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International Journal of Pharmaceutics 278 (2004) 415–422
international journal of pharmaceutics
www.elsevier.com/locate/ijpharm

Transport of leuprolide across rat intestine, rabbit intestine and Caco-2 cell monolayer

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Received 9 November 2003; received in revised form 8 March 2004; accepted 22 March 2004

Abstract

The purpose of this study was to investigate the transport mechanisms and causes of low bioavailability of leuprolide. The everted gut sac technique and Caco-2 cell monolayer were used to examine: (1) transport properties, enzyme degradation and apparent permeation coefficient (P_{app}); (2) the influence of trypsin inhibitor, EDTA, chitosan and alginate on drug transport; and (3) the effect of animal species on the intestinal transport. Results showed flux increased with increasing concentration of drug, showing a passive diffusion pathway. The enzyme degradation in rabbit gut was the highest. The P_{app} of $(4.19 \pm 1.33) \times 10^{-5}$ cm/s in rat gut was the largest and the P_{app} of $(5.20 \pm 0.20) \times 10^{-7}$ cm/s in Caco-2 cell the smallest. At a low concentration of drug, trypsin inhibitor had strong enhancement effect on the P_{app} by protecting enough drug for permeation. Chitosan had no effect on the activity of α-chymotrypsin. The increase in P_{app} was due to opening of the tight junctions and interaction with cells. In conclusion, both inhibition of proteolytic enzymes and opening the tight junctions to allow for paracellular transport improved the intestinal absorption. At low drug concentration, reduction of enzyme degradation is the most important factor.

Keywords: Leuprolide; Transport; Trypsin inhibitor; Chitosan; Everted gut sac technique; Caco-2 cell

1. Introduction

For most polypeptides, oral administration is often limited by enzyme instability in the gastrointestinal tract. Furthermore, because peptides are highly ionized and thus are mostly hydrophilic, their potential for permeating gastrointestinal absorption barriers is poor. Attempts to understand the degradation and permeation behavior in the intestine are beneficial to improve oral efficacy.

Leuprolide acetate is a synthetic superpotent agonist of luteinizing hormone releasing hormone (LHRH) receptor. It is a decapeptide hormone effective in the treatment of hormone-dependent diseases such as prostate and mammary tumors and endometriosis (Adjei et al., 1993; Fu lu and Reiland, 1994). Intramuscular and intranasal routes are the common routes of administration. Undoubtedly, oral route is the most convenient. However, plasma levels of leuprolide following oral administration of an aqueous solution to rats and humans are mostly below the limit of

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doi:10.1016/j.ijpharm.2004.03.031

detection (0.2 ng/ml) (Zheng et al., 1999; Fu lu et al., 1992; Qiu et al., 1999).

In order to investigate the transport mechanisms of leuprolide, the everted gut sac technique and Caco-2 cell monolayer are used. The everted sac is a simple and useful in vitro model to study drug transport (Barthe et al., 1999). The system provides information on drug absorption mechanisms through testing the drug content in the intestinal sac. The everted sac has been used to study the uptake of lipid vesicles (Rowland and Woodley, 1981), proteins and macromolecules with oral drug delivery potential, bioadhesive lectins and synthetic nondegradable polymers. It provides quantitative information on the uptake and absorption of the tested drug.

Alternatively, the Caco-2 cell line, a well-differentiated human intestinal cell line derived from colorectal carcinoma, may serve as a model for predicting the intestinal absorption of drugs. Although Caco-2 cells are derived from the human colon carcinoma, they differentiate into the absorptive intestine-like cells during culture, such as microvillous structure and carrier-mediated transport systems for di/tri peptides (Brandsch et al., 1994), amino acids (Hidalgo and Borchardt, 1990) and glucose. Furthermore, Caco-2 cells form the tight-junction at the apical side of monolayer and also express several kinds of brush-border enzymes (Yamada et al., 1992). These features of Caco-2 cell line are similar to those of the small intestine rather than the colon.

Zheng et al. (1999) has studied the permeability and absorption of leuprolide from various intestinal regions in rabbits and rats and showed that passive diffusion is the transport mechanism by which leuprolide is absorbed. The permeability of leuprolide in the ileum and colon was about six times higher than that in the jejunum. However, this study neither compared the difference between animal models nor differentiated the concentration of enzyme degradation and permeability in the transport process.

