Effect of Ophthalmic Preservatives on Serum Concentration and Local Irritation of Ocularly Applied Insulin

Hitoshi SASAKI,* Choyu TEI, Koyo NISHIDA, and Junzo NAKAMURA

School of Pharmaceutical Sciences, Nagasaki University, 1–14 Bunkyo-machi, Nagasaki 852, Japan.
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We previously compared hypoglycemic responses after the instillation of insulin formulations. A hypoglycemic response was actually observed after an instillation of insulin with ophthalmic preservatives. In the present study, in order to evaluate the usefulness of insulin formulation containing ophthalmic preservatives, a serum concentration of insulin and an irritation to the eye were investigated after instillation of the insulin formulation in albino rabbits. The ophthalmic preservatives used were benzalkonium chloride, paraben, 2-phenylethanol, benzyl alcohol and sorbic acid. As a result, ophthalmic preservatives, especially benzalkonium chloride and paraben, increased the serum concentration of insulin. The insulin concentration showed a significant correlation with the hypoglycemic response reported previously. This result indicates that ophthalmic preservatives increase the absorption of ocularly applied insulin, and the absorbed insulin decreases serum glucose concentration. The insulin formulation with preservatives showed little irritation on rabbit eyes according to blinking measurements. These results indicate that ophthalmic preservatives are useful for the systemic delivery of ocularly applied insulin.

Keywords insulin; drug delivery; eye; toxicity; preservative; absorption promoter

Recently, various peptide drugs have been developed and have begun to be used clinically as the result of advancements in biotechnology. In general, most peptide drugs are administered through parenteral injection because of their instability with gastrointestinal peptidase and the impermeability of the biological membrane.[1-3] Most attention has been focused on establishing a non-invasive administration route for peptide drugs including nasal, pulmonary, buccal, rectal, transdermal and vaginal routes.[1,4,5] The ocular route is another possible route for the systemic delivery of peptide drugs because the mucous membrane in the conjunctiva and nasal cavity are easily permeable to macromolecular compounds.[5,6] However, a large peptide such as insulin cannot be absorbed after its instillation as compared to small peptides.[6,7] We previously compared hypoglycemic responses after the instillation of insulin formulations. A hypoglycemic response was actually observed after the instillation of insulin with ophthalmic preservatives.[8]

In the present study, in order to evaluate the usefulness of an insulin formulation containing ophthalmic preservatives, a serum concentration of insulin and irritation to the eye were investigated after instillation of the insulin formulation in albino rabbits. The preservatives used were benzalkonium chloride, paraben, 2-phenylethanol, benzyl alcohol and sorbic acid, which are widely used in commercial ophthalmic droplets.

MATERIALS AND METHODS

Materials Monocomponent porcine insulin (26.2 U/mg, molecular weight 6000) was kindly supplied from Novo Nordisk (Gentofte, Denmark). Saponin was purchased from E. Merck (Darmstadt, Germany). Sorbic acid (SA), 2-phenylethanol (PE), methylparaben and propylparaben were purchased from Sigma Chemical Company (St. Louis, U.S.A.). Paraben (PR) was used as a mixture of methylparaben and propylparaben (13:7 w/w). Benzyl alcohol (BA), benzalkonium chloride (BK) and all other chemicals were of reagent grade, and were obtained from Nacalai Tesque, Inc. (Kyoto, Japan). Phosphate-buffered saline (pH 7.4) was prepared by mixing an isotonic phosphate buffer with an equal volume of saline.

Animals Male Nippon albino rabbits weighing 2.0—3.0 kg were used throughout the study. The animals were individually housed in cages in an air-conditioned room and maintained on a standard laboratory diet (ORC4, Oriental Yeast Co., Ltd., Tokyo, Japan). The rabbits were starved for 24 h prior to use for the experiments but had free access to water. All experiments in the present study conformed with the Guideline for Animal Experimentation in Nagasaki University.

***In Vivo* Instillation Experiment** Unanesthetized rabbits were kept in a prone position on a wooden plate. Twenty-five μl of insulin formulation (10 U/rabbit) containing preservatives (0.01% BK, 0.04% PR, 0.5% PE, 0.5% BA and 0.25% SA) was carefully applied with a micropipette (Gilson Medical Electronics, Villiers-le-Bel, France) in the lower conjunctival sac of a rabbit eye. The preservative concentration was within the range of clinical use in the ophthalmic droplet. Blood samples were withdrawn *via* a marginal ear vein 0.25, 1 and 2 h after instillation of the formulations. Serum insulin concentrations were determined by an enzyme immunoassay.

