Gastrointestinal Absorption of Recombinant Hirudin-2 in Rats

Xueying Yan, Xiangtao Wang, Xuenong Zhang, and Qiang Zhang

Department of Pharmaceutics, School of Pharmaceutical Science, Peking University Health Science Center, Beijing, People’s Republic of China

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ABSTRACT

To investigate the absorption of recombinant hirudin-2 (rHV2) after oral administration to rats and its possible absorption mechanism, a series of experiments were carried out. The degradation of $^{125}$I-rHV2 in the luminal contents and variant mucosal subcellular fractions, as well as the effect of degradation inhibition of some adjuvant was investigated. The bioavailability of rHV2, with or without degradation inhibitor after oral administration to rats was estimated, whereas the in situ loop test and everted sac experiment were also conducted to understand more about the gastrointestinal absorption of rHV2 in rats. It was demonstrated that the rHV2 was not stable in the luminal contents and subfraction of the intestinal mucosa. Some enzyme inhibitor, such as bacitracin or casein, could inhibit the degradation to certain degrees. The intact rHV2 molecules were found in the rat plasma after oral administration, and the bioavailability varies obviously, dependent on the analytical method. Some of the enzyme inhibitor could enhance the rHV2 oral absorption. There is no site difference on rHV2 absorption in different segments of small intestine. The possible transport mechanism of rHV2 across the gastrointestinal tract is concerned with the endocytosis process.

Hirudin is a peptide composed of 65-amino acid residues with a molecular mass of 7000 Da. Hirudin reacts with thrombin in a 1:1 M ratio to form an enzyme-inhibitor complex in which the proteolytic activity of the enzyme is totally blocked (Markwardt, 1970). The dissociation constant of the hirudin-thrombin complex was determined to be as $6.3 \times 10^{-11}$mol/l (Chang, 1983). Hirudin holds many advantages over the commonly used anticoagulants such as heparin. It does not need a cofactor for the interaction with the enzyme; neither is it inactivated by platelet factors. Also, it has no toxicity or antigenicity. These characteristics make hirudin a promising candidate for proteinase inhibitor therapy in clinical states associated with thrombosis and disseminated intravascular coagulation (Nowak and Markwardt, 1991). It has become possible to produce biologically active peptide and proteins that are therapeutically applicable such as hirudin by means of recombinant DNA technique. Generally, recombinant hirudin has three variants: rHV1, rHV2, and rHV3. All of them have a high degree of homology (13 variable positions only), but in contrast to the natural molecule they lack a sulfate group at Tyr$^{63}$. They all have a similar effect of anticoagulation as natural hirudin (Dolt et al., 1985; Scharf et al., 1989; Vindigni et al., 1994). Most studies on hirudin are concerned with the pharmacological evaluations and chose the intravenous injection as the administration route. In clinic, frequent injections of hirudin are needed for the purpose of therapy; therefore, patients experience great discomfort. To improve the quality of life of such patients, an alternative method of administration is desired. Oral administration is the most convenient route, and development of an oral formulation for hirudin would be of great value. rHV2 has been proved has the effect of anticoagulation when administered to the duodenal of rats (Wang et al., 1999). But there is no information on the GI absorption of rHV2 administered by direct oral administration. The oral delivery of biologically active peptides and proteins is a very attractive but tough job. There are two main barriers to the GI absorption of peptide drugs: one is the poor membrane permeability that results from their macromolecular weight and hydrophilic characteristics, and another is the degradation of peptides by proteases in the GI lumen and mucosa (Lee, 1990). Scientists have made numerous attempts in this field for decades and investigated methods to enhance the absorption of peptides and proteins, as well as the mechanisms of their absorption. In the studies reported, most of the model drugs are small peptides containing fewer than five amino acids (Berteloot et al., 1981; Shen and Xu, 2000), or endogenous peptides and proteins, such as insulin (Cheeseman and Johnston, 1982; Stoll et al., 2000), insulin like growth factor I (Due et al., 1985; Toshikiro et al., 1996; Xu and Wang, 1996; Donovan et al., 1997; Hosny et al., 1997; Nakagawa et al., 1997; Scharf et al., 1989; Vindigni et al., 1994).
fraction, whereas the other was resuspended with 15 ml of acetate/buffer (pH 7.0), and then homogenized. The homogenate was centrifuged at 100,000 × g for 20 min to separate each fraction. The experiment of 125I-rHV2 degradation was performed at 37°C. Samples were taken at 0, 1, 5, 10, 15, and 30 min and determined by trichloroacetic acid (TCA)-precipitable method.