In this study, we evaluated: (1) transport properties, enzyme degradation and apparent permeation coefficient; (2) the influence of trypsin inhibitor, EDTA, chitosan and alginate on the transport; and (3) the effect of animal species on the intestinal transport. The comparison of different intestinal models helps to understand the causes of the differences in the transport.

2. Materials and methods

2.1. Materials

Leuprolide acetate (lot number: 011015) was obtained from Lizhu Pharmaceutical Factory (China). Chitosan (1000 kDa, >90% deacetylation, 2.45 cps for a 0.1% solution at 25 °C) was supplied by Jiangshu Xinxing Pharmaceutical Factory (China). Sodium alginate (2.95 cps for a 0.1% solution at 25 °C) was presented by ISP Alginate Inc. Both trypsin inhibitor and EDTA were purchased from Sigma. Methanol, chloroform, sodium chloride and other reagents, all of analytical purity, were products of Nanjing Chemical Corporation (China).

2.2. Content determination

Concentration of leuprolide was determined by HPLC. The HPLC system consisted of a pump (Model LC-10A, Shimadzu, Japan), a shim-pack CLC-ODS column (150 mm × 6 mm i.d., Shimadzu) maintained at 25 °C, an UV detector (Model SPD-10A, Shimadzu, Japan) at 220 nm and a data station (Model SCL-10A, Shimadzu, Japan). The composition of the mobile phase was acetonitrile and 0.1% trifuroacetic acid (30:70). The mobile phase was delivered at a flow rate of 1 ml/min. The injection volume was 20 μl and the relative retention time was found to be 11.0 min.

2.3. Preparation of rat and rabbit intestinal homogenates

Male Sprague–Dawley rats (250–300 g) or rabbits (2–2.5 kg) were purchased from Central Animal Laboratory of China Pharmaceutical University. The animals were fasted overnight with free access to water and then sacrificed by CO_2 inhalation. The small intestinal segment (jejunum, 20 cm long) was removed, washed with cold normal saline, excised and rinsed with 30 mM phosphate buffer (pH 6.9). The mucosal tissue was scraped off gently with surgical blade. Tissue scrapings were homogenized in a glass homogenizer using 4–6 ml of phosphate buffer (pH 6.9) at 4 °C. The homogenate was centrifuged at 3000 × *g* for 10 min to remove cellular and nuclear debris. The resulting supernatant was used for the leuprolide degra-

dation study. Protein concentrations of the supernatant were determined with modified Lowry method, using bovine serum albumin (BSA) as the standard.

2.4. Degradation of leuprolide by intestinal homogenates

Three milliliters of supernatant was incubated with leuprolide (1 ml, 0.5 mg/ml) in 30 mM phosphate buffer (1.0 ml, pH 6.9) at 37 °C. Aliquots (0.3 ml) of the incubation mixture were removed at various times (0, 30, 60, 120, 180, 240, 300 min) and the reaction was quenched in 4.7 ml of 30% cold acetonitrile. The aliquots were centrifuged at 10000 × *g* for 10 min, and leuprolide in the supernatants was determined by HPLC.

2.5. Intestinal everted-sac experiments

Sprague–Dawley rats (weighed 200–250 g, provided by Central Animal Laboratory of China Pharmaceutical University) or rabbits (weighed 1.8–2 kg, provided by Central Animal Laboratory of China Pharmaceutical University) were anesthetized with 20% ethyl carbamate solution. A 20 cm segment of jejunum was quickly removed, rinsed with Tyrode buffer and everted (Barthe et al., 1999). This segment was tied at one end with a cotton thread, filled with oxygenated Tyrode buffer and then tied at the other end. The resultant large sac was divided into four 3–4 cm sacs by tying at intervals. Each sac contained about 0.5 ml oxygenated Tyrode buffer. The rat or rabbit were then sacrificed by injection of ethyl carbamate into the heart.