Local Toxicity The rabbit eyes were gently washed 6 h after instillation of the insulin formulation *in vivo* instillation experiment. Rabbit eyes were examined and scored according to the scale of Draize *et al.*[9] 1, 2, 3 and 7 d after the instillation.

Blinking counts were measured as a local irritation after the instillation of an insulin formulation containing preservatives (0.01% BK, 0.04% PR, 0.5% PE, 0.5% BA and 0.25% SA) in albino rabbits.[10] A pH 7.4 buffer was used as a control. 0.1% saponin and 0.1% EDTA were also used as an absorption promoter control. Blinking of the instilled eye, non-instilled eye and both eyes together

was counted for 5 min. The blinking for 0.1% saponin was measured for 2 min because of its severe irritation.

Analysis Serum insulin concentration was determined by enzyme immunoassay (Glazyme Insulin-EIA Test®; Wako Pure Chemical Industries, Ltd., Osaka, Japan). The area under the serum insulin concentration–time curve (AUC_{ins}) was calculated by a trapezoidal method.

RESULTS AND DISCUSSION

Insulin is a hypoglycemic peptide drug to be used clinically in a diabetic by injection. Christie and Hanzal (1931) first demonstrated that topical ocular administration of insulin with an acidic solution reduced rabbit blood glucose in proportion to the instillation dose.[11] Chiou and Chuang,[12] and Yamamoto *et al.*[13] demonstrated that insulin was absorbed and showed a hypoglycemic response after its instillation with several absorption enhancers. However, the long-term efficacy and safety of this method have yet to be defined. There have been some reports on the effect of ophthalmic preservatives on corneal irritability and the enhancement of drug penetration into the eye.[14,15] Camber and Edman demonstrated that some preservatives significantly increased the corneal permeability of pilocarpine and dexamethasone.[16] We previously compared hypoglycemic responses after the instillation of insulin formulations. A hypoglycemic response was actually observed after the instillation of insulin with ophthalmic preservatives.[8]

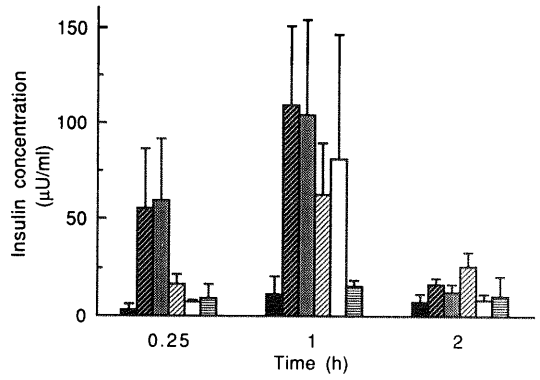

Fig. 1. Serum Insulin Concentration after Instillation of Insulin (10 U) with Preservatives

Key: ■, no preservative; ▨, 0.01% BK; ▩, 0.04% PR; ▨, 0.5% PE; □, 0.5% BA; ▤, 0.25% SA.
Each value presents an average of at least three experiments and the vertical bar means S.E.

The effect of ophthalmic preservatives on insulin absorption through an ocular route was examined by measuring the insulin concentration in serum. The serum glucose concentration is affected by various stresses and fasting during experiment. The preservative concentration used was within the range of clinical use for ophthalmic droplets in view of the safety of formulation. The results are shown in Fig. 1. The serum insulin level before the experiment was $4.6 \pm 0.5\,\mu$U/ml. Preservatives increased the serum concentration of ocularly applied insulin. Especially, BK and PR showed higher insulin levels than insulin instilled alone. These results agreed with the effect of preservatives on the hypoglycemic response reported previously.[8]

The hypoglycemic responses to insulin were previously reported as a minimum serum glucose concentration (C_{min}) and as an area under the decrease of serum glucose concentration from control–time curve (AUC_{glu}) calculated by the trapezoidal rule.[8] The AUC_{ins} was also calculated. AUC_{ins} is plotted with the hypoglycemic responses in Fig. 2. There is a linear relationship between the logarithmic value of AUC_{ins} and hypoglycemic responses. This result indicates that ophthalmic preservatives increase the absorption of ocularly applied insulin and the absorbed insulin then decreases serum glucose concentration.

