

Effects of permeation enhancers BL-9 and Brij-78 on absorption of four peptide eyedrops in rabbits¹

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ABSTRACT Systemic absorption of 4 peptide drugs through ocular route was enhanced by permeation enhancers, BL-9 and Brij-78. Gonadorelin (LHRH) was the smallest molecule ($M_r=1200$) and its systemic delivery was most efficiently enhanced by BL-9 (17.0-20.8 times) and Brij-78 (13.9-21.5 times). Although bombesin ($M_r=1620$), atrial natriuretic peptides (ANP) ($M_r=3240$), and adrenocorticotrophic hormone (ACTH) ($M_r=4540$) had molecular weight ranged widely, their systemic absorption through ocular route was enhanced by BL-9 (6.1-8.3 times) and Brij-78 (6.5-9.0 times) in about the same degree. BL-9 enhanced systemic absorption of peptide drugs faster and reached peak peptide concentrations in 5-20 min. On the other hand, Brij-78 took 20-60 min to reach to peak concentration of peptide drugs in the blood. These results indicate that systemic delivery of peptide drug through ocular route is a feasible one particularly when the absorption enhancers are used.

KEY WORDS permeability; gonadorelin; bombesin; atrial natriuretic peptides; adrenocorticotrophic hormone; ophthalmic solutions; absorption

Small peptide drugs of $M_r < 5000$ can be absorbed systemically through ocular route⁽¹⁻⁵⁾. Ever since the development of biotechnology, numerous peptide drugs have become available in the clinics with affordable prices. However, these drugs have to be

administered by parenteral injections which are painful, expensive, and complicated. Several alternative methods have been investigated including rectal, vaginal, buccal, nasal tracheal, and transdermal⁽⁹⁻¹⁵⁾. However, none of them have been accepted enthusiastically. Therefore, it would be desirable to improve the systemic absorption by using permeation enhancers⁽⁶⁻⁸⁾. It is known that larger peptide drug such as insulin with a molecular weight of higher than 5000 can be absorbed much more efficiently with the addition of permeation enhancers in the eyedrops⁽¹⁻⁴⁾. The feasibility of peptide drug administration through ocular route has been investigated recently with promising results⁽¹⁻⁵⁾. In this study, 2 absorption enhancers, BL-9 and Brij-78, were used to test their ability to enhance the systemic absorption of smaller peptides such as gonadorelin (LHRH) ($M_r=1200$), bombesin ($M_r=1620$), ANP ($M_r=3240$), and ACTH ($M_r=4540$). This study raises the feasibility even higher by using the absorption enhancers to reduce the drug doses required for reducing drug prices.

MATERIALS AND METHODS

Materials Gonadorelin (LHRH), bombesin, atrial natriuretic peptides (ANP), adrenocorticotrophic hormone (ACTH), bovine serum albumin (BSA, fraction V, RIA grade), polyoxyethylene 9-lauryl ether (BL-9), polyoxyethylene-20-stearyl ether (Brij-78), aprotinin (Trasylol), and Triton X-100 were purchased from Sigma Chem Co (St Louis MO, USA). Dextran T-70

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(Pharmacia, Sweden), activated charcoal (C-190N, Nuchar, Matheson Coleman & Bell, East Rutherford NJ, USA), and lamb serum (North America Biologicals, Miami FL, USA) were purchased commercially. Radioimmunoassay (RIA) kits for bombesin and ANP were purchased from Incstar Co (Stillwater MN, USA). The antibody for LHRH determination and ^{125}I -LHRH were purchased from Amersham Co. (Arlington Heights IL, USA). The RIA kit for ACTH assay was purchased from Nichols Institute Diagnostics (San Juan Capistrano CA, USA).

Rabbit experiments New Zealand white rabbits weighing 2.5–3.0 kg were anesthetized with katamine ($40 \text{ mg} \cdot \text{kg}^{-1}$) and xylazine ($5 \text{ mg} \cdot \text{kg}^{-1}$) injected im. Half of the dose was then given every hour to maintain anesthesia. The femoral artery was cannulated with polyethylene tubing (PE-90) for blood sampling. At 0, 5, 10, 20, 30, 60, 120, and 180 min after administration of peptide eyedrops, a 3-ml aliquot of blood was collected into a pre-chilled tube containing EDTA (1.44 mg/ml blood) and aprotinin, a peptidase inhibitor, (500 KIU/ml blood). The sample was mixed and centrifuged immediately at $1500 \times g$ for 10 min at 4°C . The plasma was immediately stored in the freezer at -20°C until RIA.