Materials and Methods

Materials. Recombinant hirudin (rHV2, RVH-Lys47; College of Life Science, Peking University, Beijing, China) was obtained by polymerase chain reaction-directed mutagenesis and expressed in Escherichia coli. The specific activity is 10,347 ATU/mg determined by chromogenic assay. Bacitracin, casein, bovine serum albumin (BSA), sodium deoxycholate (SDCh), poly-L-lysine hydrobromide (molecular weight about 1000), Tris, and colchicine were purchased from Sigma-Aldrich (Shanghai, China). Chromoyzm TH was from Roche Diagnostics (Mannheim, Germany), and Carbopol 941 was a product of Noveon Chemical Company (Cleveland, OH). Hydroxypropyl-β-cyclodextrin (HP-β-CD) was supplied by Xi’an Deli Biological and Chemical Engineering Co., Ltd. (Xi’an, China), 2,4-Dinitrophenol (DNP) was purchased from Beijing Chemical reagent factory (Beijing, China). All other chemicals were analytical grade and used as received.

Animals. Male Sprague-Dawley rats weighing 270 to 300 g were provided by Vital Laboratory Animal Center (Beijing, China). All care and handling of animals were performed with the approval of Institutional Authority for Laboratory Animal Care.

Preparation of Luminal Contents and Mucosal Subcellular Fractions. The luminal contents of the GI tract were prepared according to the method of Asada et al. (1994). Rats were euthanized with an overdose intraperitoneal injection of urethane, and the GI tract was excised. The intestinal tissues of the small intestine were prepared according to the method of Bai and Chang (1995). The isolated jejunum was 20 cm long), were collected by flushing with 15 ml of the fresh PBS (pH 7.4) incubation media, which contained 100 mM NaCl, 10 ml) for 10 min. After recovery of the serosal solution, the everted sac was twice immersed in 20 ml of the fresh PBS (pH 7.4, 4°C) for 5 min to remove the nonspecifically adsorbed rHV2 on the mucosal surface. To remove the rHV2 bound on the membrane, the everted sac was immersed in the ice-cold basic washing solution (0.05 M NaOH/0.5 M NaCl, 10 ml) for 10 min. After recovery of the serosal solution, the tissue was homogenized and mixed with 4% acetic acid. The mixture was centrifuged at 2000g for 10 min. The rHV2 in the resulting supernatant and in the serosal solution were determined by chromogenic assay.

Assay Procedures. The 125I-rHV2 in the blood or GI luminal contents was determined by TCA precipitation method (Toshikiro et al., 1996). For GI luminal contents, 200 μl of 15% TCA was added to 200 μl of the incubation mixture, as for plasma, 1 ml of 15% TCA was added to the mixed solution after 1 ml of Krebs-Henseleit bicarbonate buffer (119 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl2, 1.2 mM MgSO4, 1.2 mM KH2PO4, 25 mM NaHCO3) containing 5% BSA was added to 50 μl of each plasma sample. Afterward, the mixtures were centrifuged at 2000g for 15 min and then the radioactivity in the precipitate was counted by a gamma-counter (SNB-69513; Rihuan Instrument Factory, Shanghai, China).

Chromogenic assay was performed according to Grotzsch et al. (1991) with a little modification. The blood samples were denatured by heating for 15 min at 65°C and then 0.05 ml of HCl (1 M) was put
into 0.5-ml blood sample. The samples were neutralized after cooling by addition of 0.05 ml NaOH (1 M) and then centrifuged at 3000g for 10 min. Supernatant (100 μl) was added to 200 μl of Tris buffer (50 mM, containing 154 mM NaCl and 0.12 U of thrombin), and incubated at 37°C for 5 min. Afterward, 16 μl of chromozym TH (1.9 mM) was added into the incubation mixture and kept on incubating for another 10 min at 37°C. The reaction is terminated by addition of 200 μl of acetic acid (33%) and then the absorbance of the sample was read at 405 nm with a spectrophotometer (TU-1901; Puxi Versatile Instrument Co., Beijing, China).

