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International Journal of Pharmaceutics 117 (1995) 129–137

international journal of pharmaceutics

Research Papers

The effect of soybean-derived sterol and its glucoside as an enhancer of nasal absorption of insulin in rabbits in vitro and in vivo

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Received 9 May 1994; revised 25 July 1994; accepted 2 September 1994

Abstract

The effect of a soybean-derived sterol mixture (SS) and its glucoside mixture (SG) as an enhancer for the nasal bioavailability of insulin was studied in rabbits. SG possesses excellent properties in a peanut oil suspension as an enhancer and is superior to the known absorption enhancers. The absorption-enhancing effect of SG was increased by the combined use of peanut oil and L-glutamic acid. When SG was used as an absorption enhancer for insulin in peanut oil, the bioavailability amounted to 11.6%, and was increased to 13.3% on the addition of L-glutamic acid. The addition of calcium led to a significant reduction in the extent of absorption enhancement of insulin by SG; therefore, it was suggested that chelation may be a possible mechanism for the enhancement of the absorption of SG. SG appears to be more effective in the enhancement of insulin absorption in peanut oil than SS, since SG shows an enhancement effect at lower concentrations than SS. To elucidate the contribution of both SG and SS in peanut oil to enhancement, insulin permeation through an artificial membrane and the nasal mucosa was investigated in vitro.

Keywords: Nasal absorption; Insulin; Peanut oil; Sterol; Sterol glucoside; L-Glutamic acid

1. Introduction

Interest in the drug delivery of peptides and proteins through nonparenteral routes has recently been increasing. Presently, most peptides and proteins are not effective when administered orally and so must be administered by injections. A nonparenteral way of administration is highly desirable because injections are poorly accepted by most patients. Insulin is the polypeptide that has received the most attention with this method. The nonparenteral routes that have been investigated for insulin delivery include the nasal, buccal, rectal, vaginal, pulmonary and transdermal routes. The results to date indicate that the nasal route is considered to be the most promising (Aungst et al., 1988).

In an attempt to increase the bioavailability of insulin after nasal administration, a number of

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SSDI 0378-5173(94)00293-2

enhancers have been investigated. Among them, bile salts (Hirai et al., 1981a; Uchida et al., 1991), sodium taurodihydrofusidate (Lee et al., 1991) and didecanoyl-L-α-phosphatidylcholine (Bechgaard et al., 1993) are effective as enhancers for nasal absorption of insulin but are also reported to alter nasal morphology (Gizurarson et al., 1990; Hermens et al., 1990).

We have already reported that peanut oil is an effective vehicle for nasal absorption of insulin and that the bioavailability for peanut oil was 6.0% (Yamamoto et al., 1994). This bioavailability needs to be increased. Therefore, a soybean-derived sterol mixture (SS) and its glucoside mixture (SG) were used as enhancers in various vehicles. SG, an abundant plant sterylglucoside, exhibits an inhibitory effect on vascular permeability, antiulcerogenic and hemostatic effects. To date, neither SG nor SS has been reported as an enhancer. They are natural products and the histological change is expected to be small.

The purpose of this study was to evaluate the bioavailability of insulin in oil suspensions using SG and SS as enhancers. Also, the mechanism of enhancement of insulin absorption by SG was investigated by an insulin permeation study through the nasal mucosa.

2. Materials and methods

2.1. Chemicals

Bovine insulin (24.4 IU/mg) was purchased from Sigma Chemical Co. (USA). Peanut oil and liquid paraffin were of guaranteed reagent grade from the Japan Pharmaceutical Codex XII (1991). L-Glutamic acid was purchased from Wako Pure Chemical Ind. Ltd (Japan). All other chemicals were obtained from commercial sources and were of analytical reagent grade. SG and SS were kindly supplied by Ryukakusan Co., Ltd. SG is a mixture of the glucoside of β-sitosterol (49.9%), campesterol (29.1%), stigmasterol (13.8%), and brassicasterol (7.2%). SS was obtained by hydrolysis of the glucoside bond of SG, i.e., SS is the aglycon of SG (Muramatsu et al., 1994).