RIA of LHRH RIA buffer was made with phosphate buffer $0.05 \text{ mol} \cdot \text{L}^{-1}$ at pH 7.2 plus 0.1% bovine serum albumin and 0.1% Triton-X 100. The rabbit antibody solution was prepared by adding 2 ml of RIA buffer into the vial of lyophilized powder obtained from Amersham Co (sufficient for 250 assays) and stored at -70°C . The stock solution was further diluted 15 times with RIA buffer upon use. The ^{125}I -LHRH was diluted with RIA buffer to a final concentration of approximately $6500 \text{ dpm} / 100 \mu\text{l}$ as a tracer. The standard curve was prepared at a range of

$3.9\text{--}1000 \text{ pg} \cdot \text{ml}^{-1}$. The charcoal suspension was prepared by stirring the mixture of charcoal and 0.25 g dextran in 100 ml b for at least 20 min at 4°C before using.

The RIA of LHRH was performed by adding 200 μl of sample or standard into $\times 75 \text{ mm}$ siliconized glass tubes, to which $100 \mu\text{l}$ of the antibody was added. The mixture was vortexed gently, and incubated at 4°C for 24 h. Then, 100 μl of ^{125}I -LHRH was added to each tube and the mixture was further incubated at 4°C for 24 h. The separation of bound and free ^{125}I -LHRH was accomplished by adding 200 μl of lamb serum and 500 μl of charcoal suspension, vortexed and stood for 20 min at room temperature. The mixture was centrifuged at $2000 \times g$ for 15 min at 4°C and the radioactivity of the precipitate was counted with an Auto-Gamma Counter. The concentration of LHRH in each sample was calculated from the standard curve.

RIA of Bombesin The bombesin concentration in plasma was determined using a RIA kit purchased from Incstar Co. To 200 μl of sample or standard, 100 μl of rabbit antiserum of bombesin and 100 μl of ^{125}I -bombesin were added. After incubation for 24 h at 4°C , 0.2 ml normal lamb serum and 0.5 ml charcoal-dextran mixture (0.5 g charcoal and 0.5% dextran T-70 in phosphate buffer $0.01 \text{ mmol} \cdot \text{L}^{-1}$ at pH 7.4) were added for the separation of free tracer from bound tracer. The test tubes were shaken and let stand at room temperature for 30 min. Then, the mixtures were centrifuged at $1500 \times g$ at 4°C for 15 min. After discarding the supernatant, the charcoal pellet was counted.

RIA of ANP Plasma 1 ml were mixed carefully with 4% acetic acid 3 ml, and primed to ODS-Silica column activated in advance by acetic acid 5 ml in 86% ethanol 5 ml, distilled water 5 ml, and acetic acid 5 ml separately. Then, the column was washed using distilled water

ANP adsorbed on the column was eluted by 3 ml of 4% acetic acid in 86% ethanol. The fractions were placed in a 37°C water bath for 1 h to evaporate the solvent under the steam of compressed air. One hour later, 95% ethanol 1 ml was added and continued to evaporate to dryness. The residues of dried samples were dissolved in 1 ml of RIA buffer for radioimmunoassay.

The ANP concentration in plasma was determined using RIA kit obtained from Amersham Pharmacia Biotech Co in a nonequilibrium system. To 100 µl of extract or standard ANP in the 12 × 75 mm disposable polystyrene tube, ANP antibody 200 µl was added. After 24 h of incubation at 4°C, 200 µl of ¹²⁵I-ANP were added, and incubated for another 24 h at 4°C.

The free tracer was separated from antibody-bound tracer by adding 0.5 ml of horse anti-sheep precipitating complex. The tubes were vortexed gently, and stood at room temperature for 25 min. They were then centrifuged at 1500 × g for 25 min. The supernatant was discarded, and the pellet was counted using the Auto-gamma counter.

