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Effect of Preservatives on Systemic Delivery of Insulin by Ocular Instillation in Rabbits

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Abstract—The effects of absorption promoters and ophthalmic preservatives on the systemic absorption of insulin through the ocular route were investigated in albino rabbits. Insulin absorption was evaluated by its hypoglycaemic response. Although ocular instillation of insulin alone did not decrease the serum glucose concentration, instillation of insulin with absorption promoters such as EDTA and saponin decreased it. The promoting effect depended on the concentration of the absorption promoters. Of ophthalmic preservatives investigated (benzalkonium chloride, paraben, 2-phenylethanol, benzyl alcohol and sorbic acid), benzalkonium, chloride and paraben showed promoting effects. The promoting effect for benzalkonium chloride was reversible and dependent on concentration of both benzalkonium chloride and insulin in the formulation.

Recently, various peptide drugs have been developed by advancements in biotechnology and have been used clinically. In general, most peptide drugs are administered through parenteral injection because of their instability towards gastrointestinal peptidase and impermeability through biological membranes (Banga & Chien 1988; Pitt 1990; Talmadge 1993). Injections are painful and poorly accepted by most patients, and numerous attempts have been made to find alternative administration routes of peptide drugs including nasal, pulmonary, buccal, rectal, transdermal and vaginal routes (Banga & Chien 1988; Zhou & Li Wan Po 1991a, b). These approaches have been useful for some stable small peptides, but not for most peptide drugs. Absorption promoters have also been investigated for further improvement of peptide drug absorption (Lee et al 1991; Swenson & Curatolo 1992).

The ocular route is another possible route for systemic delivery of peptide drugs, since the mucous membrane in the conjunctiva and nasal cavity are permeable to macromolecular compounds (Chiou 1991; Zhou & Li Wan Po 1991b). Small polypeptides such as thyrotropin-releasing hormone (mol. wt 360) and luteinizing hormone-releasing hormone (mol. wt 1200) are absorbed systemically by the ocular route (Chiou & Chuang 1988), and the amount of these polypeptides absorbed into the eyeball itself is almost negligible. However, the amount of insulin (mol. wt 6000) absorbed is small in comparison with the absorption of small polypeptides (Chiou 1991).

Chiou & Chuang (1989) and Yamamoto et al (1989) demonstrated that insulin was absorbed and showed a hypoglycaemic response after its instillation with several absorption enhancers. Camber & Edman (1987) reported that some ophthalmic preservatives showed an enhancing effect on the penetration of ophthalmic drugs through ocular membranes.

In this study, the effects of absorption promoters and preservatives on systemic delivery of insulin by the ocular route were investigated, by comparing the hypoglycaemic response after insulin instillation in albino rabbits.

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Materials and Methods

Materials

Monocomponent porcine insulin (26·2 units mg^{-1}, mol. wt 6000) was kindly supplied by Novo Nordisk (Gentofte, Denmark). Saponin was purchased from Merck (Darmstadt, Germany). 2-Phenylethanol, sorbic acid, methylparaben and propylparaben were purchased from Sigma Chemical Co. (St Louis, MO). Paraben was used as a mixture of methylparaben and propylparaben (13:7 w/w). EDTA was purchased from Hayashi Pure Chemicals Co. Ltd (Tokyo, Japan). Benzyl alcohol, benzalkonium chloride, *o*-toluidine and all other chemicals were of reagent grade obtained from Nacalai Tesque Inc. (Kyoto, Japan). Phosphate-buffered saline (pH 7·4) was prepared by mixing isotonic phosphate buffer with an equal volume of 0·9% NaCl.

Animals

Male Nippon albino rabbits, 2·0–3·0 kg, 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 before use but had free access to water.

All experiments in the present study conformed to the Guideline for Animal Experimentation in Nagasaki University.