Data Analysis. The plasma concentration of rHV2 was calculated by dividing the total volume of plasma isolated. The plasma concentration-time data were analyzed noncompartmentally on the basis of the statistical moment theory. The area under the curve0-360 was estimated by the trapezoidal method and the bioavailability was calculated by comparing the area under the curve0-360 obtained after intraintestinal or oral administration to that obtained after intravenous injection. Statistical evaluations were performed using the Student’s t test. Differences with a p value less than 0.05 were considered significant.

Results

Degradation of rHV2 in the GI Contents and Mucosa. Degradation of rHV2 in the GI contents is given in Fig. 1. 125I-rHV2 rapidly degraded into the TCA-soluble form in the gastric contents and small intestinal contents. The residual percentages of 125I-rHV2 in the GI contents are summarized in Table 1. The maximal degrading activity is in the stomach; only 27.04% of 125I-rHV2 remained after 30 min of degradation. The degrading activity in distal ileum is almost the same as in stomach, whereas those in jejunum and proximal ileum are much lower. Some adjuvant could inhibit the degradation, especially bacitracin and casein, with the residual percentage of hirudin of 62 and 58%, respectively.

Table 1 shows the degrading activity in the intestinal mucosa. Degrading activity in a subfraction of the intestinal mucosa is nearly the same as in GI contents, except that the activity in the cytosol fraction is a little lower than in other parts of the subfraction. There was no site difference through the entire small intestine.

Oral Administration Experiments. The plasma concentration-time curves of TCA-perceptible radioactivity after oral administration of 125I-rHV2 (1.0 mg/kg) with or without bacitracin (4.0 mg/kg) and casein (10.0 mg/kg) are shown in Fig. 2. The bioavailability of rHV2 without adjuvant was 21.23 ± 3.73%. When coadministered with bacitracin or casein, the bioavailability was increased to 37.41 ± 3.55% (p < 0.05) and 29.23 ± 3.08% (p > 0.05), respectively.

To further confirm the above-mentioned absorption results obtained from the 125I-rHV2 oral administrations, chromogenic assay of rHV2 in the plasma was also performed after oral administration of unlabeled rHV2. The plasma concentration-time curves obtained are shown in Fig. 3. The bioavailability values of rHV2 administered alone, with bacitracin or casein were 6.99 ± 0.32%, 12.70 ± 1.00% (p < 0.01), and 8.36 ± 1.02% (p > 0.05), respectively.

In Situ Loop Experiments. The site difference of rHV2 absorption in the intestine was also examined by an in situ loop method. There was no significant variance of absorption in different segments of small intestine. The bioavailabilities of rHV2 in various parts of the intestine are expressed in Fig. 4, and the values range from 6.33 to 8.98%. The rank order of the absorption was proximal ileum > distal ileum > jejunum with no statistical differences, whether with or without adjuvant (p > 0.05).

In Vitro Everted Sac Experiments. To clarify the transport mechanism of rHV2 across the GI tract, we examined the effect of variant inhibitors on the uptake and transport of rHV2 across the jejunal mucosa. The results are summarized in Table 2. The transport of rHV2 to the serosal side was significantly inhibited by low temperature, DNP (an uncoupler of oxidative phosphorylation), carbopol 941 (a polyanion), and colchicines (an inhibitor of microtubular assembly), but not by poly-L-lysine (a polycation). The amount of rHV2 found in the intestinal tissue was also decreased, and the total amount of rHV2 taken in and transported across the GI tract tended to be inhibited by these treatments, except for the addition of poly-L-lysine.