2.2. Preparations of dosage form

SG and SS were passed through a 200 mesh sieve. The insulin suspension for the liquid dosage form was prepared by suspending 40 mg of the insulin that passed through the 200 mesh sieve with or without SG and SS in 10 ml of peanut oil, liquid paraffin and a pH 7.31 phosphate-buffered saline solution (PBS) with stirring (97.6 IU/ml). The powder sample for the powder dosage form was prepared by mixing 1 mg of insulin and 9 mg of SG or SS for one dose (10 IU/kg).

2.3. Administration methods

Preparations were administered to female Japanese white rabbits weighing 2.5–3.0 kg (Saitama Experimental Animal Supply Co.). A polyethylene tube with a diameter of 1.05 mm and a length of 10.0 cm was installed at the top of a syringe and inserted into the nose of a rabbit. A 250 μl dose of the liquid form or 10 mg of the powder dosage form was loaded into the syringe and administered through the tube into the nasal cavity of a rabbit (e.g., 10 IU/kg). In the powder dosage form, spraying was effected by the attachment of a sprayer (rubber bulb with reservoir) as previously reported (Maitani et al., 1989).

2.4. Analysis of insulin

1 ml of blood sample was collected serially from the ear vein. Plasma was separated after centrifugation for 2 min at 3000 rpm. The plasma glucose was assayed by the glucose oxidase method using the Wako glucose B-test (Wako Pure Chemicals Co. Ltd); glucose assay. The insulin concentration in the plasma was determined using the EIA method employing the Insulin EIA insulin assay kit (Dainabot Co. Ltd).

2.5. Bioavailability

The area under the curves of the insulin concentration-time (AUC) and the total decrease of the glucose reduction-time ($D\%$) from 0 to 6 h after administration of the insulin were calculated using the trapezoidal method, respectively.

The bioavailability is expressed on the basis of AUC and $D\%$. It was calculated according to the following equation in which $AUC_{i.v.}$ and $D_{i.v.}\%$ were determined, respectively, after i.v. administration of 0.5 IU/kg of insulin:

bioavailability

$$= (AUC/dose)/(AUC_{i.v.}/dose_{i.v.})$$
$$= (D\%/dose)/(D_{i.v.}\%/dose_{i.v.}) \quad (1)$$

The bioavailability by insulin and glucose assays shows a linear relationship, as previously reported (Yamamoto et al., 1994).

2.6. Insulin permeation study with SG and SS in PBS and peanut oil in vitro

To prepare the artificial membrane, a type CN membrane filter (SM 11378, Sartorius Co. Ltd) was soaked with a mixture of *n*-caprylic acid and lauryl alcohol (4:0.92) at 30°C for 5 min. The nasal mucosae were detached from the rabbit according to a method used in a previous study (Maitani et al., 1991). They were mounted in two-chamber cells at 37°C, respectively, as reported by Maitani et al. (1992). 6 ml of the insulin PBS and peanut oil suspension (4 mg insulin/ml, C_d) with 1% (w/v) of SG and SS was used for the donor solution and PBS for the receiver solution. The insulin concentration in the receiver (C_r) was determined using the EIA method.

The C_r values were plotted as a function of time (t) from 0 to 3 h. The permeability coefficient (K) is calculated according to Eq. 2:

$$J = (dC_r/dt) \cdot V/S = K \cdot C_d \quad (2)$$

where J is the flux, S denotes the available area of the nasal mucosa for permeation (0.503 cm^2), and V is the volume of the receiver solution (6 ml). The C_d value is the solubility of insulin in PBS or peanut oil; 0.031 and 0.00353 mg/ml, respectively (Yamamoto et al., 1994).

Statistical analysis was accomplished using ANOVA. A p value of 0.05 was considered to be significant.

3. Results and discussion

3.1. Comparison of SG and SS in the absorption of insulin

SG is expected to show an enhancement effect, since it liquidizes the phospholipid in liposomes (Muramatsu et al., 1994). To increase the bioavailability of insulin, SG and SS were used as enhancers.

