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Effects of cholic acid and other enhancers on the bioavailability of insulin from a subcutaneous site

Xin Hua Zhou and Alain Li Wan Po

Drug Delivery Research Group, The School of Pharmacy, The Queen's University of Belfast, 97 Lisburn Road, Belfast BT9 7BL (U.K.)

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Summary

The effects of aprotinin, hyaluronidase and cholic acid and its analogues on the absorption of subcutaneously injected insulin were investigated. The enhancement of insulin absorption by sodium cholate was more pronounced than those produced by the other compounds tested. The enhancing effect of cholic acid on insulin absorption was attributed not only to its inhibition of proteolytic enzymes but also to its binding to insulin. Aprotinin produced a significant increase in the effect of insulin on plasma glucose most probably due to its inhibition of the local degradation of exogenous insulin at the injection site. Hyaluronidase was ineffective in increasing subcutaneous insulin bioavailability.

Introduction

Insulin, discovered in 1922 by Banting and Best (Banting and Best, 1922), is an indispensable pancreatic peptide hormone. It is well known that the hormone is degraded by gastrointestinal protease and that it poorly penetrates the gastrointestinal mucosa due to its high molecular weight. Therefore, the hormone is currently administered on a routine basis only by intravenous, subcutaneous or intramuscular injection.

Recently, insulin has been shown to cross intestinal (Galloway and Root, 1972; Ziv et al., 1987), rectal (Kamada et al., 1981; Morimoto, et al., 1983), respiratory (Wigley et al., 1971; Shin-Wei and Sciarra, 1976), nasal (Hirai et al., 1981a, b; Moses et al., 1983) and oral mucosae (Earle, 1972) in animal models and human subjects. Compared with parenteral administration, insulin preparations for nonparenteral administration are of low efficacy and there is continued effort being made to increase the absorption of insulin administered by those routes. Improved absorption of insulin from the gastrointestinal tract is possible when admixed with non-ionic surfactants such as polyoxyethylene ethers (Shichiri et al., 1978; Hirai et al., 1981b). Bile acids which physiologically solubilize dietary fat do so through their surfactant properties. For this reason, bile acids have been widely used to promote the absorption of insulin by the rectal (Ziv et al., 1981, 1987; Aungst et al., 1988a), nasal (Hirai et al., 1981a, b; Moses

Correspondence: A. Li Wan Po, Drug Delivery Research Group, The School of Pharmacy, The Queen's University of Belfast, 97 Lisburn Road, Belfast BT9 7BL, U.K.

et al., 1981; Aungst et al., 1988a) and buccal (Aungst et al., 1988a) routes.

The protease inhibitor aprotinin has been shown to enhance insulin absorption via the intestinal wall (Ziv et al., 1987) and from a subcutaneous injection site (Berger et al., 1980; Linde and Gunnarsson, 1985; Owens et al., 1988).

Hyaluronidase is an enzyme derived from animal tissues and is present in many animal secretions including snake and insect venoms. It causes hydrolytic depolymerization of the viscous mucopolysaccharide, hyaluronic acid, which is present in the interstitial ground substance of tissues. In this way, the enzyme promotes increased diffusion of the injected drug into surrounding tissues (Meyer, 1972).

The purpose of the work described here is specifically to compare the insulin absorption enhancing effects, if any, of sodium cholate and its analogues and hyaluronidase following concomitant subcutaneous injection with a view to clarifying the mechanisms involved. More generally, the work was aimed at improving understanding of peptide drug diffusion and disposition from a subcutaneous site.

Experimental

Materials

Insulin-Actrapid 100 I.U./ml was obtained from Novo Industry (Copenhagen, Denmark). Sodium cholate, sodium deoxycholate, bile acids (oxidized), sodium glycocholate, sodium caprate, sodium caprylate and sodium laurate were purchased from Sigma (St. Louis, MO). Aprotinin (Trasyl^R) 10000 KIU/ml (Kallikrein inactivator units) was obtained from Bayer (Leverkusen, Germany), hyaluronidase (800 U/mg) was purchased from Sigma, and glucose oxidase (218.8 U/mg) and peroxidase (227 U/mg) were obtained from Boehringer (Mannheim, Germany). Insulin crystals from porcine origin were obtained from Nordisk, Gentofte, Denmark. The red blood cells were obtained from Travenol Laboratories Ltd (Thetford, Norfolk, U.K.). All other reagents used were of analytical-reagent grade. Acetonitrile for high-performance liquid chromatography was HPLC grade and was obtained from Analytical sciences (Labscan Limited, Dublin, Ireland). The Teflon membrane for the binding test (molecular weight cut-off of approx. 3500) was obtained from Spectrum Medical Industries, Inc., Los Angeles, U.S.A. An ODS-C18 column (Spherisorb, 5 μ, ODS II) was obtained from AGB Co. (MidGlam, U.K.).

Methods

Preparation of solutions

Protein precipitant was prepared as follows: 10 g sodium tungstate, 10 g disodium hydrogen phosphate and 9 g sodium chloride were dissolved in 800 ml distilled water; 125 ml of 1.0 M HCl were then added to bring the pH to 3.0. The final volume was made up to 1 l with distilled water.