Each sac was individually placed in a 15 cm high glass tube containing 3 ml oxygenated Tyrode buffer kept in a water bath at 37 °C. After equilibration at this temperature for 5 min, 1 ml of drug was added to each tube to initiate the experiment. Different concentrations of leuprolide from 0.004 to 0.2 mg/ml were tested. The entire volume of inside medium was taken at predetermined time points of 15 min, 30 min, 1 h, 1.5 h and 2 h. Then, fresh oxygenated Tyrode buffer was added to the sacs and the incubation was continued. The remaining amount of drug in the serosal side at the end of the experiment was also determined. All the samples collected were extracted by mixing with methanol. The mixtures were centrifuged at 5000 × *g* for 10 min and the supernatants were analyzed by HPLC.

2.6. Caco-2 cell culture model

The Caco-2 cell line was obtained from the American Type Culture Collection. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/l glucose and 10% fetal bovine serum in an atmosphere of 5% CO_2 and 90% relative humidity. Cells were passaged every 3–4 days at a split ratio of 1–5 (1.0 ml of 0.05% trypsin and 0.02% EDTA in HBSS per flask was used for trypsinization) and confluency was reached within 4–5 days. For transport uptake studies, cells were seeded at a density of 10^4 cells/ml onto in 12 wells/plate transwells (3.0 μm pore size: Costar). Cells of 21 day-old were used in the transport study. Caco-2 cells between passage numbers (40–45) were used in this study.

2.7. Measurement of transepithelial electrical resistance

Transepithelial electrical resistance (TEER) was measured using an EVOM^{TM} epithelial volt ohmmeter (World Precision Instrument, Sarasota, FL). Measurements started 1 h prior to incubation on the apical side of the cells with leuprolide solution in serum-free DMEM. After the completion of transport, leuprolide solution was removed and cells were washed three times with and placed in fresh DMEM. Following this, the TEER was again measured. Average TEER values for untreated cells were in the range of 350–400 Ω/cm^2.

2.8. Cellular transport studies

Leuprolide solutions were prepared in serum-free DMEM. The pH of all of the solutions was adjusted to pH 6.0 with 0.1 M HCl. The drug concentration was ranged from 0.065 to 1 mg/ml. The medium on the basolateral side was DMEM buffered at pH 7.40 with HEPES. Samples taken from the basolateral side were replaced with an equal volume of DMEM with HEPES. All experiments were done in triplicate in an atmosphere of 95% air and 5% CO_2 at 37 °C. Drug transported to the basolateral

side was measured by HPLC. Results were corrected for dilution and expressed as cumulative transport at time *t*.

2.9. Influence of trypsin inhibitor, EDTA, chitosan and alginate on the transport

Leuprolide (0.0125, 0.05 and 0.08 mg/ml) with trypsin inhibitor (0.5 mg/ml) were co-incubated in the rabbit sac to investigate the effect of enzyme degradation of leuprolide.

Leuprolide (0.5 mg/ml) with EDTA (1 mM) in serum-free DMEM (pH 6.0)was incubated apically to investigate the function of calcium in Caco-2 monolayer. Leuprolide (0.5 mg/ml) with alginate (0.1%) in serum-free DMEM (pH 6.0) was incubated apically to investigate the possible enhancement effect of alginate. Chitosan was added to 1% acetic acid to get 1.0% (w/v) stock solution. Chitosan (0.1%) was prepared by dilution of stock solution in serum-free DMEM, then the enhancement effect of chitosan on the transport of leuprolide in Caco-2 cell was studied.

2.10. Viability of Caco-2 cell monolayer

Both the apical and basolateral sides of the cell monolayer were rinsed twice with 0.01 M phosphate-buffered saline (PBS, pH 7.4) after completion of the transport experiments. The cell monolayer was incubated apically with a solution of 0.1% trypan blue (Sigma) in PBS. The basolateral medium was PBS. After 30 min, the medium was removed from both sides of the cell monolayer and examined by light microscopy for exclusion of the marker. Cells excluding trypan blue were considered to be viable.

2.11. Data analysis

The apparent permeability coefficients (P_{app}) are calculated from the following equation: $P_{app} = (dQ/dt)/C_0/A$ (cm/s), where dQ/dt is the flux across the monolayer or intestinal sac (μg/s), C_0 the initial concentration in the mucosal side of sac or on the apical side of cell monolayer (μg/ml), and A the surface area of the sac or transwell.