RIA of ACTH Immediately prior to

RIA of ACTH, the plasma was thawed and centrifuged to remove any fibrin clots which might interfere with the assay system. The RIA of ACTH was carried out with RIA kit. Two hundred microliters of plasma or standard was incubated at room temperature for 20 h with ¹²⁵I-ACTH, biotin coupled antibody and avidin coated plastic head. At the end of incubation, the heads were washed twice with washing solution and the radioactivity of each tube was counted with Auto-gamma counter. The concentration of ACTH in plasma was calculated from the standard curve.

Statistical analysis All data were analyzed with *t* test.

RESULTS

When LHRH eyedrops were instilled into eyes, the LHRH peaks of systemic blood concentrations were attained at 10–20 min after drug administration (Tab 1). When 0.5% BL-9 was added to the eyedrops, the LHRH peaks in the systemic circulation were attained faster at 5 min after drug administration. With 0.5% Brij-78, the LHRH peaks in the

Tab 1. Concentration of LHRH in plasma (pg · ml⁻¹) after systemic delivery of LHRH via ocular route and its enhancement by BL-9 and Brij-78. $\bar{x} \pm s$.

Time/ min	10 µg LHRH eyedrops (n=5)	10 µg LHRH +0.5% BL-9 eyedrops (n=5)	10 µg LHRH +0.5% Brij-78 eyedrops (n=5)	20 µg LHRH eyedrops (n=5)	20 µg LHRH +0.5% BL-9 eyedrops (n=4)	20 µg LHRH +0.5% Brij-78 eyedrops (n=5)
0	29 ± 18	26 ± 33	30 ± 4	27 ± 13	69 ± 47	33 ± 33
5	34 ± 17	714 ± 266	39 ± 8	54 ± 60	2 450 ± 1 756	75 ± 70
10	42 ± 25	586 ± 239	160 ± 35	62 ± 64	1 202 ± 702	181 ± 118
20	39 ± 17	220 ± 168	584 ± 289	118 ± 181	772 ± 442	1 716 ± 630
30	32 ± 16	96 ± 40	543 ± 254	103 ± 96	364 ± 427	2 532 ± 829
60	38 ± 18	44 ± 45	69 ± 41	45 ± 39	94 ± 26	788 ± 676
120	34 ± 13	32 ± 24	31 ± 3	13 ± 4	40 ± 33	120 ± 134
180	31 ± 10	29 ± 23	37 ± 10	16 ± 18	74 ± 34	57 ± 59
Absorption ^a	—	17.0 times	13.9 times	—	20.8 times	21.5 times

^a Peak concentration with absorption enhancer / Peak concentration without absorption enhancer.

systemic circulation were attained 10 min later than those attained by LHRH without absorption enhancer. It is interesting to note that at lower doses of LHRH (10 μg), BL-9 and Brij-78 enhance the LHRH absorption for 17.0 times and 13.9 times, respectively, whereas at higher dose of LHRH (20 μg), the LHRH absorption was enhanced further by BL-9 and Brij-78 for 20.8 times and 21.5 times, respectively (Tab 1).

Similar results were obtained with 15 μg bombesin eyedrops (Tab 2). The systemic absorption of bombesin eyedrops was enhanced less effectively by 0.5% BL-9 and 0.5% Brij-78 for 8.3 times and 6.5 times, respectively, and reached the peak blood concentrations at 5 min and 60 min, respectively, after drug instillation.

Tab 2. Concentration of Bombesin in plasma ($\text{pg} \cdot \text{ml}^{-1}$) after systemic delivery of bombesin via ocular route and its enhancement by BL-9 and Brij-78. $n=4$, $\bar{x} \pm s$.