The promoting mechanism of preservatives on insulin absorption was not clear. Some ophthalmic preservatives enlarged the intercellular spaces and disrupted the cytoplasmic membrane in superficial cells.[17,18] A conjunctival permeability of β-blocker was also reported to be enhanced by a preservative as well as by bile salts.[19] The quantitative cytotoxicity of preservatives was demonstrated using cultured human conjunctival cells.[20] On the other hand, the nasal membrane in comparison with the conjunctival membrane was reported to be the predominant contributor to the systemic delivery of insulin instilled with benzalkonium chloride.[8]

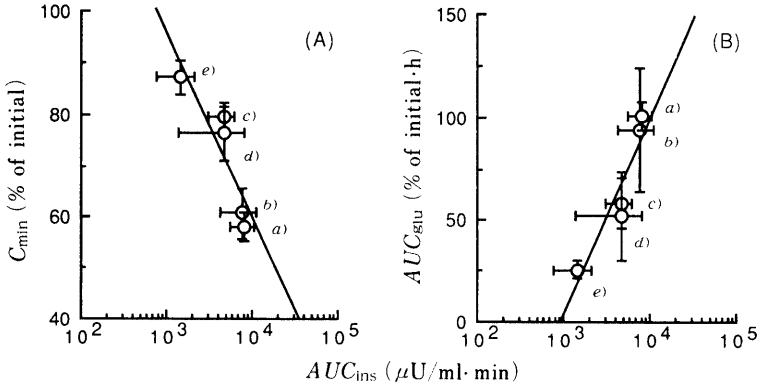

Fig. 2. Relationship between AUC_{ins} and C_{min} (A) or AUC_{glu} (B) after Instillation of Insulin (10 U) with Preservatives

a) 0.01% BK; *b*) 0.04% PR; *c*) 0.5% PE; *d*) 0.5% BA; *e*) 0.25% SA. (A) $C_{min} = 209 - 37 \times \log(AUC_{ins})$, correlation coefficient (γ) = 0.890 (significantly correlative, $p < 0.05$). (B) $AUC_{glu} = -282 + 95 \times \log(AUC_{ins})$, correlation coefficient (γ) = 0.925 (significantly correlative, $p < 0.05$).
Each value presents an average of at least three experiments and the horizontal and vertical bars mean S.E.

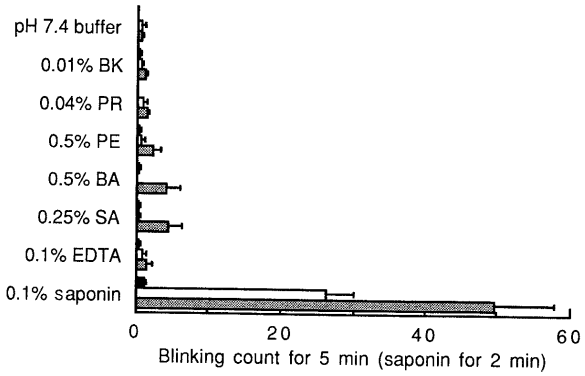

Fig. 3. Rabbit Blinking Count after Instillation of Insulin (10 U) with EDTA, Saponin and Preservatives

Key: ■, non-instillation eye; □, both eyes; ▨, instillation eye.
Each value presents an average of at least four experiments and the horizontal bar means S.E.

After the *in vivo* instillation experiment, the damage of the eye by insulin formulations was evaluated according to Draize scoring,[9] but no damage was observed. Local irritation by preservatives was also examined using the blinking count method. The results are shown in Fig. 3. The blinking counts for all preservatives showed no significant difference from the pH 7.4 buffer.

Saponin was found to be the best enhancer of insulin absorption *via* the ocular route.[12] However, the irritation by saponin was more severe than that of preservatives (Fig. 3). EDTA, a known calcium chelator, also showed an enhancing effect on insulin absorption.[21] EDTA showed no significant irritation (Fig. 3). However, it was shown to penetrate the cornea, conjunctiva, and iris/ciliary body from a topically applied dose.[21] On the other hand, ophthalmic preservatives should be safe as an absorption promoter for insulin delivery because they have already been repeatedly used in the instilled droplet form for clinical ophthalmic disease for an extended period. BK and PR, which showed high promoting activity, are used widely because of their sufficient bactericidal efficacy and lower toxicity. Corneal exposure to multiple drops of BK leads to epithelial accumulation but no penetration into the anterior chamber.[15]

In conclusion, ophthalmic preservatives are useful for the systemic delivery of ocularly applied insulin. Especially, BK and PR showed low irritation and high promoting effects.

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