2.12. Determination of the activity of α-chymotrypsin in the presence of chitosan and alginate

It has been reported leuprolide is highly susceptible to degradation by the intestinal protease α-chymotrypsin (Haviv et al., 1992). EDTA has long been known to inhibit this enzyme's activity, however, little is known about the effect of chitosan and alginate on the activity of α-chymotrypsin. Therefore, the activity of α-chymotrypsin in the absence and presence of chitosan (0.1%) and alginate (0.1%) was determined as the amount of tyrosine produced from the hydrolysis of casein by α-chymotrypsin per minute. The formation of tyrosine was measured by the modified Lowry method.

3. Results and discussion

3.1. Determination of leuprolide

The regression equation for leuprolide content (μg/ml) in the intestinal sac ranging from 0.5 to 5 μg ml^{-1} was $A = 33992C - 1399$ ($r = 0.997$). The absolute recovery was 91.0 ± 7.4%. Precision assay showed the average of the relative standard deviations within 1 day was 3.51% and among every other day was 4.64%.

The regression equation for leuprolide content (μg/ml) in the serum-free DMEM (pH 7.4) ranging from 0.1 to 5 μg/ml was $A = 38569C - 2834$ ($r = 0.9999$). The absolute recovery was 98.32 ± 1.62%. Precision assay showed the average of the relative standard deviations within 1 day was 1.35% and among every other day was 4.02%.

The analytic method fitted the requirement of content determination.

3.2. Comparison of degradation in intestinal homogenate

The protein concentrations of the supernatant in rat and rabbit were 0.17 ± 0.03 and 0.71 ± 0.13 mg/ml, respectively. The protein content in rabbit intestinal homogenate was four times larger than that in rat intestinal homogenate. Fig. 1 showed the degradation of leuprolide in rat intestinal homogenate was relatively slow. The remaining amount was 24.78 ± 3.10% at

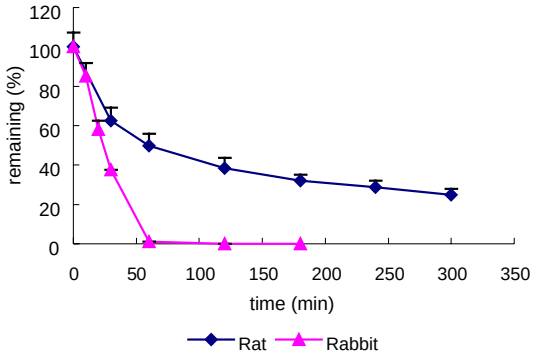

Fig. 1. Degradation of leuprolide in rat and rabbit intestinal homogenates ($n = 3$; mean ± S.D.).

5 h. While in rabbit intestinal homogenate, the degradation of leuprolide was faster, leaving only 1.1% left at 1 h.

3.3. The flux versus concentration

Figs. 2–4 show the cumulative permeation of leuprolide across everted gut of rat, rabbit and Caco-2 cell. It can be seen that the cumulative permeation amount of drug increased proportionally with time. The lag time in Caco-2 cell monolayer was 20 min, much longer than that of rat and rabbit gut. Furthermore, the cumulative permeated amount of drug across Caco-2 cell monolayer was only 1/10 of that across rabbit intestine.

It can be seen from Fig. 5 that the flux in rat and rabbit intestine increased with the increasing drug con-

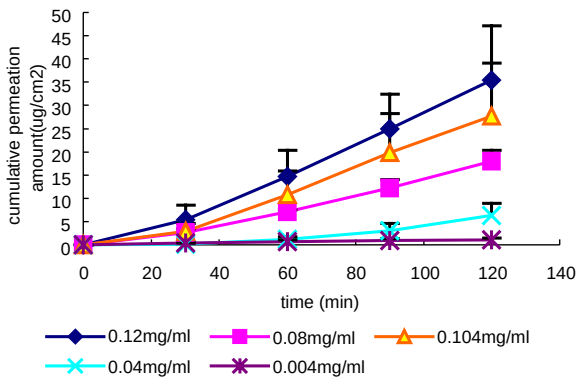

Fig. 2. Cumulative permeation of leuprolide across everted gut of rat. ($n = 4$; mean ± S.D.).

