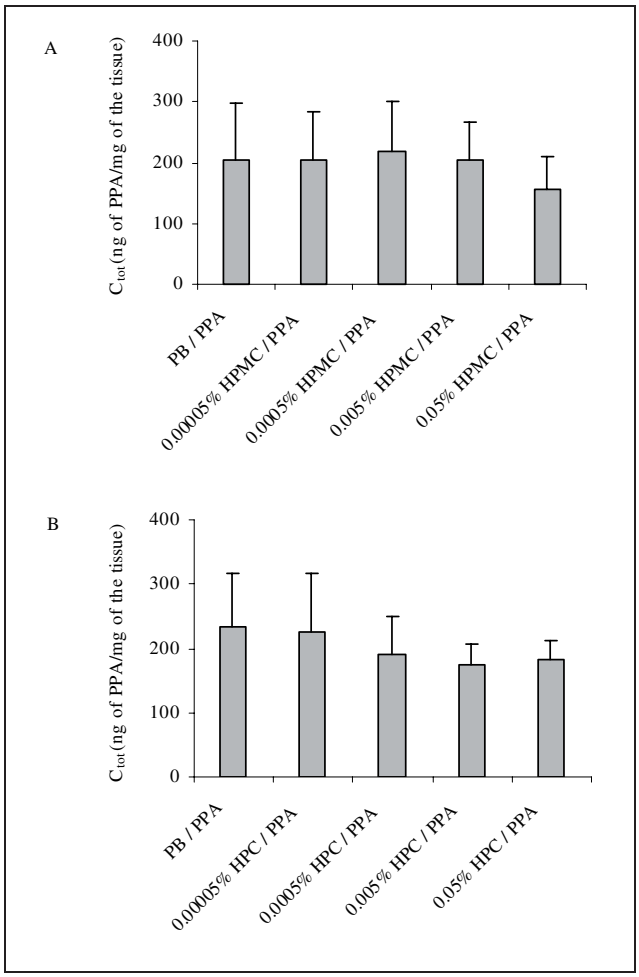

Fig. 8: The cumulative amounts of pipemidic acid (PPA) that permeated into the urinary bladder wall when the tissue was first exposed for 45 minutes to a phosphate buffer (PB) (a control), a dispersion of hydroxypropyl methylcellulose (HPMC) (Fig. A) or hydroxypropyl cellulose (HPC) (Fig. B) and then for additional 45 minutes to a solution of pipemidic acid (mean ± S.D., n = 5)

the same time as HPMC, and no significant effect of the polymer on the drug permeation was determined at any concentration tested (Fig. 7). As with anionic polymers, in the further experiments pipemidic acid was applied to the tissue after it was exposed to HPMC or HPC. In this case also, the tested polymers did not significantly increase the amounts of pipemidic acid that permeated into the bladder wall (Fig. 8). The obtained results are in accordance with those of Hosoya et al. (1998), who determined that the nonionic polymers HPC and polyethyleneoxide did not significantly influence the permeation of morphine and salicylic acid through excised hairless rat skin. On the contrary, the addition of HPC to the solution of doxorubicin increased the amounts of the drug detected in normal and tumorous urinary bladder walls (Ueda et al. 1992). However, increased tissue amounts of doxorubicin in the presence of HPC were more probable to be the consequence of prolonged residence time of the drug within the urinary bladder due to the mucoadhesiveness of HPC rather than the influence of HPC on the permeability of the urinary bladder wall. In contrast to positively charged polymers that can form ionic interactions with negatively charged glycosaminoglycans on the surface of the urothelium, nonionic polymers form weaker interactions with the luminal bladder surface. Moreover, they do not possess any functional groups that would bind calcium ions and consecutively influence paracellular permeability. Therefore, nonionic polymers are not promising absorption enhancers for the urinary bladder wall.

