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Rectal Absorption of Lysozyme and Heparin in Rabbits in the Presence of Non-surfactant Adjuvants

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Ethylacetoacetate enamines of phenylalanine and phenylglycine enhanced the rectal absorption of macromolecular drugs such as lysozyme and heparin in rabbits. Glycerine-1,3-diacetoacetate, 1,2-isopropylideneglyceryl-3-acetoacetate and ethylacetoacetylglycolate also enhanced the rectal absorption of lysozyme and heparin. Lysozyme is a basic compound and heparin is an acidic compound, so the non-surfactant adjuvants used in this study can promote the rectal absorption of macromolecular compounds irrespective of their electrochemical properties. The coadministration of calcium ion inhibited the adjuvant action, so complex formation of the adjuvant with calcium ion may occur, reducing the efficacy of these compounds as adjuvants.

Keywords——macromolecular drug; lysozyme; heparin; nonsurfactant adjuvant; enamine; acetoacetate ester; rectal absorption suppository; calcium effect

There are many problems associated with current drug therapy, especially with macromolecular drugs which are poorly absorbed from the gastrointestinal tract. In general, both polypeptide and mucopolysaccharide drugs are degraded or poorly absorbed after oral administration. To overcome these problems, considerable effort have been made to develop new dosage forms suitable for oral or rectal delivery.

It has been reported recently that rectal absorption of macromolecular drugs such as insulin and growth hormone is enhanced by co-administration with non-surfactant adjuvants such as enamine derivatives,[1,2] glycerine derivatives[3] and salicylates[4–6] as well as surfactants.[7,8] Rectal administration of macromolecular drugs may be useful to overcome the inconvenience of injection and the antigenicity of parenterally administered foreign macromolecules.

In this paper, rectal absorption of lysozyme and heparin was studied, with enamine derivatives of ethyl acetoacetate and glycerine esters of acetoacetic acid as adjuvants, and the effect of calcium ion on the enhancing activity was studied.

Experimental

Materials——Commercially available lysozyme chloride and heparin sodium (173 U.S.P. units/mg) were supplied by Toho Pharmaceutical Co., Ltd. (Osaka, Japan) and Teijin Co., Ltd. (Tokyo, Japan), respectively. Antilysozyme antibody was supplied from Eizai Co., Ltd. (Tokyo, Japan). Enamine derivatives of amino acids were routinely synthesized by reacting the sodium salt of the amino acid with ethyl acetoacetate in ethanol at room temperature.[9] Acetoacetate esters were routinely synthesized by adding diketene to either glycerol, 1,2-isopropylidene glycerol, or ethyl glycolate in the presence of potassium acetate (a catalyst) at 100—110 °C. The crude products obtained were purified by liquid chromatographic methods.[3] Thus, glycerine-1,3-diacetoacetate (GlyDA), 1,2-isopropylidene glyceryl-3-acetoacetate (GlyIA), and ethyl acetoacetylglycolate (EtGlcA) were synthesized. Other reagents used were of analytical grade.

Preparation of Suppositories——To facilitate drug dispersion, lysozyme chloride was passed through a 100-mesh sieve and sodium heparin was freeze-dried before use. Suppositories were prepared by combining the adjuvant (5% w/w) and either lysozyme chloride or sodium heparin with a triglyceride base (Witepsol H-15, Chemische Werk, Witten, Germany) by a fusion method at 40 °C.[1] The molten liquid was poured into disposable plastic molds (Nichii Packing Co., Osaka, Japan). The suppositories were allowed to stand for 2 h at room temperature and were then stored in a refirigerator until use.

***In Vivo* Studies**——Male albino rabbits, 2.2—2.8 kg, were fasted for 16 h prior to experiments, but water was given *ad libitum*. Three ml control blood samples for calibration curve of lysozyme were collected from the marginal ear vein of each rabbit before the rectal administration of the suppository. After rectal administration of the suppository at a dose of 0.2 g suppository/kg, the anus was closed with a plastic crimp to prevent leakage of the rectal contents during the experiments. Blood samples were collected at designated intervals. For intramuscular administration of lysozyme, a saline solution of lysozyme chloride was administered into the femoral muscle. Blood samples were centrifuged at 3000 rpm for 10 min and the plasma fraction was kept in a refrigerator until required for assay.