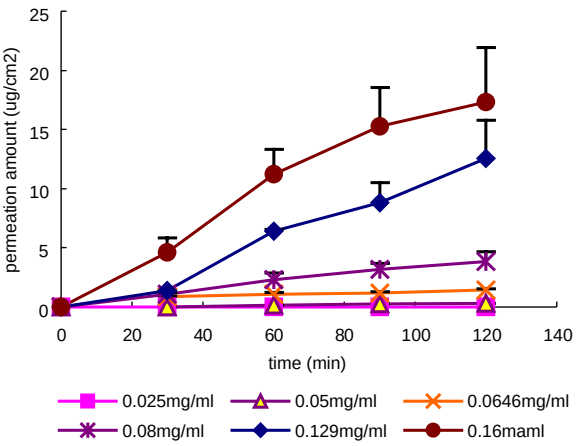

Fig. 3. Cumulative permeation of leuprolide across everted gut of rabbit. ($n = 4$; mean ± S.D.).

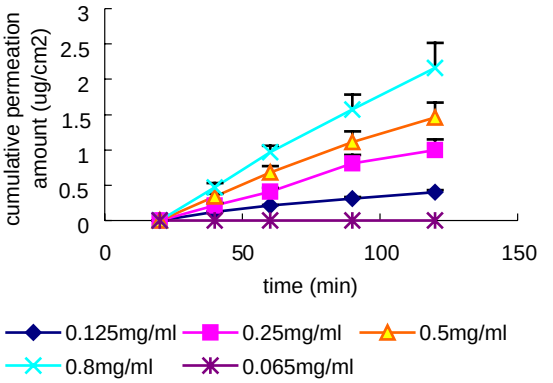

Fig. 4. Cumulative permeation of leuprolide across Caco-2 cell monolayers ($n = 3$; mean ± S.D.).

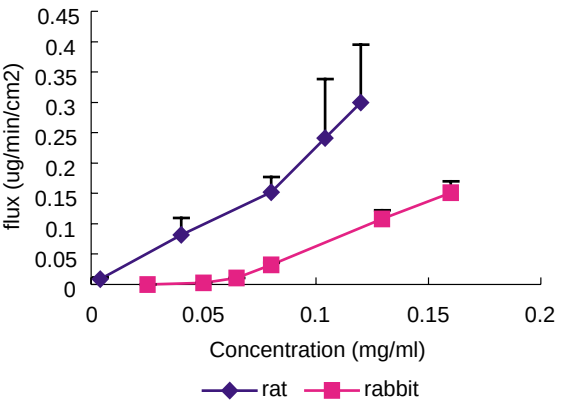

Fig. 5. Transepithelial fluxes of leuprolide across everted gut of rat and rabbit ($n = 4$; mean ± S.D.).

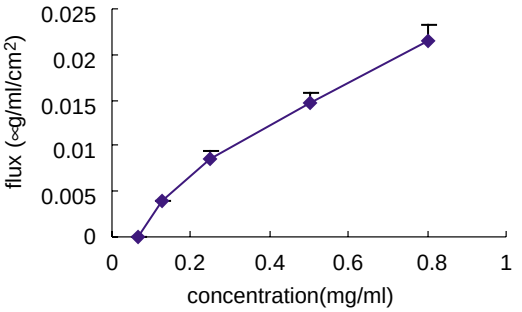

Fig. 6. Transepithelial fluxes of leuprolide across Caco-2 monolayer ($n = 3$; mean ± S.D.).

centration from 0.004 to 0.16 mg/ml, but not proportionally. The flux was larger in rat intestine and the recovery of drug was 78.18 ± 4.99%. While in rabbit intestine, the flux was nearly 0 when the concentration increased from 0 to 0.05 mg/ml. This is probably because at low drug concentration, the leuprolide was totally degraded by enzyme and resulted in no permeation. As the drug concentration increased from 0.005 to 0.16 mg/ml, proteolysis was saturated and led to an increase in flux. The recovery of drug at the end of the experiment was only 20 ± 6.6%. The proteolysis in rabbit intestine was more prominent than that in rat intestine, which affects the permeation of leuprolide.