In-vivo experiment

Non-anaesthetized rabbits were kept in a prone position on a wooden plate. Twenty-five microlitres of insulin formulation (2, 5 or 10 units/rabbit) containing absorption promoters (0·05, 0·1 or 0·5% saponin or EDTA) or preservatives was carefully applied with a micropipette (Gilson Medical Electronics, Villiers-le-Bel, France) in the lower conjunctival sac of the eye. The preservative concentration was within the range of clinical use in commercial ophthalmic droplets. At 7, 15, 30, 60, 90, 120, 180, 240 and 300 min after application of the insulin formulation, blood samples were withdrawn via the marginal ear vein and the serum glucose concentrations were determined. In the experiment to investigate the interval between insulin and

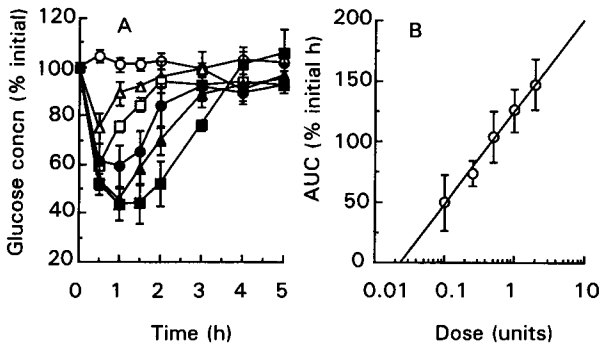

FIG. 1. A. Change in serum glucose after intravenous administration of insulin. ○ Control, △ 0·1, □ 0·25, ● 0·5, ▲ 1·0 and ■ 2·0 units. B. Plot of AUC for serum glucose against insulin dose ($y = 77{\cdot}2 \times +124{\cdot}8$, $r = 0{\cdot}997$). Each value represents the average of at least four experiments ± s.e.m.

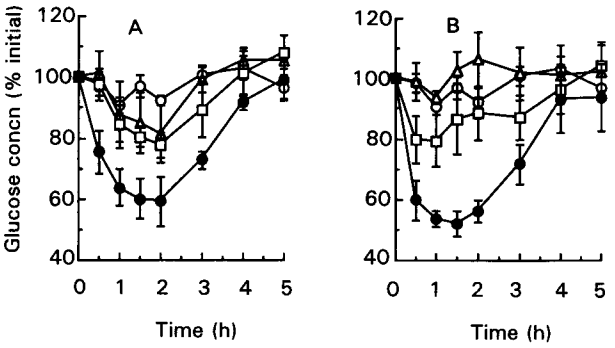

FIG. 2. A. Change in serum glucose after instillation of 10 units insulin with EDTA. ○ Control, △ 0·05, □ 0·1, ● 0·5% EDTA. B. Change in serum glucose after instillation of 10 units insulin with saponin. ○ Control, △ 0·05, □ 0·1, ● 0·5% saponin. Each value represents the average of four experiments ± s.e.m.

preservative administration, the insulin formulation without preservatives was instilled 1, 5 or 30 min after instillation of 0·01% benzalkonium chloride.

Intravenous injection (25 μL) of insulin (0·1, 0·25, 0·5, 1 or 2 units/rabbit) was carried out via the marginal ear vein. Nasal administration of the insulin formulation was carried out using a spray into the nasal cavity (3·5 cm depth insertion of polyethylene tubing SP67, Natsume Seisakusho Co. Ltd, Tokyo, Japan). In the experiments on the administration route, the insulin formulation without preservatives was administered nasally 1 min after instillation of 0·01% benzalkonium chloride.

An insulin instillation experiment using a rabbit with the drainage duct plugged (Patton & Robinson 1976) was also carried out. The punctum of rabbit eye for tear drainage was closed by insertion of polyethylene tubing (SP45, Natsume Seisakusho Co. Ltd). A filter paper (5 mm × 5 cm; No. 41, Whatman International Ltd, Maidstone, UK) was gently inserted on the punctum for tear drainage immediately after instillation of the insulin formulation. An excess of tear fluid was completely collected with the filter paper during the experiment. In the rabbit with the drainage duct plugged, conjunctival absorption, rather than nasal absorption, contributed to the hypoglycaemic response.

Analysis

Serum glucose was measured spectrophotometrically (595 nm, UV-160A UV-vis spectrophotometer, Shimadzu Co. Ltd, Kyoto, Japan) after staining by the *o*-toluidine-boric acid method (Glucose Test Kit, Wako Pure Chemical Industries Ltd, Osaka, Japan). The hypoglycaemic response of insulin was calculated as the minimum serum glucose concentration and the area under the concentration change-time curve from 0 to 5 h (AUC) calculated by the trapezoidal rule.

All data were analysed by Student's *t*-test. $P < 0{\cdot}05$ was considered significant.

Results

Intravenous injection of insulin and instillation of insulin with absorption promoter

The serum glucose concentration decreased according to the dose of insulin administered intravenously (Fig. 1A). There was a linear relationship between the logarithmic value of the insulin dose and the AUC value (Fig. 1B) and between the logarithmic value of the insulin dose and the minimum glucose concentration.