Assay Method——Assay of lysozyme in plasma was carried out by a lytic assay method[10] and an enzyme immunoassay method.[11] For the lytic assay, 60 mg of dry cells of *Micrococcus lysodeikticus* was suspended in 100 ml of 1/15 M phosphate buffer at pH 6.2 and allowed to swell for 24 h at 4 °C. The absorbance of the suspension was adjusted at 10% at 640 nm by the addition of the phosphate buffer. A 100 μl aliquot of plasma sample was mixed with 3 ml each of the cell suspension and phosphate buffer and the whole was incubated for 7 min at 37 °C. Then the suspension was rapidly cooled at 0 °C and the decrease in turbidity at 640 nm was measured. To obtain a standard curve, 100 μl of lysozyme solution dissolved in the phosphate buffer at various concentrations was mixed with 100 μl of control plasma and 2.9 ml of phosphate buffer. These standard solutions were incubated and the turbidity was measured as described above. The measurement range was 0.5—30 μg/ml in plasma. Thus, a concentration of lysozyme in plasma of less than 0.5 μg/ml was designated as ND (not detectable). Enzyme immunoassay of lysozyme was carried out following the method of Yuzuriha *et al.*[11]

Assay of heparine in plasma was carried out by an activated partial thromboplastin time (ATPP) method[12] using an assay kit distributed by Parke-Davis-Sankyo Co., Ltd.

Results

After intramuscular injection of a saline solution of lysozyme chloride at a dose of 5 mg/kg or rectal administration of a lysozyme suppository containing 5% ethyl acetoacetate enamine of sodium DL-phenylalanine (PheEtAA) at a dose of 15 mg/kg in rabbits, the plasma

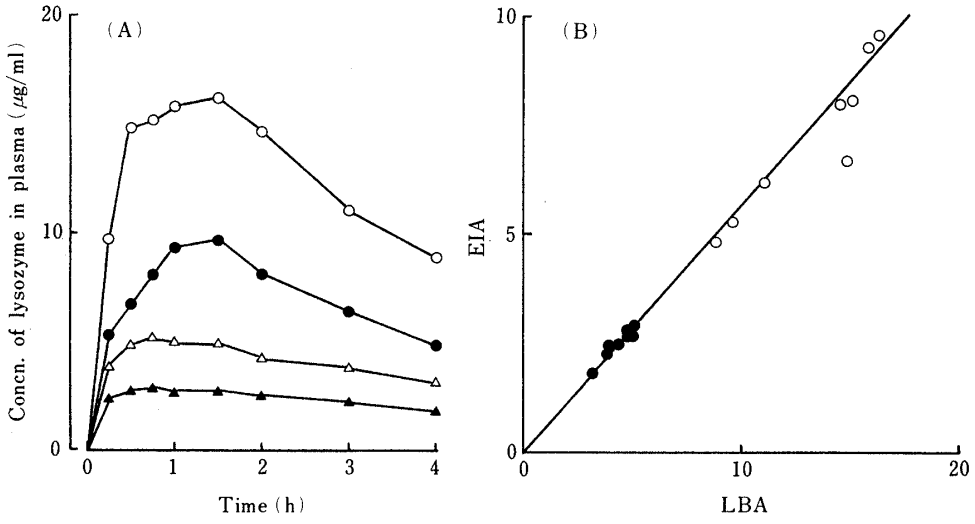

Fig. 1

Plasma lysozyme levels (A) determined by LBA (● and ▲) and EIA (○ and △) after intramuscular injection of lysozyme chloride at a dose of 5 mg/kg (○ and ●) and after rectal administration of lysozyme at a dose of 15 mg/kg in a suppository containing 5% (w/w) PheEtAA (△ and ▲).

The relationship between plasma levels of lysozyme determined by LBA and those determined by EIA is shown (B).

$y=0.572x\pm0.083$, $r=0.986$.

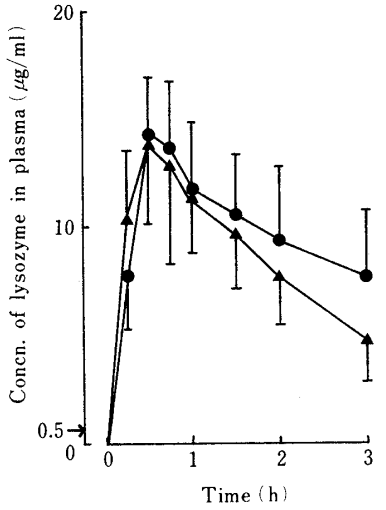

Fig. 2

Plasma concentration profiles of lysozyme after rectal administration of lysozyme chloride at a dose of 15 mg/kg with a suppository containing 5% (w/w) adjuvant; PheEtAA (●), PGEtAA (▲), ethyl acetoacetate, phenylalanine and phenylglycine. Plasma lysozyme levels were not detectable when lysozyme was coadministered with ethyl acetoacetate, phenylalanine or phenylglycine. Each value is the mean ±S.D. ($n \geq 4$).