It can be seen from Fig. 6 that in Caco-2 monolayer, the flux increased with the increasing concentration of drug from 0.065 to 0.80 mg/ml, showing the character of passive diffusion. However, at the concentration of 0.065 mg/ml, the flux was too small to be detected by HPLC.

3.4. Permeation coefficient

Table 1 displays the permeation coefficients of leuprolide in rat gut, rabbit gut and Caco-2 monolayer. At a concentration of 0.12 mg/ml, permeation coefficients in rat gut, rabbit gut and Caco-2 monolayer were $(4.19 \pm 1.33) \times 10^{-5}$ cm/s, $(1.4 \pm 0.18) \times 10^{-5}$ cm/s and $(5.20 \pm 0.20) \times 10^{-7}$ cm/s, respectively. In rat intestine, the permeation coefficients were always the largest, indicating the relatively large permeability of the rat intestine. Probably due to enzyme degradation, the permeation coefficients were rather small in rabbit gut at low drug concentration (<0.1 mg/ml). As the concentration increased, due to the saturation of enzyme degradation, the permeation coefficients increased to a relatively steady value. In Caco-2 monolayer, the permeation coefficients were the smallest, always at the level of 10^{-7}. There is no chymotrypsin present in Caco-2 cells, so the influence of enzyme degradation was relatively small. The poor permeability explains the low permeation coefficient.

3.5. Effect of trypsin inhibitor

In rabbit intestine, after addition of trypsin inhibitor, the permeation coefficients increased from 0, $(9.33 \pm 0.33) \times 10^{-7}$ cm/s and $(6.73 \pm 0.75) \times 10^{-6}$ cm/s to $(1.14 \pm 0.11) \times 10^{-5}$ cm/s, $(1.27 \pm 0.21) \times 10^{-5}$ cm/s and $(1.09 \pm 0.16) \times 10^{-5}$ cm/s, respectively. Then, there is no significant difference among the permeation coefficients ($P > 0.05$) for the experiments when a trypsin inhibitor was included. Therefore, trypsin

Table 1
The permeation coefficients (cm/s) of leuprolide in rat, rabbit intestine and Caco-2 monolayer ($n = 4$; mean ± S.D.)

Concentration (mg/ml)	Rat intestine	Rabbit intestine	Caco-2 monolayer
0.004	$(3.75 \pm 1.21) \times 10^{-5}$	–	–
0.04	$(3.40 \pm 1.19) \times 10^{-5}$	–	–
0.05	–	$(9.33 \pm 0.33) \times 10^{-7}$	–
0.065	–	$(2.71 \pm 0.09) \times 10^{-6}$	0
0.08	$(3.17 \pm 0.51) \times 10^{-5}$	$(6.73 \pm 0.75) \times 10^{-6}$	–
0.104	$(3.86 \pm 156) \times 10^{-5}$	–	–
0.12	$(4.19 \pm 1.33) \times 10^{-5}$	$(1.4 \pm 0.18) \times 10^{-5}$	$(5.20 \pm 0.20) \times 10^{-7}$
0.2	–	$(1.57 \pm 0.20) \times 10^{-5}$	–
0.25	–	–	$(5.62 \pm 0.56) \times 10^{-7}$
0.5	–	–	$(4.87 \pm 0.36) \times 10^{-7}$
0.8	–	–	$(4.50 \pm 0.16) \times 10^{-7}$

–: not determined.

inhibitor increased the permeation coefficients by protecting against drug degradation allowing more drug to permeate.

3.6. Effect of EDTA, chitosan and alginate

In order to determine the relative contribution of transcellular and paracellular transport for leuprolide across Caco-2 cell monolayer, drug transport was studied in the serum-free DMEM (pH 6.0) containing EDTA (Gan et al., 1993). Results indicated that a more than 15-fold increase in flux (from 0.015 ± 0.002 to $0.23 \pm 0.022\,\mu g\,min^{-1}\,cm^{-2}$) was observed with inclusion of EDTA in the medium. It has been reported that the integrity of the tight junctions can be modulated by varying the calcium concentrations in the media (De Boer et al., 1991). Depletion of Ca^{2+} ions from the extracellular cell medium by EDTA has been shown to reduce the TEER and consequently to increase the paracellular permeability of epithelial cell monolayers (Noach et al., 1993). The increase in flux was significant ($P < 0.05$), which suggested that paracellular passive diffusion was the major pathway for the transport of leuprolide.