Discussion

Chang demonstrated that hirudin is a very stable compound; it may be irreversibly denatured unless under ele-
Degradation of $^{125}$I-rHV2 in the contents and mucosal subfractions of rat GI tract and its inhibition by some adjuvant

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Adjuvant</th>
<th>Stomach</th>
<th>Jejunum</th>
<th>Proximal ileum</th>
<th>Distal ileum</th>
</tr>
</thead>
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<tr>
<td>Luminal contents</td>
<td>None</td>
<td>27.04 ± 1.28</td>
<td>36.94 ± 5.56</td>
<td>41.80 ± 2.21</td>
<td>30.29 ± 2.80</td>
</tr>
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<td></td>
<td>Bacitracin</td>
<td>47.17 ± 0.59**</td>
<td>55.59 ± 3.73*</td>
<td>62.54 ± 5.10**</td>
<td>52.16 ± 8.22*</td>
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<td></td>
<td>Casein</td>
<td>39.29 ± 1.89*</td>
<td>50.75 ± 4.91</td>
<td>58.54 ± 9.92</td>
<td>42.01 ± 2.35*</td>
</tr>
<tr>
<td></td>
<td>HP-β-CD</td>
<td>63.62 ± 4.49*</td>
<td>50.67 ± 3.10*</td>
<td>52.10 ± 0.59*</td>
<td>39.28 ± 5.81</td>
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<tr>
<td></td>
<td>SDCCh</td>
<td>38.95 ± 2.24*</td>
<td>47.75 ± 4.87**</td>
<td>41.84 ± 8.44</td>
<td>40.50 ± 4.57*</td>
</tr>
<tr>
<td>BBM fraction of mucosal cells</td>
<td>None</td>
<td>40.45 ± 13.28</td>
<td>35.48 ± 5.92</td>
<td>31.54 ± 0.62</td>
<td>32.17 ± 0.53</td>
</tr>
<tr>
<td></td>
<td>Bacitracin</td>
<td>65.58 ± 3.96**</td>
<td>59.34 ± 2.11*</td>
<td>46.82 ± 3.20**</td>
<td>44.32 ± 4.07**</td>
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<tr>
<td></td>
<td>Casein</td>
<td>48.07 ± 7.00*</td>
<td>60.05 ± 4.17**</td>
<td>46.41 ± 9.04</td>
<td>41.70 ± 4.08**</td>
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<tr>
<td></td>
<td>HP-β-CD</td>
<td>55.56 ± 14.70*</td>
<td>50.41 ± 2.82*</td>
<td>37.35 ± 0.34</td>
<td>37.65 ± 3.57</td>
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<tr>
<td></td>
<td>SDCCh</td>
<td>45.76 ± 2.91*</td>
<td>35.48 ± 2.99</td>
<td>31.70 ± 1.50</td>
<td>32.17 ± 1.53</td>
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<td>Lysosome fraction of mucosal cells</td>
<td>None</td>
<td>38.45 ± 1.71</td>
<td>42.86 ± 2.99</td>
<td>31.70 ± 1.50</td>
<td>32.17 ± 1.53</td>
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<tr>
<td></td>
<td>Bacitracin</td>
<td>61.39 ± 4.75</td>
<td>51.74 ± 4.24*</td>
<td>44.50 ± 2.08*</td>
<td>44.50 ± 2.08*</td>
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<td></td>
<td>Casein</td>
<td>56.59 ± 1.80**</td>
<td>51.29 ± 0.02*</td>
<td>53.88 ± 4.38*</td>
<td>54.34 ± 4.78*</td>
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<td></td>
<td>HP-β-CD</td>
<td>50.85 ± 4.01</td>
<td>57.09 ± 3.22**</td>
<td>49.34 ± 0.77**</td>
<td>49.34 ± 0.77**</td>
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<td></td>
<td>SDCCh</td>
<td>52.17 ± 2.46**</td>
<td>44.61 ± 4.53</td>
<td>41.15 ± 1.53*</td>
<td>41.15 ± 1.53*</td>
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<tr>
<td>Cytosol fraction of mucosal cells</td>
<td>None</td>
<td>39.61 ± 6.33</td>
<td>46.28 ± 2.84</td>
<td>59.68 ± 3.26</td>
<td>59.68 ± 3.26</td>
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<tr>
<td></td>
<td>Bacitracin</td>
<td>49.39 ± 1.44</td>
<td>74.41 ± 1.80*</td>
<td>59.74 ± 5.95</td>
<td>59.74 ± 5.95</td>
</tr>
<tr>
<td></td>
<td>Casein</td>
<td>47.29 ± 7.67</td>
<td>62.62 ± 2.83*</td>
<td>60.42 ± 1.14</td>
<td>60.42 ± 1.14</td>
</tr>
<tr>
<td></td>
<td>HP-β-CD</td>
<td>48.19 ± 3.71</td>
<td>53.45 ± 8.73</td>
<td>56.87 ± 3.32</td>
<td>56.87 ± 3.32</td>
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<tr>
<td></td>
<td>SDCCh</td>
<td>45.48 ± 1.88</td>
<td>53.72 ± 1.81</td>
<td>64.04 ± 13.55</td>
<td>64.04 ± 13.55</td>
</tr>
</tbody>
</table>

*p < 0.05, **p < 0.01 versus none. Data represent mean ± S.D.; n = 3.