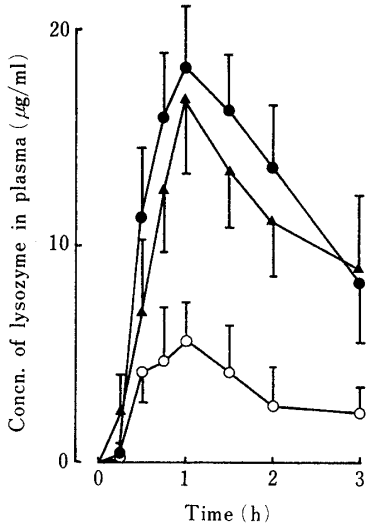

Fig. 3

Plasma concentration profiles of lysozyme after rectal administration of lysozyme chloride at a dose of 15 mg/kg with a suppository containing 5% (w/w) adjuvant; GlyDA (○), GlyIA (●) and EtGlcA (▲). Each value is the mean ±S.D. ($n \geq 4$).

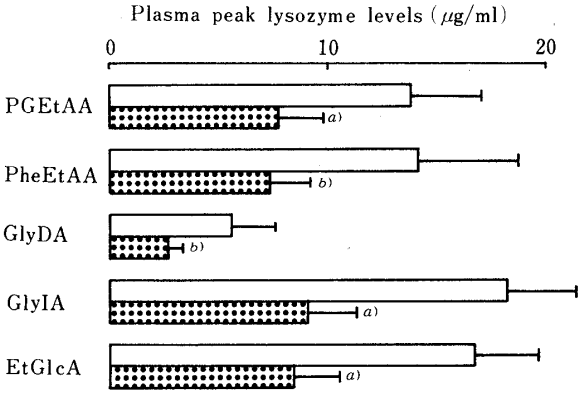

Fig. 4

Effect of calcium gluconate (⊡) in a suppository on the adjuvant action of various adjuvants in enhancing rectal lysozyme absorption (□). Doses of lysozyme chloride and adjuvants were as described in Figs. 1 and 2, and the concentration of calcium gluconate was 5% (w/w) in this study. The effect of calcium gluconate was determined by comparing the plasma peak lysozyme levels after rectal administration. Each value is the mean ±S.D. ($n \geq 4$). *a*) $p < 0.01$ against no calcium gluconate (Student's *t*-test), *b*) $p < 0.05$ against no calcium gluconate.

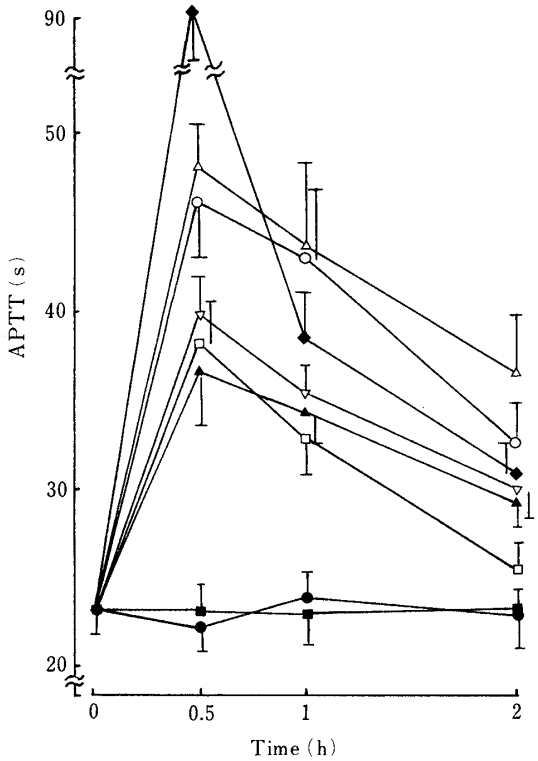

Fig. 5

APTT after rectal administration of heparine at a dose of 500 U.S.P.U/kg with a suppository containing 5% (w/w) adjuvant; ● no adjuvant, ■ EtAA, ▽ PheEtAA, □ PGEtAA, ▲ GlyDA, △ GlyIA, and ○ EtGlcA. APTT after intravenous injection at a dose of 500 U.S.P.U/kg is shown by ◆. APTT was not affected by administration of each adjuvant without heparine (APTT between 21.5 to 25.3 s at any experimental period). Each value is the mean ±S.D. ($n \geq 4$).

concentration of lysozyme was measured by enzyme immunoassay (EIA) and lytic assay (LBA) as described above. Although the plasma concentrations determined by LBA were higher than those determined by EIA, the values obtained by the two methods were linearly correlated with a correlation coefficient of 0.986 (Fig. 1). Although it was not clear why LBA showed higher lysozyme levels in plasma, the subsequent study was carried out by using the LBA method.