Chitosan has long been recognised as a safe and effective enhancing agent to open tight junctions between cells. The mechanism has been attributed to the interaction of the positively charged amino groups with the negatively charged sialic groups of the membrane-bound glycoproteins. Several studies have highlighted the potential use of chitosan as an absorption enhancing agents for the administration of hydrophilic drugs (Kotze et al., 1998; Kas, 1997). Chitosan was investigated in this study to explore the possibility of enhancement of absorptoin of leuprolide. Results showed chitosan increased the permeation coefficients by a factor of 5.2 (from $(4.87 \pm 0.36) \times 10^{-7}$ to $(2.53 \pm 0.17) \times 10^{-6}$ cm/s). These results are significantly different ($P < 0.05$).

Like chitosan, alginate is also a mucoadhesive polysaccharide. However, it has little effect on the permeability of leuprolide. There was no significant difference ($P > 0.05$) between permation coefficients before and after the use of alginate (from $(4.87 \pm 0.36) \times 10^{-7}$ to $(4.95 \pm 0.42) \times 10^{-7}$ cm/s). Alginate is negatively-charged and therefore, would be expected to have little effect on the negatively-charged glycoproteins.

The activity of α-chymotrypsin in the absence and presence of alginate (0.1%) and chitosan (0.1%) were determined to be 0.2058, 0.2035 and 0.1975 μmol/(min ml) respectively. Neither polysaccharides provided protection against proteolysis.

3.7. TEER value and viability of cell

After completion of transport, the TEER value was approximately 90% of the original value. The TEER value was reduced to 50% by inclusion of EDTA or chitosan in the medium., indicating the opening of tight junctions. Reversibility of the TEER value could be demonstrated after removal of the chitosan solution. Ninety-five percent of cells were free of trypan blue dying after completion of the transport studies, which is an indication that the cells remained undamaged and functionally intact. However, when EDTA was added, there was only 80% of cells were viable, approximately 20% of cells being stained by the trypan blue dye were stained. EDTA may have a harmful effect on the cells.

4. Conclusion

In conclusion, we quantitatively evaluated the transport mechanisms of leuprolide across rat intestine, rabbit intestine and Caco-2 monolayer. Passive diffusion via the paracellular route was the rate determing factor for leuprolide. Compared with other charged hydrophilic molecules, like polyamines: putrescine (MW 161.1), spermidine (MW 254.6), and spermine (MW 348.2), their transepithelial transports occur similarly by passive diffusion through the paracellular pathway (Milovic et al., 2001).

We clarified the contribution of the enzymatic and permeation barriers to the transport of leuprolide. At low drug concentration, proteolysis determined the extent of drug transport. Leuprolide acetate is very potent and is available for daily s.c. administration (1 mg) in the palliative treatment for advanced cancer of the prostate (Adjei et al., 1992). Suppression of enzyme degradation is a key factor in substantially enhancing the intestinal permeation of these relatively low-molecular weight peptides that are susceptible to hydrolysis by enzymes.

Furthermore, we demonstrated the transport of leuprolide can be improved by the addition of trypsin inhibitor, EDTA and chitosan. Trypsin inhibitor can protect the drug from enzyme degradation, thus increasing the amount of drug available for permeation. EDTA and chitosan increase permeability by widening the tight junctions. Since this study is just a prelimilary one, a complete explanation for the experimental results may be found after more extensive investigation of the intracellular responses to the enzyme inhibitor and permeation enhancer.

There are often differences among species in characteristics such as biochemical properties, enzymatic activities and intestinal membrane structure. The impact of these factors on transport can often be drug dependent. In the present study, the higher extent and rate of transport from rat intestine may be attributed to the low enzymatic activity and high apparent permeability. Thus, appropriate selection of animal models to simulate humans needs careful consideration and further investigation.

Acknowledgements

This research work is granted by the key project of National Natural Science Foundation of China, with project number of 39930200.

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