Time / min	15 μg Bombesin eyedrops	15 μg Bombesin +0.5% BL-9 eyedrops	15 μg Bombesin +0.5% Brij-78 eyedrops
0	81 \pm 23	120 \pm 33	112 \pm 54
5	84 \pm 31	699 \pm 161	90 \pm 50
10	78 \pm 32	585 \pm 104	145 \pm 69
20	65 \pm 15	372 \pm 166	261 \pm 131
30	69 \pm 18	243 \pm 83	514 \pm 245
60	70 \pm 12	180 \pm 94	548 \pm 304
120	62 \pm 24	112 \pm 27	120 \pm 39
180	80 \pm 21	113 \pm 140	130 \pm 142
Absorption ^a	—	8.3 times	6.5 times

^a Peak concentration with absorption enhancer / Peak concentration without absorption enhancer

Although the M_r of ANP is as approximately 3 times as that of LHRH and 2 times as that of bombesin, its systemic absorption was enhanced by 0.5% BL-9 and 0.5% Brij-78 for 7.8 times and 9.0 times,

respectively, and reached the peak blood concentrations at 10 min and 30 min after drug instillation, respectively (Tab 3). The absorption enhancement was less effective than LHRH but was about the same as bombesin.

Tab 3. Concentration of ANP in plasma ($\text{pg} \cdot \text{ml}^{-1}$) after systemic delivery of ANP via ocular route and its enhancement by BL-9 and Brij-78. $n=5$, $\bar{x} \pm s$.

Time / min	15 μg ANP eyedrops	15 μg ANP +0.5% BL-9 eyedrops	15 μg ANP +0.5% Brij-78 eyedrops
0	31 \pm 13	42 \pm 8	39 \pm 7
5	41 \pm 25	316 \pm 146	54 \pm 14
10	31 \pm 10	321 \pm 174	100 \pm 44
20	30 \pm 7	152 \pm 58	245 \pm 86
30	25 \pm 5	80 \pm 20	368 \pm 166
60	26 \pm 8	65 \pm 16	179 \pm 82
120	32 \pm 8	72 \pm 7	121 \pm 50
180	32 \pm 9	51 \pm 15	84 \pm 30
Absorption ^a	—	7.8 times	9.0 times

^a Peak concentration with absorption enhancer / Peak concentration without absorption enhancer

The largest molecule of peptide drug studied is ACTH with a M_r as approximately 4 times as that of LHRH, 3 times as that of bombesin and 1.5 times as that of ANP. The systemic absorption of ACTH via ocular route was enhanced by 0.5% BL-9 and 0.5% Brij-78 for 6.1 folds and 7.7 folds, respectively, and was very similar to the results obtained with bombesin and ANP. The blood concentration peaks were attained at 20 min after drug administration with both permeation enhancers (Tab 4).

DISCUSSION

Among 4 peptides studied, LHRH is the smallest and its systemic absorption is most efficiently enhanced by BL-9 and Brij-78. The systemic absorption of bombesin,

Tab 4. Concentration of ACTH in plasma ($\mu\text{g} \cdot \text{ml}^{-1}$) after systemic delivery of ACTH via ocular route and its enhancement by BL-9 and Brij-78. $n=5$, $\bar{x} \pm s$.

Time / min	25 μg ACTH eyedrops	25 μg ACTH +0.5% BL-9 eyedrops	25 μg ACTH +0.5% Brij-78 eyedrops
0	4 \pm 4	8 \pm 8	18 \pm 16
5	3 \pm 3	572 \pm 123	972 \pm 254
10	15 \pm 7	936 \pm 335	1 090 \pm 313
20	168 \pm 67	1 030 \pm 342	1 300 \pm 122
30	139 \pm 27	944 \pm 397	1 042 \pm 236
60	84 \pm 28	686 \pm 200	914 \pm 114
90	58 \pm 21	408 \pm 150	706 \pm 151
120	45 \pm 33	309 \pm 149	567 \pm 204
180	17 \pm 8	132 \pm 41	221 \pm 150
Absorption ^a	—	6.1 times	7.7 times

^a Peak concentration with absorption enhancer / Peak concentration without absorption enhancer

ANP, and ACTH was enhanced by BL-9 and Brij-78 in a similar extent and was about half of that of LHRH.

It is interesting to note that BL-9 enhances the systemic absorption of peptides faster than Brij-78. Peptide concentrations reached peaks by BL-9 at 5-20 min after drug instillation whereas Brij-78 took 20-60 min to do the same. As for the potency, both BL-9 and Brij-78 were about the same. In general, Brij-78 has longer duration than BL-9. Therefore, for faster and shorter peptide delivery, BL-9 can be used as an absorption enhancer. On the other hand, Brij-78 can be used for a slower and longer enhancement of peptide drug delivery.

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