Absorption of lysozyme was not observed during the experimental period of 4 h after the rectal administration of a lysozym chloride suppository at a dose of 15 mg/kg without an adjuvant. Thus, it is clear that PheEtAA enamine is effective as an adjuvant for the rectal absorption of macromolecules with a molecular weight up to 14300 which are not absorbed by the rectal route in the absence of an adjuvant.

Coadministration of the ethyl acetoacetate enamine of either DL-phenylalanine or D-phenylglycine (PGEtAA) with lysozyme chloride at a dose of 15 mg/kg significantly enhanced the rectal absorption in rabbits (Fig. 2). However, coadministration of lysozyme chloride with either ethyl acetoacetate or the sodium salt of phenylalanine or phenylglycine did not enhance the absorption. No significant difference was observed between the two enamine adjuvants.

To study the structural requirements for an enamine adjuvant, acetoacetate esters of glycerine, GlyDA and GlyIA, and ethyl glycolate, EtGlcA, were studied (Fig. 3). At 15 mg/kg dose of lysozyme chloride with 5% acetoacetate adjuvant, the peak lysozyme levels of GlyIA and EtGlcA suppositories were significantly higher than those of enamine adjuvants. The enhancing efficacy of GlyDA was the poorest among the adjuvants studied.

To study the factors required for adjuvant, enhancement of rectal drug absorption, calcium gluconate was coadministered with lysozyme and adjuvant. The coadministration of calcium resulted in a significant decrease but not in complete loss of the enhancing activity for all adjuvants studies (Fig. 4).

Enamine derivatives and acetoacetate esters also enhanced the rectal absorption of heparin, an anionic mucopolysaccaride with a molecular weight 6000 to 20000, at a dose of 500 U.S.P. units/head (Fig. 5).

Discussion

The enhancing activities of PheEtAA and PGEtAA on rectal absorption of lysozyme chloride in rabbits were significant (Fig. 2). The activities of two enamines were similar, with a rapid increase in plasma levels, reaching the maximum level of 15 μg/ml at 30 min after administration at a dose of 15 mg/kg. When EtAA, phenylalanine or phenylglycine was administered with lysozyme chloride, no enhancement of drug absorption was detected within the limit of the LBA method (0.5 μg/ml). These results suggest the importance of enamine structure for adjuvant efficacy. Enamines are considered rather ineffective in aqueous solution,[13] but the enamine used in this study seem to be more stable in rectal fluide than would be expected from the previous report.[13] We are planning a detailed study on the stability of enamines in aqueous solution.

To investigate the structural requirements for adjuvant activity, acetoacetic esters of glycerol, GlyDA and GlyIA, and an ester of ethyl glycolate (EtGlcA) were studied (Fig. 3). The plasma levels of lysozyme administered with these ester adjuvants peaked at about 60 min after rectal administration. This rather slow attainment at maximum level with a 10—15 min lag time in comparison to that of enamines can be partly explained on the basis of the affinity of esters to the triglyceride base, inhibiting their quick release from the suppository,[3] while enamine adjuvants might be released rapidly since they are present as sodium salts with very high water solubilities.[1]

The enhancing activities of GlyIA and EtGlcA were significantly stronger than those of

enamine adjuvants but the efficacy of GlyDA was much weaker than those of other adjuvants. In a previous paper, it was reported that GlyDA and GlyIA showed similar enhancing activities on the rectal absorption of insulin.[3] The present results with lysozyme and insulin may be partly explained by the fact that insulin molecules dissolve in water under alkaline conditions as the monomer with a molecular weight of 6000, but under acidic condition, a considerable amount of dimer is present.[14] The molecular weight of lysozyme is about 14000 daltons. Although it is not yet clear whether the adjuvant action occurs through a paracellular route or transcellular route, the actions of each adjuvant may be different and there may be limit to the size of macromolecule which can be absorbed. This should be investigated.