In Conclusion the charge of the polymer affects its ability to enhance permeability of the urinary bladder wall, but only to a certain extent. Cationic polymers were established as the most promising absorption enhancers for the urinary bladder wall. For significant absorption enhancement, higher concentrations of poly-L-arginine were needed compared to chitosan, and over 90 min the permeability of the bladder wall increased constantly in the presence of poly-L-arginine, while chitosan reached the plateau of its absorption enhancement activity after 60 min. In contrast to polycarbophil, two other anionic polymers, NaCMC and alginate, did not significantly enhance the permeation of pipemidic acid into the tissue. Interactions between the polymers and pipemidic acid might prevail over the potential effect of NaCMC and alginate on tissue permeability. Moreover, nonionic polymers HPMC and HPC were not proven useful to increase the permeation of drugs into the urinary bladder wall. Therefore, the selection of absorption enhancers for intravesical drug delivery systems is limited and cannot be done exclusively on the basis of a polymer charge.

3. Experimental

3.1. Materials

Poly-L-arginine hydrochloride (in the text referred to as poly-L-arginine) with molecular weight 70–150 kDa was obtained from Sigma-Aldrich, St. Louis, USA. Hydroxypropyl methylcellulose (HPMC) (Metolose 65SH-400,

Table: Solutions used in the permeability experiments

1st series; n = 6		2nd series; n = 6	
• PPA (60)		• PPA + 0.005% poly-L-arginine (15)	
• PPA + 0.0005% poly-L-arginine (60)		• PPA + 0.005% poly-L-arginine (30)	
• PPA + 0.005% poly-L-arginine (60)		• PPA + 0.005% poly-L-arginine (60)	
• PPA + 0.05% poly-L-arginine (60)		• PPA + 0.005% poly-L-arginine (90)	
3rd series; n = 9		**4th series; n = 7**	
• PPA (60)		• PPA (60)	
• PPA + 0.005% NaCMC (60)		• PPA + 0.005% alginate (60)	
• PPA + 0.05% NaCMC (60)		• PPA + 0.05% alginate (60)	
• PPA + 0.5% NaCMC (60)		• PPA + 0.5% alginate (60)	
• PPA + 1% NaCMC (60)			
5th series; n = 6		**6th series; n = 5**	
• PB (45)	• PPA (45)	• PB (45)	• PPA (45)
• 0.05% NaCMC (45)	• PPA (45)	• 0.005% alginate (45)	• PPA (45)
• 0.5% NaCMC (45)	• PPA (45)	• 0.05% alginate (45)	• PPA (45)
• 1% NaCMC (45)	• PPA (45)	• 0.5% alginate (45)	• PPA (45)
7th series; n = 5		**8th series; n = 5**	
• PPA (60)		• PB (45)	• PPA (45)
• PPA + 0.00005% HPMC (60)		• 0.00005% HPMC (45)	• PPA (45)
• PPA + 0.0005% HPMC (60)		• 0.0005% HPMC (45)	• PPA (45)
• PPA + 0.005% HPMC (60)		• 0.005% HPMC (45)	• PPA (45)
• PPA + 0.05% HPMC (60)		• 0.05% HPMC (45)	• PPA (45)
9th series; n = 5			
• PB (45)	• PPA (45)		
• 0.00005% HPC (45)	• PPA (45)		
• 0.0005% HPC (45)	• PPA (45)		
• 0.005% HPC (45)	• PPA (45)		
• 0.05% HPC (45)	• PPA (45)		

PB is a phosphate buffer, PPA a solution of pipemidic acid in PB; solutions of the tested polymers (poly-L-arginine, sodium carboxymethyl cellulose (NaCMC), alginate, hydroxypropyl methylcellulose (HPMC), hydroxypropyl cellulose (HPC)), were prepared in PB or in the solution of pipemidic acid (PPA + polymer); different polymer concentrations (% (w/v)) were tested; in 5th, 6th, 8th and 9th series of the experiments the tissue was first exposed to one solution, rinsed and then the second solution was applied; the minutes of the tissue exposure to a particular solution are given in parentheses; n indicates the number of the urinary bladders used in a particular series of the experiments

an apparent viscosity of 2% aqueous dispersion 468 mPas) was purchased from Shin-Etsu Chemical co., Tokyo, Japan. Hydroxypropyl cellulose (HPC) (Klucel, GXF Pharm) and sodium carboxymethyl cellulose (NaCMC) (Blanose CMC, 7M8SXF PH) were supplied by Aqualon, France. The apparent viscosity of 2% aqueous dispersion was 295 mPas for HPC and 655 mPas for NaCMC. Sodium alginate (in the text referred to as alginate) (Protanal LF 240 D, an apparent viscosity of 1% aqueous dispersion 70–150 mPas) was obtained from FMC BioPolymer, Zürich, Switzerland. The model drug pipemidic acid was kindly provided by Lek, Ljubljana, Slovenia.