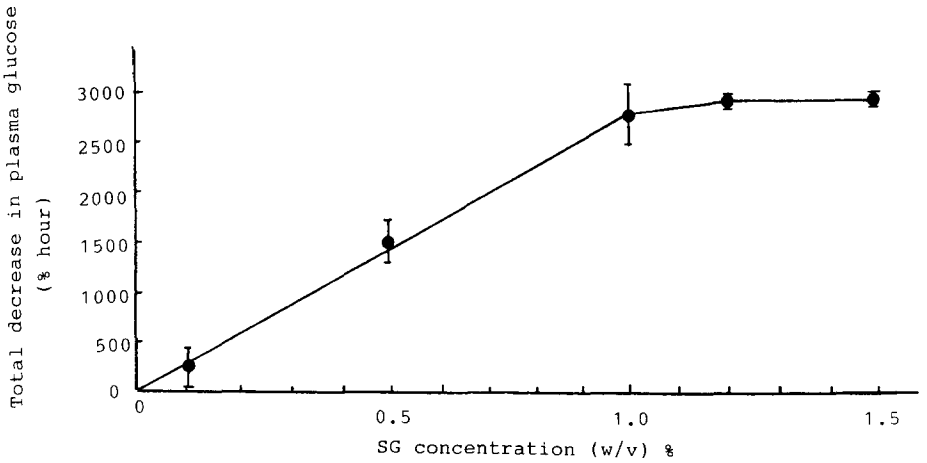

Fig. 1. Effect of the concentration of SG in an insulin suspension in phosphate-buffered saline on total decrease in plasma glucose. The curve was determined by the linear least-squares method using experimental mean values (10 IU/kg). Each value represents the mean ± S.D. ($n = 3$).

The effect of the concentration of SG for insulin (10 IU/kg) in PBS on the total decrease in plasma glucose was examined as shown in Fig. 1. The glucose level decreased as the concentration of SG increased, reaching a plateau at 1.0% (w/v) . This may be because the surface of the nasal mucosa is limited and 1% (w/v) of SG is the saturation amount. Therefore, 1% (w/v) of SG was used for comparing the vehicle in the liquid dosage form.

The effect of SG in liquid and powder dosage forms as an enhancer was compared to that of SS. The bioavailabilities of the liquid dosage form in PBS, liquid paraffin and peanut oil, and the powder dosage form (10 IU/kg) are demonstrated in Fig. 2.

Peanut oil and liquid paraffin were used as a vehicle as they have an enhancing effect on the absorption of insulin (Yamamoto et al., 1994). The bioavailability of insulin was increased by adding either SS or SG to the vehicles, and SG was significantly more effective than SS, except for the powder dosage form. The highest bioavailability by glucose assay with SG was in peanut oil (10.4%).

The powder dosage form has been reported to be more effective than the liquid dosage form of peptides (Nagai et al., 1984; Farraji et al., 1990). Schipper et al. (1993) reported that the improved

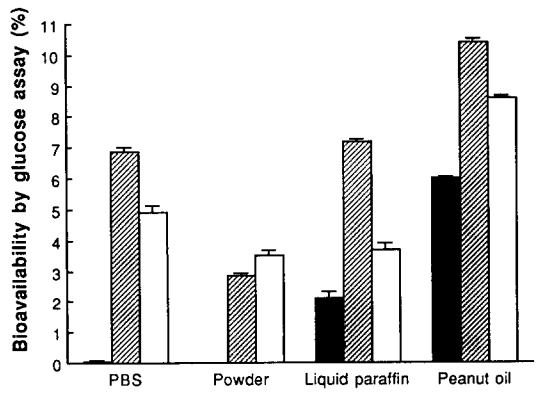

Fig. 2. Bioavailability by glucose assay after nasal administration of the insulin suspension in phosphate-buffered saline, liquid paraffin and peanut oil with 1% (w/v) of SG and SS and insulin powder dosage forms with SG and SS as a vehicle (10 IU/kg). Each value represents the mean ± S.D. ($n = 3$). * Not detected, (filled bars) control, (hatched bars) SG, (empty bars) SS.