**Fig. 2.** Plasma concentration profiles after oral administration of $^{125}$I-rHV2 alone or with adjuvant in rats, determined by TCA-precipitable method. $^{125}$I-rHV2 (●), +bacitracin (■), +casein (▲). Data represent mean ± S.E.; n = 5.

**Fig. 3.** Plasma concentration profiles after oral administration of rHV2 alone or with adjuvant in rats, determined by chromogenic assay. $^{125}$I-rHV2 (●), +bacitracin (■), +casein (▲). Data represent mean ± S.E.; n = 5.

above-mentioned studies only one kind of protease acts with hirudin in each experiment; however, the effects of various digestive enzymes in the GI tract should be more complex, especially in small intestine. Therefore, the luminal contents and mucosal subcellular fractions were used in our investigation to simulate the complicated surroundings in GI tract.

It was indicated from the degradation experiments that rHV2 was degraded rapidly by the GI tract luminal contents and mucosal subcellular fraction. The rHV2 concentration decreased below 50% of the initial within 30 min. The significant inhibitory effects of bacitracin and casein on rHV2 degradation have also been noticed. Xian et al. (1995) reported that casein can protect IGF-I from degradation in the stomach or duodenal flushing, but the mechanisms of this protection are not clear. The inhibitory effect of HP-β-CD and SDCCh are relatively weak compared with bacitracin and casein, although some studies suggested that DM-β-CD and SDCCh have the ability of inhibiting the enzyme activities (Kakemi et al., 1970; Hovgaard and Brondsted, 1995). Regardless, it seems possible to use some protease inhibitors to...
protect rHV2 from the degradation by digestive enzymes in GI tract.

The bioavailability of oral administration of $^{125}$I-rHV2 in rats is $21.23 \pm 3.73\%$, estimated by TCA-precipitable method, which is not consistent with the value of $6.99 \pm 0.32\%$ determined by chromogenic assay. The variance was due to the different methods used and to the two methods’ advantages and drawbacks. TCA-precipitable method is more sensitive and could prove the transport of the intact rHV2 molecules into the body circulation, whereas the chromogenic assay could demonstrate at least the absorption of biologically active segments of rHV2 molecules into the blood of the animals. With coadministration of rHV2 with bacitracin or casein, the bioavailability was increased markedly, no matter which of the two analytical methods was applied.

The results of in situ loop indicate that the absorption of rHV2 is more slow than in that of the oral administration. This finding may be related to the route of administration and the state of the animals. In the perfusion process, the drug solution was pumped into intestinal tract slowly, whereas in oral administration the drug solution was administered very rapidly. Another reason might be that the animals used in the in situ loop experiment were under anesthesia, and their systemic circulation was much slower than that of the normal states (Holzer et al., 2003). Therefore, the absorption of drug in the in situ loop experiment was also slow. On the other hand, no significant differences in the rHV2 absorption among different parts of small intestine were found, suggesting that rHV2 has no preference on the absorption sites. Coadministration with bacitracin or casein could attain a small increment in bioavailability of rHV2, possibly due to the fact that intestinal tract was washed well and cleared of most digestive enzymes before perfusion studies.

Furthermore, as demonstrated in the experiments using the everted sac of jejunum, the uptake of rHV2 was inhibited by polyanion (carbopol 941) and low temperature but not by polycation (poly-L-lysine). The significant decrease of rHV2 transport under 4°C suggested that the transport was energy-dependent, so the transport routine may be related to transcellular way. The pollysine was reported to bind anionic sites of gall bladder epithelial cell membranes, thereby producing morphological changes such as collapse of the microvillar structure, membrane folding from the apical border into the terminal web, or "fused" membranes with pentilaminar substructure (Quinton and Phillpot, 1973). These changes limited the absorption by transcellular way. Madara (1989) reported that pollysine could interact with anionic components of the glycoproteins on the surface of the epithelial cells. Moreover, the interiors of the tight junction (pores) are highly hydrated and negative charged. An alteration in the relative concentration of specific ion species in the pore would result in substantial changes in tight junction resistance, which might lead to loosening or opening of the pore and enhancing the absorption by paracellular routine.