For chromatographic determination of pipemidic acid in the tissue samples methanol and acetonitrile were of HPLC grade (PChromasolv, Sigma-Aldrich Laborchemikalien) and trichloroacetic acid was of analytical grade (Merck, Darmstadt, Germany).

The phosphate buffer saline (PBS) (Ph. Eur. IV) consisted of 0.944 g Na_2HPO_4, 0.19 g KH_2PO_4, and 8 g NaCl in 1 L of deionised water (pH = 7.4). The phosphate buffer (PB) consisted of 0.472 g Na_2HPO_4, 0.095 g KH_2PO_4, and 1.6 g NaCl in one litre of deionised water (pH adjusted to 4.5). All chemicals used were of analytical grade.

3.2. Tissue preparation

Pig urinary bladders were obtained from a local slaughterhouse. Until used, the bladders were kept in PBS cooled to 5 °C and saturated with carbogen (95% O_2, 5% CO_2). All experiments were performed within 3 h after sacrifice. The corpus of the urinary bladder was cut into pieces with an approximate size of 25 × 25 mm. Each piece was mounted into a diffusion cell, developed at the Faculty of Pharmacy, Ljubljana, Slovenia (Kerec et al. 2005). The donor chamber of the diffusion cell had a volume of 10 mL and the tissue exposure area was 4.5 cm^2. In diffusion cells the luminal side of the urinary bladder wall was exposed to tested solutions.

3.3. Permeability studies

In the permeability studies poly-L-arginine, NaCMC, alginate, HPC, and HPMC were tested for their ability to increase the permeability of the urinary bladder wall. All the tested solutions were prepared in PB and due to the comparability to the results of our previous studies their pH was adjusted to 4.5. A pH of 4.5 is within the normal range of urine pH (Thomas 1998), and it was proven in our previous study (Kerec et al. 2005) that a one-hour exposure of luminal urinary bladder surface to PB with pH 4.5 did not affect the morphology of the tissue. Pipemidic acid was used as a model drug in all the experiments in a concentration of 0.014% (w/v). Permeability studies were performed at room temperature within 4 h after the pigs were sacrificed.

Nine series of the permeability experiments were performed. In some series the tested polymer was applied on the tissue together with pipemidic acid, while in the other experiments the tissue was preliminarily exposed the tested polymer and then separately to pipemidic acid only. Between both applications the tissue was rinsed three times with PB. The solutions used in the experiments as well as the time of the tissue exposure to the particular solution are shown in the Table.

3.4. Sample preparation and HPLC analyses

At the end of the permeability experiments the tissue was rinsed three times with PB. Afterwards it was placed between two parallel stainless steel plates, whose distance was regulated regarding the tissue thickness, and rapidly frozen with liquid nitrogen. The tissue was then sectioned by cryostat (Leica CM 1850, Nussloch, Germany) in sections of 20 μm thickness parallel to luminal surface up to 1.2 mm of the tissue depth. Three consecutive sections were pooled and 500 μL of mobile phase (0.2% trichloroacetic acid/acetonitrile/methanol, volume ratio 76/20/4) was added to each sample. To ensure complete extraction, the samples were first vortexed until all the tissue sections were sunken into the mobile phase and then shaken (225 cpm, 2 h, room temperature). After centrifugation of the samples (45.000 g, 10 min, room temperature) concentration of pipemidic acid in the supernatant was determined by HPLC. A PRP-1 column (150 × 4.1 mm, 5 μm particles; Hamilton, Reno, USA) and a precolumn of the same type were used. The flow of the mobile phase was 1 mL/min and diode array detection at 275 nm was applied. The cumulative amounts of pipemidic acid that permeated into the urinary bladder wall were calculated.

3.5. Statistical evaluation

The data obtained within a particular series of the permeability experiments were evaluated for statistically significant differences by ANOVA for repeated measures using the Bonferroni *post hoc* test ($\alpha = 0.05$).

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