insulin absorption from the powder formulation may be due to a different distribution in the nose which might influence drug absorption. The powder dosage form of insulin appears to be less effective on enhancement compared with the liquid dosage form. SS in the powder dosage form shows significantly greater bioavailability by glu-

Table 1
D%, AUC and bioavailability after nasal administration of insulin in liquid paraffin and peanut oil containing the SG or SS in rabbits (10 IU/kg)

Enhancer	*D*% glucose assay (% h)	AUC_{0-6} insulin assay (μIU ml^{-1} h)	Bioavailability (%)	
			Glucose assay	Insulin assay
i.v. (0.5 IU/kg)	1899.6 ± 7.4	87.9 ± 9.1		
Liquid paraffin systems				
None	800.5 ± 80.3	40.6 ± 6.4	2.1 ± 0.21	2.3 ± 0.71
1.0% SG	2727.0 ± 33.5	146.2 ± 3.6	7.2 ± 0.09	8.3 ± 2.22
1.0% SS	1404.5 ± 72.2	75.5 ± 2.6	3.7 ± 0.19	4.3 ± 1.15
Peanut oil sytems				
None	2264.5 ± 97.1	121.8 ± 6.5	6.0 ± 0.04	6.9 ± 1.88
1.0% SG	3947.5 ± 47.6	204.8 ± 1.5	10.4 ± 0.13 [a]	11.6 ± 3.10
3.5% SG	3516.1 ± 78.2	195.8 ± 2.6	9.3 ± 0.21	11.1 ± 2.97
1.0% SS	3257.5 ± 30.5	165.4 ± 3.9	8.6 ± 0.09	9.4 ± 2.52
3.5% SS	3958.0 ± 40.1	206.7 ± 3.8	10.4 ± 0.21 [b]	11.8 ± 3.14

Each value represents the mean ± S.D. ($n = 3$–4).
[a,b] Values are not significant ($p > 0.05$).

cose assay than SG. The factors contributing to the effectiveness of enhancers might be different between the powder and liquid dosage forms. This may be because the interaction of vehicle with water in the mucus and the effective concentration of SG and SS are different.

3.2. Effect of concentration of SG and SS in peanut oil and liquid paraffin on bioavailability

The AUC and bioavailability after nasal administration of insulin (10 IU/kg) in liquid paraffin and peanut oil containing SG or SS and the AUC after i.v. administration of 0.5 IU/kg of insulin in rabbits are summarized in Table 1. The data indicate that 1% (w/v) SG and 3.5% (w/v) SS in peanut oils show significantly higher AUC values and bioavailability and that the differences in bioavailability of either one are not significant. This difference in concentration of SG and SS in the most effective enhancement might be due to a difference in the solubility of SG and SS in peanut oil. SS is more soluble than SG in peanut oil. Because SG functions as an enhancer in peanut oil at lower concentrations than SS, SG might induce less morphological change in the nasal mucosae; therefore, SG appears to be a more effective enhancer than SS.

To increase the bioavailability, L-glutamic acid was used to improve the moisture content in the mucus as amino acids have been used in cosmetic products for this purpose. Aluminum stearate, which is known as a thixotropic agent in oleaginous suspensions, was added to increase the viscosity of peanut oil in order to prolong the residence time in the nasal cavity. The data on the AUC and bioavailability of insulin in peanut oil containing 1.0% (w/v) SG and L-glutamic acid or aluminum stearate are summarized in Table 2. L-Glutamic acid (0.5% (w/v)) added to 1.0% (w/v) SG had a significant effect on the bioavailability compared with 1.0% (w/v) SG. The reason why the addition of 1% L-glutamic acid decreased the bioavailability as compared with the addition of 0.5% might reside in the fact that the interaction of peanut oil with L-glutamic acid causes the optimal concentration of L-glutamic acid for bioavailability to be around 0.5%. 1.0% (w/v) aluminum stearate added to 1.0% (w/v) SG decreased the bioavailability. This may be due to the fact that insulin could not be released from such a highly viscous vehicle.