Fig. 2 shows the serum glucose concentration after an instillation of 10 units insulin with various concentrations of absorption promoters. The instillation of insulin in the absence of promoter (control) did not show a significant decrease in the glucose level. Instilled insulin with EDTA and saponin showed a hypoglycaemic response which was dependent on the concentration of EDTA or saponin. Table 1 shows bioavailability data calculated from the AUC.

Insulin absorption after instillation with preservative

Fig. 3 shows the serum glucose concentration after instillation of insulin with ophthalmic preservatives. Benzalkonium

Table 1. Hypoglycaemic response and bioavailability after instillation of insulin (10 units) with absorption promoters.

Promoter	n	Minimum glucose concn (% of initial) ± s.e.m.	AUC (% of initial h)	Bioavailability (%)
EDTA 0·05%	4	79·1 ± 8·6	26·1 ± 17·8	0·5
0·1%	4	73·4 ± 2·8**	47·9 ± 19·9	1·0
0·5%	4	56·0 ± 6·1**	124·6 ± 11·9**	10·0
Saponin 0·05%	4	87·9 ± 4·6	2·4 ± 31·7	0·3
0·1%	4	74·0 ± 7·4*	59·7 ± 34·5	1·4
0·5%	4	50·5 ± 3·6**	147·0 ± 27·7**	19·4

Bioavailability was calculated from an average of AUC. *$P < 0{\cdot}05$, **$P < 0{\cdot}01$ compared with insulin without promoter.

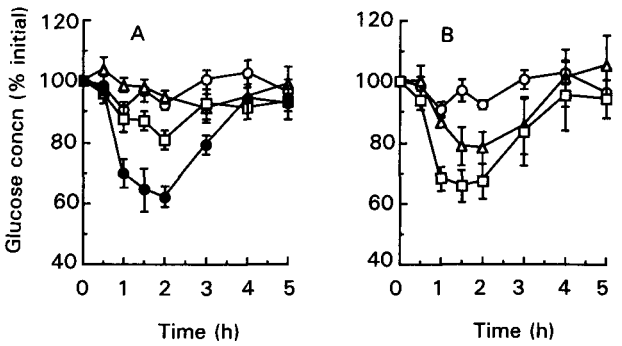

FIG. 3. Change in serum glucose after instillation of 10 units of insulin with preservatives. A. ○ Control, △ 0·25% sorbic acid, □ 0·5% 2-phenylethanol, ● 0·01% benzalkonium chloride, B. ○ Control, △ 0·5% benzyl alcohol, □ 0·04% paraben. Each value represents the average of at least four experiments ± s.e.m.

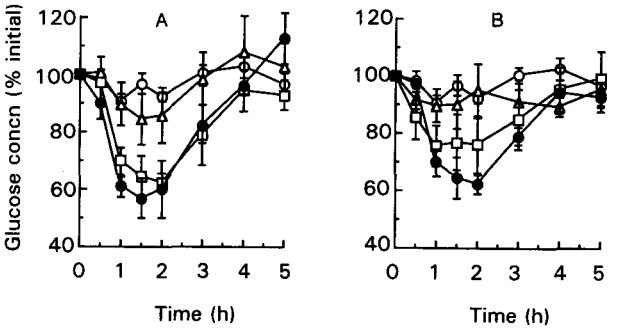

FIG. 4. Effect of insulin dose and benzalkonium chloride concentration on the change in serum glucose produced by insulin. A. 10 units insulin with benzalkonium chloride. ○ Control, △ 0·005, □ 0·01, ● 0·05% benzalkonium chloride. B. 0·01% Benzalkonium chloride with insulin. ○ Control, △ 2, □ 5, ● 10 units insulin. Each value represents the average of at least four experiments ± s.e.m.

chloride and paraben showed high promoting effects on the insulin hypoglycaemic response. Table 2 shows the calculated bioavailability.

Factors influencing the effect of benzalkonium chloride

The enhancing effect of benzalkonium chloride was investigated under various conditions. Serum glucose concentration was dependent on both benzalkonium chloride concentration and insulin dose in the ophthalmic formulation (Fig. 4). The hypoglycaemic response of instilled insulin was determined at various intervals after benzalkonium chloride instillation (Table 3). The bioavailability of instilled insulin decreased to 20·4% at 30 min after benzalkonium chloride instillation in comparison with that when insulin and benzalkonium chloride were instilled together.