Presuming rHV2 was absorbed by endocytosis, the transcellular way would be blocked, whereas the paracellular routine opened after pollysine was added. These two adverse effects on the epithelial cells would result in no increase of the rHV2 transport. Pollysine and rHV2 are being oppositely charged and might interact with one another, so the amount of transport of rHV2 in the mucosal tissue has a little decrease. The results of our experiments are consistent with the above-mentioned presumption, so it seems logical to deduce that rHV2 was absorbed mainly by endocytosis.

The transport of rHV2 was inhibited by DNP and colchicine. DNP is an uncoupler of oxidative phosphorylation, whereas colchicine is an inhibitor of microtubular assembly. Both of them can damage the functions of epithelial cells and inhibit the transport of rHV2 through transcellular way.

It was reported that carbopol showed a clear effect on opening of intercellular junctions, thereby enhancing the paracellular permeability for hydrophilic macromolecules (Luessen et al., 1996). Carbopol also displays strong mucoadhesive properties (Bai et al., 1995; Mortazavi, 1995) and may therefore be able to localize its enzyme inhibiting and absorption enhancing activities to a confined area in the intestinal tract. However, it was showed in this study that carbopol inhibited the rHV2 transport. The possible reason is that a stagnant layer formed by a highly viscous carbopol solution and repulsion between both negatively charged carbopol and rHV2 may prevent rHV2 molecules from getting close to epithelial cell membrane, thus leading to the decrease of transport of rHV2 across the membrane.

Toshikiro demonstrated that rhIGF-I could be absorbed into systemic circulation (Toshikiro et al., 1996). The molecular weight of rhIGF-I is 7000 Da, which is almost equal to that of rHV2. The transport mechanism of rhIGF-I was inferred to be absorptive-mediated endocytosis. It was supposed from the above-mentioned results that rHV2 has the similar absorption mechanism as rhIGF-I, i.e., endocytosis might be the main route by which rHV2 molecules were absorbed into the circulation.

In conclusion, the maximum degradation was found in the stomach and distal ileum, slightly higher than that in proximal ileum, jejunum, and mucosal subfraction. Some enzyme inhibitors, such as bacitracin or casein, could inhibit the degradation to a certain degree. The bioavailability after oral

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**TABLE 2**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration</th>
<th>Amount Transported in 30 min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nM</td>
<td>Serosal Solution</td>
</tr>
<tr>
<td>None</td>
<td>141.4 ± 16.1</td>
<td>738.3 ± 70.1</td>
</tr>
<tr>
<td>None (4°C)</td>
<td>71.8 ± 4.1***</td>
<td>484.7 ± 17.8**</td>
</tr>
<tr>
<td>DNP</td>
<td>72.4 ± 4.7***</td>
<td>460.6 ± 30.9*</td>
</tr>
<tr>
<td>Colchicine</td>
<td>17.4 ± 3.0***</td>
<td>464.6 ± 15.6*</td>
</tr>
<tr>
<td>Carbopol 941</td>
<td>156.8 ± 9.7</td>
<td>499.4 ± 6.1*</td>
</tr>
</tbody>
</table>

*p < 0.05, **p < 0.01, ***p < 0.001 versus none. Data represent mean ± S.D.; n = 5.
administration of rHV2 to rats varies and is dependent on the analytical method, and some of the enzyme inhibitor could enhance rHV2 oral absorption. There is no site difference in rHV2 absorption in different segments of the small intestine. The possible transport mechanism of rHV2 across the GI tract is concerned with the endocytosis process.

References


Address correspondence to: Dr. Qiang Zhang, Department of Pharmaceutics, School of Pharmaceutical Science, Peking University, Health Science Center, 38 XueYuan Rd., Beijing 100083, P.R. China. E-mail: zqdodo@mail.bjmu.edu.cn.