3.3. Duration of effect of SG

Enhancers show the effect of enhancement together with inducing irreversible mucosal damage. The histological change induced by SG was examined as 8 mg of SG was administered along with 2 mg of insulin (20 IU/kg). SG appeared to affect the nasal mucosa very rapidly after administration, since the peak of glucose levels occurred 1 h after administration. Pre-administration of SG before insulin administration led to a significant reduction in the AUC values, indicating that the permeable state disappeared immediately at 60 min after administration of SG as shown in Table 3. The enhancement effect of SG is readily restored to the normal level. SG may be considered to be safe on the nasal mucosa.

Table 2
D%, AUC and bioavailability after the nasal administration of insulin with 1.0% (w/v) SG in peanut oil containing additives in rabbits (10 IU/kg)

Additives	*D*% glucose assay (% h)	AUC_{0-6} insulin assay (μIU ml^{-1} h)	Bioavailability (%)	
			Glucose assay	Insulin assay
1.0% SG	3947.5 ± 47.6	204.8 ± 1.5	10.4 ± 0.13 [a]	11.6 ± 3.10
0.5% glutamic acid (+1.0% SG)	4372.0 ± 46.0	233.5 ± 2.9	11.5 ± 0.13 [a]	13.3 ± 3.54
1.0% glutamic acid (+1.0% SG)	1115.0 ± 32.2	55.8 ± 1.2	2.9 ± 0.09	3.2 ± 0.85
1.0% Al stearate (+1.0% SG)	494.0 ± 20.0	61.2 ± 3.2	3.0 ± 0.08	3.5 ± 0.95

Each value represents the mean ± S.D. (n = 3–4).
[a] $p < 0.05$.

Table 3
Duration of the effect of SG in the powder dosage form on the bioavailability by glucose assay

Time (min)	$D\%$ (% h)	Bioavailability (%)
0	1504.0±93.0	2.0±0.12
30	1067.0±57.2	1.4±0.08
60	284.0±75.2	0.4±0.10

SG (8 mg) was administered at 0, 30, or 60 min prior to the administration of insulin (2 mg, 20 IU/kg). Each value represents the mean±S.D. ($n = 3$).

3.4. Effect of chelation of SG and L-glutamic acid on glucose level

An enhancer takes on many roles in increasing the permeation of insulin through the nasal mucosa. It reduces the viscosity of mucus, inhibits proteolytic enzymes and alters the membrane structure (Hirai et al., 1981b). One of the alterations in membrane structure is due to the chelation that removes calcium ion from the tight junctions in cell-cell binding found with EDTA (Bhat et al., 1993). We investigated the glucose level after pre-administration of calcium ion and then after administration of insulin to examine the effect of SG and L-glutamic acid in PBS and peanut oil.

Fig. 3 and 4 show the change in the glucose concentration after administration of insulin (10 IU/kg) in PBS with 1% (w/v) SG or with 1% (w/v) SG and 0.5% (w/v) L-glutamic acid 5 min after the pre-administration of 0.05 M calcium chloride, respectively. The presence of 0.05 M calcium chloride led to a significant reduction in the extent of absorption enhancement of insulin by SG. SG is supposed to promote the chelation of calcium ion. Based on this result, the observed absorption-enhancing effect of SG is probably due to alteration of the membrane integrity as a result of chelation. L-Glutamic acid may have had no effect on chelation of the calcium ion because the glucose level did not decrease significantly after pre-administration of calcium chloride.