It was suggested in our previous paper[1] that chelating activity with calcium and magnesium ions could be involved in the adjuvant action on the rectal absorption of insulin, because incorporation of these ions suppressed the adjuvant action of enamine derivatives[1] and glycerine esters.[3] To clarify this inhibitory action of calcium ions on the enhanced rectal lysozyme absorption, calcium gluconate was incorporated into suppositories which were administered rectally to rabbits (Fig. 4). The peak plasma level of lysozyme was significantly reduced by the incorporation of calcium ion with all adjuvants studied. These findings lead us to speculate that chelating agents such as EDTA remove calcium ions from the membrane structure and change the membrane permeability to polar compounds.[15] This speculation is supported by our previous finding[16] that disodium EDTA enhanced the rectal absorption of ampicillin in rabbit but calcium EDTA did not.

Since EtAA did not enhance the rectal absorption of lysozyme even though it possesses strong chelating activity with metal ions including calcium, some other mechanism may be involved, however. Since it has been reported[17] that amino components in the cell or cell membrane may be involved in the mechanism of salicylate action enhancing the uptake of water-soluble compounds into red blood cells, such components could also be involved in the case of adjuvants used in this study. Since the adjuvants used in this study are chelating agents, the observed inhibitory effect of calcium ion on the adjuvant action may be due to complex formation with adjuvant, reducing the adjuvant activity.

Since the isoelectric point of lysozyme is 10.5—11.0, lysozyme can be considered as a rather strong base and is present as the cation at the physiological pH of rectal fluid. To examine the enhancing action of adjuvants, heparine sodium was selected as an anionic macromolecular drug (Fig. 5). As was the case with lysozyme, stronger enhancing activity of acetoacetate esters than enamine derivatives was observed.

In this work, we confirmed that enamine derivatives and acetoacetate esters can facilitate the rectal absorption of macromolecular compounds irrespective of the electrochemical properties of the latter. Further work is necessary, but it seems likely that the use of these adjuvants will permit the development of a new rectal delivery method for macromolecular drugs which are at present difficult to administer other than by parenteral injection.

References

1) A. Kamada, T. Nishihata, S. Kim, M. Yamamoto, and N. Yata, *Chem. Pharm. Bull.*, **29**, 2012 (1981).
2) S. Kim, A. Kamada, T. Higuchi, and T. Nishihata, *J. Pharm. Pharmacol.*, **35**, 100 (1983).
3) T. Nishihata, S. Kim, S. Morishita, A. Kamada, N. Yata, and T. Higuchi, *J. Pharm. Sci.*, **72**, 280 (1983).
4) T. Nishihata, J. H. Rytting, T. Higuchi, and L. Caldwell, *J. Pharm. Pharmacol.*, **33**, 334 (1981).
5) T. Nishihata, J. H. Rytting, L. Caldwell, S. Yoshioka, and T. Higuchi, "Optimization of Drug Delivery," Alfred Benson Symposium 17, ed. by H. Bundgaard, A. B. Hansen, and H. Kofod, Munksgaad, Copenhagen, 1982, p. 19.
6) T. Nishihata, J. H. Rytting, A. Kamada, T. Higuchi, M. Routh, and L. Caldwell, *J. Pharm. Pharmacol.*, **35**, 148 (1983).
7) E. Touitou, M. Donbrow, and E. Azaz, *J. Pharm. Pharmacol.*, **30**, 662 (1987).
8) Y. Yamasaki, M. Shichiri, R. Kawamori, M. Kikuchi, T. Hakui, T. Yagi, and H. Abe, *J. Pharm. Pharmacol.*,

30, 806 (1979).
9) T. Murakami, H. Tamauchi, M. Yamazaki, K. Kubo, A. Kamada, and N. Yata, *Chem. Pharm. Bull.*, **29**, 1986 (1981).
10) G. Litwack, *Proc. Soc. Exptl. Biol. Med.*, **89**, 401 (1955).
11) T. Yuzuriha, K. Katayama, J. Tsutsumi, and M. Kimura, *Chem. Pharm. Bull.*, **27**, 2802 (1979).
12) J. G. Lenahan, S. Frye, and G. Philips, *Clin. Chem.*, **12**, 263 (1966).
13) K. Kamikawa, T. Nishihata, H. Takahata, and A. Kamada, *J. Pharm. Dyn.*, **7**, 143 (1984).
14) W. D. Lougheed, S. U. Fischer, K. Perlman, and A. M. Albisser, *Diabetologia*, **20**, 51 (1981).
15) H. Kunz, G. Rehbock, and W. Vogt, *Naunyn-Schiedeberg's Arch. Pharmacol.*, **273**, 331 (1972).
16) T. Nishihata, H. Takahagi, and T. Higuchi, *J. Pharm. Pharmacol.*, **35**, 124 (1983).
17) T. Nishihata, A. Kamada, and T. Higuchi, *Life Sciences*, **34**, 437 (1984).