Fig. 5 shows the serum glucose concentration after intranasal administration of insulin. Intranasal administration of insulin alone showed little effect on glucose concentration, but the addition of benzalkonium chloride to the insulin formulation induced the insulin hypoglycaemic response. After instillation of benzalkonium chloride alone, insulin administered intranasally also decreased serum glucose. Fig. 6 shows the serum glucose concentration after instillation of insulin with benzalkonium chloride in the rabbit with the drainage duct plugged. The instillation showed only a little effect on decrease in serum glucose.

Discussion

Recently, it has been claimed that a painless, simple and practical method has been designed for the systemic delivery of insulin by the ocular route (Chiou & Chuang 1989; Yamamoto et al 1989), although long-term efficacy and safety have yet to be defined. Saponin was found to be the best enhancer of insulin absorption via this route although saponin causes eye irritation at the 0·5% level (Chiou & Chuang 1989). EDTA, fusidic acid, bile salts and some surfactants also showed an enhancing effect on insulin absorption (Grass et al 1985; Marsh & Maurice 1971; Green 1993). EDTA, a known calcium chelator, was shown to penetrate the cornea, conjunctiva, and iris/ciliary body from a topically applied dose (Grass et al 1985). Bile salts and surfactants caused irritation of the eye and the nasal mucosa (Green 1993; Merkus et al 1993). Instillation of insulin with saponin and EDTA decreased serum glucose (Fig. 2) and the bioavailability of insulin showed dependency on the concentration of saponin or EDTA.

There are some reports on the effect of ophthalmic preservatives on corneal irritability and enhancement of drug penetration into the eye (Burstein 1984; Green 1993). Camber & Edman (1987) demonstrated that some preservatives significantly increased the corneal permeability of pilocarpine and dexamethasone. Some ophthalmic preservatives enlarged intercellular spaces and disrupted cytoplasmic membranes in superficial cells (Green & Tønjum 1971; Tønjum 1975). Preservative and bile salts were also reported to increase conjunctival permeability of β-blockers (Ashton et al 1991). These ophthalmic preservatives may be considered safe as absorption promoters for insulin delivery since they have been used as instilled droplets for clinical ophthalmic disease over long periods. Therefore, the effect of ophthalmic preservatives on insulin absorption through

Table 2. Hypoglycaemic response and bioavailability after instillation of insulin (10 units) with preservatives.

Preservative	n	Minimum glucose concn (% of initial) ± s.e.m.	AUC (% of initial h)	Bioavailability (%)
Benzalkonium chloride (0·01%)	6	58·0 ± 2·6**	100·8 ± 7·1**	4·9
Paraben (0·04%)	6	60·6 ± 4·8**	94·0 ± 30·1*	4·0
2-Phenylethanol (0·5%)	4	79·8 ± 2·5*	58·2 ± 12·5**	1·4
Benzyl alcohol (0·5%)	4	76·3 ± 5·3*	51·7 ± 21·6	1·1
Sorbic acid (0·25%)	4	87·3 ± 3·4	25·9 ± 4·2	0·5

Bioavailability was calculated from an average of AUC. *$P < 0·05$, **$P < 0·01$ compared with insulin without preservative.

Table 3. Effect of interval between instillations of 0·01% benzalkonium chloride and insulin (10 units) on hypoglycaemic response and bioavailability.

Interval (min)	n	Minimum glucose concn (% of initial) ± s.e.m.	AUC (% of initial h)	Bioavailability (%)
0	6	58·0 ± 2·6**	100·8 ± 7·1**	4·9
1	4	63·5 ± 5·9**	82·4 ± 12·1**	2·8
5	6	68·9 ± 7·0*	69·7 ± 24·7*	1·9
30	5	73·0 ± 5·9*	47·7 ± 20·5	1·0

Bioavailability was calculated from an average of AUC. *$P < 0.05$, **$P < 0.01$ compared with insulin without preservative.

the ocular route was studied by measuring the hypoglycaemic response. Insulin instilled with these ophthalmic preservatives, especially benzalkonium chloride and paraben, showed a significant hypoglycaemic response (Fig. 3, Table 2).