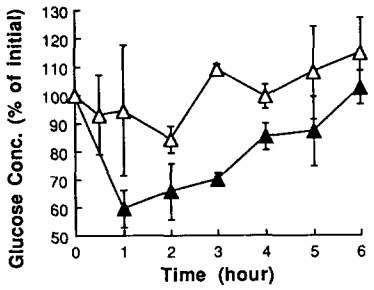

Fig. 3. Plasma glucose level after nasal administration of the insulin suspension in phosphate-buffered saline with 1% (w/v) of SG 5 min after pre-administration of 0.05 M $CaCl_2$ (10 IU/kg). Each value represents the mean±S.D. ($n = 3$). (▲) No pre-administration; (△) pre-administration of $CaCl_2$.

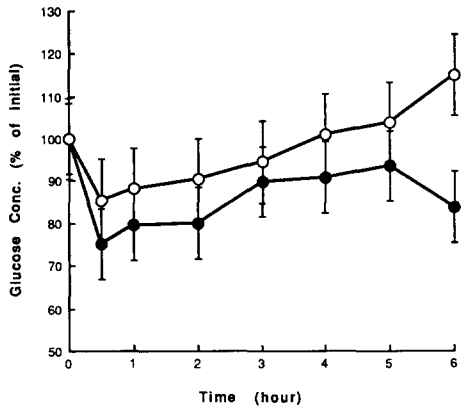

Fig. 4. Plasma glucose level after nasal administration of the insulin suspension in phosphate-buffered saline with 1% (w/v) of SG and 0.5% (w/v) L-glutamic acid 5 min after pre-administration of 0.05 M $CaCl_2$ (10 IU/kg). Each value represents the mean±S.D. ($n = 3$). (●) No pre-administration; (○) pre-administration of $CaCl_2$.

The change in plasma glucose concentration after administration of insulin (10 IU/kg) in peanut oil with 1% (w/v) SG and 0.5% L-glutamic acid 5 min after the pre-administration of 0.05 M calcium chloride is demonstrated in Fig. 5. The bioavailability from glucose assay decreased significantly after pre-administration of calcium chloride. The chelation of SG appeared to increase to a greater extent in peanut oil than in PBS. This may be because peanut oil also exerts a chelation action and/or a synergistic effect with L-glutamic acid, since the following in vitro experiment showed that peanut oil and SG have no special interaction.

3.5. Mechanism of enhancement of SG and SS for in vitro permeation of insulin in peanut oil

In vitro permeation of insulin through an artificial membrane and nasal mucosa was investigated to elucidate the absorption-enhancing mechanism of SG and SS in peanut oil compared with PBS. Comparing the ratio of the K values in the nasal mucosa with those in the artificial membrane, the stability of insulin by SG and SS and the effect of the enhancer on the nasal mucosa may be estimated separately. Firstly, the effect of SG and SS on the K values in PBS was compared with that in peanut oil to examine the stability of insulin by SG and SS.

Table 4 summarizes the K values in PBS and peanut oil with and without SG and SS calculated by linear regression using Eq. 2 which was reported previously in an in vitro permeation study (Yamamoto et al., 1994). The K value of SG in PBS was almost 2-fold greater than that of SS through the artificial membrane and the nasal mucosa, whereas the K value of SG in peanut oil was only slightly higher than that of SS. SG appears to stabilize insulin in PBS but not in peanut oil. This result may correspond to the in vivo experiment where SG displayed a more effective enhancement in PBS than in peanut oil compared with SS since the ratio of bioavailability with SG to that with SS in PBS is higher than that in peanut oil (Fig. 2). Therefore, the in vitro experiment may be reflected in the in vivo experiment regardless of washing out the mucus layer and hydrating the nasal mucosa.

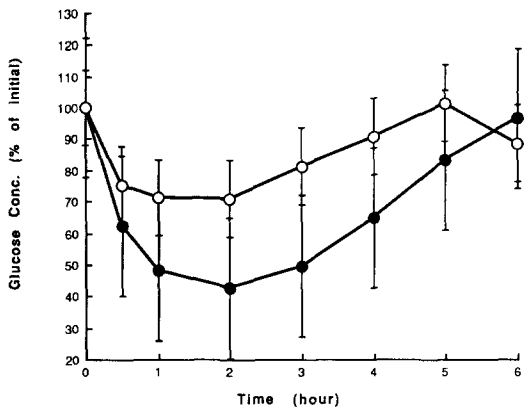

Fig. 5. Plasma glucose level after nasal administration of insulin suspension in peanut oil with 1% (w/v) of the SG and 0.5% (w/v) L-glutamic acid 5 min after pre-administration of 0.05 M $CaCl_2$ (10 IU/kg). Each value represents the mean ± S.D. ($n = 3$). (●) No pre-administration; (○) pre-administration of $CaCl_2$.

Secondly, the ratio of the K value of the nasal mucosa to that of the artificial membrane of SG was compared with that of SS to examine the effect of SG and SS on the nasal mucosa. The K value ratio of SG and SS in PBS was not significantly different in peanut oil. This may indicate that SG and SS in peanut oil may exert almost the same effect on permeation of insulin through the nasal mucosa. The mechanism by which SG and SS enhance the absorption of drugs through the nasal mucosa appears not to be due to a synergistic effect with peanut oil. However, the K value ratios in peanut oil (1.86 for SG, 1.88 for

Table 4
Permeability coefficients (K) of insulin from PBS and peanut oil suspensions with 1.0% SG and SS through the artificial membrane and the nasal mucosa in vitro

Vehicle	Enhancer	K ($\times 10^6$) (cm/s)		Ratio of K of nasal/artificial
		Artificial membrane	Nasal mucosa	
PBS	control	0.00	0.00	–
	SG	2.89 ± 0.03	4.72 ± 0.07	1.63
	SS	1.33 ± 0.05	2.29 ± 0.16	1.72
Peanut oil	control	3.51 ± 0.01	8.55 ± 1.62	2.44
	SG	7.36 ± 0.01	13.7 ± 0.37	1.86
	SS	6.49 ± 0.37	12.2 ± 0.18	1.88

Each value represents the mean ± S.D. ($n = 3$).

SS) were slightly higher than those in PBS. This may indicate that SG and SS in peanut oil could affect the nasal mucosa more strongly than in PBS.

To date, high bioavailability of insulin after nasal absorption with 1% sodium glycocholate led to a decrease in the glucose level in blood of about 33% compared with i.v. administration in rats and dogs (Hirai et al., 1981a; Nagai et al., 1984). The nasal absorption of insulin with 1% sodium taurodihydrofusidate in powder dosage form showed a decrease in glucose level of about 37% in sheep (Lee et al., 1991). Schipper et al. (1993) reported that nasal insulin delivery with 5% dimethyl-β-cyclodextrin resulted in 13% bioavailability in rabbits. These differences simply cannot be attributed to differences in the species and in structural and physical factors of the nasal cavity. However, Corbo et al. (1990) indicated that nasal mucosae in rabbits have a structure similar to that of humans, both physiologically such as in mucus flow and enzyme activity and with respect to anatomical characteristics such as the conchae structure. The experimental study using rabbits in nasal administration of insulin might be extrapolated correctly to that in humans (Gizurarson, 1993). In our case, nasal administration of insulin with SG and L-glutamic acid in peanut oil led to 13.3% bioavailability. It also showed that SG possesses excellent properties in peanut oil as an absorption enhancer and is superior to the known absorption enhancers.

SG is a mixture of steryl-β-glucoside, and SS is the aglycon of SG. It will be very interesting to determine which component is the most effective enhancer. These results will be reported in the next paper.

It can be concluded that SG appears to be a potentially effective absorption enhancer for nasal absorption of insulin. This effect is enhanced considerably when used in combination with peanut oil and L-glutamic acid. Peanut oil appeared to have a synergistic effect with L-glutamic acid but not with SG and SS. The mechanism of absorption enhancement on addition of SG may be related to chelation by calcium ion. The histological change in SG is currently under investigation for practical use in pharmaceutical areas.

Acknowledgements

The authors are grateful to Ms Kuniko Ichikawa for assistance in the experimental work.

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