In ophthalmic formulations, benzalkonium chloride is widely used as a preservative because of its bactericidal efficacy and low toxicity. Instillation of 0·01% benzalkonium chloride (100 μL) showed no irritation according to the Draize score (Griffith et al 1980; Draize et al 1944), and at 0·004 to 0·01% had no influence on epithelial aerobic metabolism (Burton & Hill 1981). Corneal exposure to multiple drops of benzalkonium chloride leads to epithelial accumulation but no penetration into the anterior chamber (Green 1993).

Benzalkonium chloride was demonstrated to induce dose-dependent morphological changes in the epithelium, causing partial loss of surface microvilli (Green & Tønjum 1971; Tønjum 1975). The promoting effect of benzalkonium chloride depended on concentrations both of benzalkonium chloride and insulin in the formulation. Green & Tønjum (1975) also reported that a physiological change—the inhibition of the electrical potential difference across the corneal epithelium—occurred within 1 min and disappeared at 2 h after 0·02% benzalkonium chloride. An increase of interval between benzalkonium chloride instillation and insulin instillation decreased the hypoglycaemic response. This decrease is thought to reflect the recovery of mucosal membrane from the enhancing effect. Dilution and washing out of benzalkonium chloride by tear fluid turnover may contribute to this reversibility.

Upon instillation of an ophthalmic drug, most of the instilled amount is rapidly eliminated from the precorneal area due to drainage by the naso-lacrimal duct and is readily absorbed through conjunctival and nasal membranes into the systemic circulation (Chrai et al 1973; Himmelstein et al 1978). Instilled benzalkonium chloride also enhanced systemic absorption of insulin administered nasally.

The instillation of insulin with benzalkonium chloride showed a slight hypoglycaemic response in the rabbit with drainage duct plugged; where the insulin is absorbed only through the conjunctival membrane. These results indicate that the nasal membrane in comparison with conjunctival membrane is the predominant contributor to the systemic delivery of insulin instilled with benzalkonium chloride.

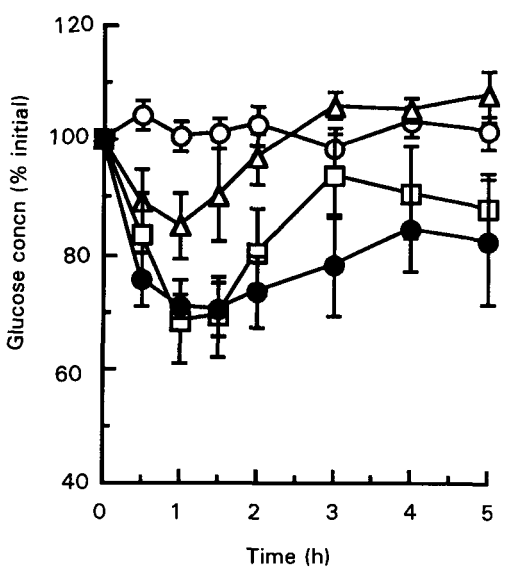

FIG. 5. Change in serum glucose after intranasal administration of insulin with benzalkonium chloride. ○ Phosphate-buffered saline, △ 10 units insulin alone, □ 10 units insulin with 0·01% benzalkonium chloride, ● 10 units insulin after 0·01% benzalkonium chloride instillation. Each value represents the average of at least four experiments ± s.e.m.

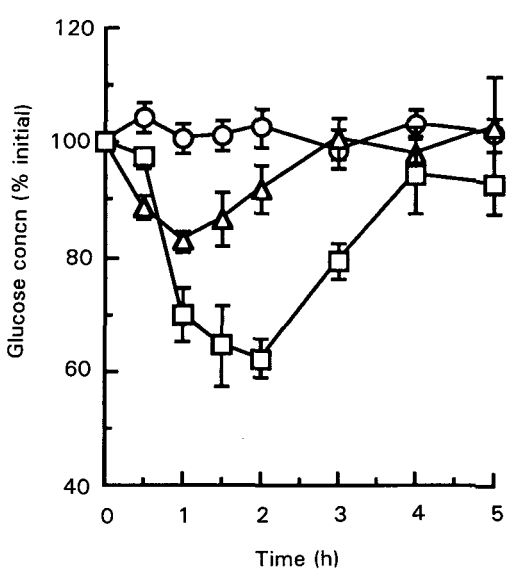

FIG. 6. Effect of plugging the drainage duct in rabbit on the change in serum glucose after administration of 10 units insulin with 0·01% benzalkonium chloride. ○ Phosphate-buffered saline, △ drainage-plugged rabbit, □ intact rabbit. Each value represents the average of at least four experiments ± s.e.m.

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