Enzyme reagent was prepared by the method developed by Trinder (1969) and modified by Wideroe et al. (1983). 7500 U glucose oxidase, 2000 U peroxidase, 0.4 mmol 4-aminoantipyrine and 0.2 mmol 2,4-dichlorophenol were dissolved in 0.5 l of 0.1 M Na_2HPO_4/NaH_2PO_4 buffer at pH 7.0. The final reagent was then stored at 4°C prior to usage.

The substrate solution for the determination of leucine-aminopeptidase was prepared using the method developed by Takennaka and Takahashi (1962) and modified by Hirai et al. (1981b). 40 mg L-leucylnaphthylamide hydrochloride was dissolved in 100 ml of 0.1 M phosphate sodium buffer, pH 7.0, as substrate solution. 0.2 N HCl-4% *p*-dimethylaminobenzaldehyde ethanolic solution was used as the acidic colour reagent for quenching the enzymatic reaction.

Animal studies

Male Wistar rats weighing 250–330 g were fed with pelleted chow. All the animals were starved for 16 h before insulin administration.

Insulin absorption

Several drug mixtures, i.e. insulin alone, insulin/aprotinin/hyaluronidase, insulin/aprotinin/cholic acid, insulin/cholic acid, insulin/bile acid, insulin/glycocholic acid, insulin/deoxycho-

TABLE 1

Composition of the enhancer solutions used in the study of enhanced absorption of subcutaneously injected insulin

Enhancer studied	Concentration (mg/ml or U/ml [a])	Insulin concentration in saline (IU/ml)
None [b]	–	0.10
Sodium cholate	5	0.10
Bile salts	solution saturated	0.10
Sodium glycocholate	5	0.10
Sodium deoxycholate	5	0.10
Aprotinin	2000	0.10
Sodium cholate and aprotinin	5 2000	0.10
Hyaluronidase and aprotinin	400 2000	0.10
Insulin alone B [c]		0.12
Hyaluronidase I	560	0.12
Hyaluronidase II	280	0.12
Hyaluronidase III	210	0.12
Hyaluronidase IV	140	0.12

[a] For hyaluronidase, the standard enzyme unit was used and for aprotinin, kallikrein inactivator unit (KIU) was used.
[b] This solution was used as control in the study of enhancement of absorption of insulin by cholate and its analogues as well as aprotinin.
[c] Solution B was used as control in the study of enhancement effect of hyaluronidase on uptake of insulin.

lic acid, insulin/hyaluronidase, were prepared (Table 1); 0.1 ml of each mixture per 100 g body weight was administered by hypodermic injection to the rats, anaesthetized with pentobarbitone sodium at a dose of 60 mg/kg. Samples of 0.1 ml of blood were taken from the tail-vein at zero time and at 1, 1.5, 2, 3 and 4 h and transferred into glass tubes containing 100 μl of protein precipitant and then mixed thoroughly for analysis of blood glucose.

Determination of plasma glucose concentration

Blood glucose was measured using a modification of the Trinder procedure (Trinder et al., 1969; Bauminger, 1974). The blood samples were centrifuged at 500 × *g* for 3–5 min; 20 μl of the supernatant plasma were withdrawn and added to a tube containing 2 ml enzyme reagents. The tubes were then incubated for 10 min, at 37 °C and shaken briefly two or three times during incubation to ensure adequate aeration. The tubes were cooled immediately to room temperature by shaking in cold water and the absorbance read at 505 nm.

Dialysis of sodium cholate

Determination of equilibration time The time necessary to achieve equilibrium in a dialysis experiment with sodium cholate was determined at 37 °C, using a Spectrum Equilibrium Dialyser (Spectrum Medical Industries, Inc., Los Angeles, U.S.A.); 1 ml of 21.4 mM cholic acid in 0.02 M phosphate buffer with a pH of 7.4 and containing 0.15 M NaCl, was dialyzed against the same volume of phosphate buffer. Samples from each compartment of the dialysis system were assayed using high-performance chromatography at appropriate intervals to determine the equilibration time for sodium cholate.

Measurement of sequestration of insulin by sodium cholate All the Teflon cells and Teflon membranes used in the dialysis experiment were presaturated with insulin solution before use (Wideroe et al., 1983) to avoid the binding of the hormone to the membranes during the experiment. Two insulin-cholic acid solutions were used to study the sequestration of sodium cholate to insulin. Solution A was prepared by adding 61 mg of pork insulin (0.214 mM) and 500 mg sodium cholate (21.4 mM) to a 50 ml volumetric flask and making up to volume with a 0.02 M phosphate buffer with a pH of 7.4, containing 0.15 M NaCl. Solution B contained 1.07 mM insulin and 21.4 mM sodium cholate in a phosphate buffer as described above. These solutions were then separately dialyzed against the same volumes of phosphate buffer. A volume of 21.4 mM cholic acid in a phosphate buffer, pH 7.4, was used against the same volume of buffer as a control. Aliquots (500 μl) were withdrawn from all the receptor compartments at 3 and 5 h beyond equilibration time. The samples were diluted by addition of internal standard solution (500 μl) and then analyzed by

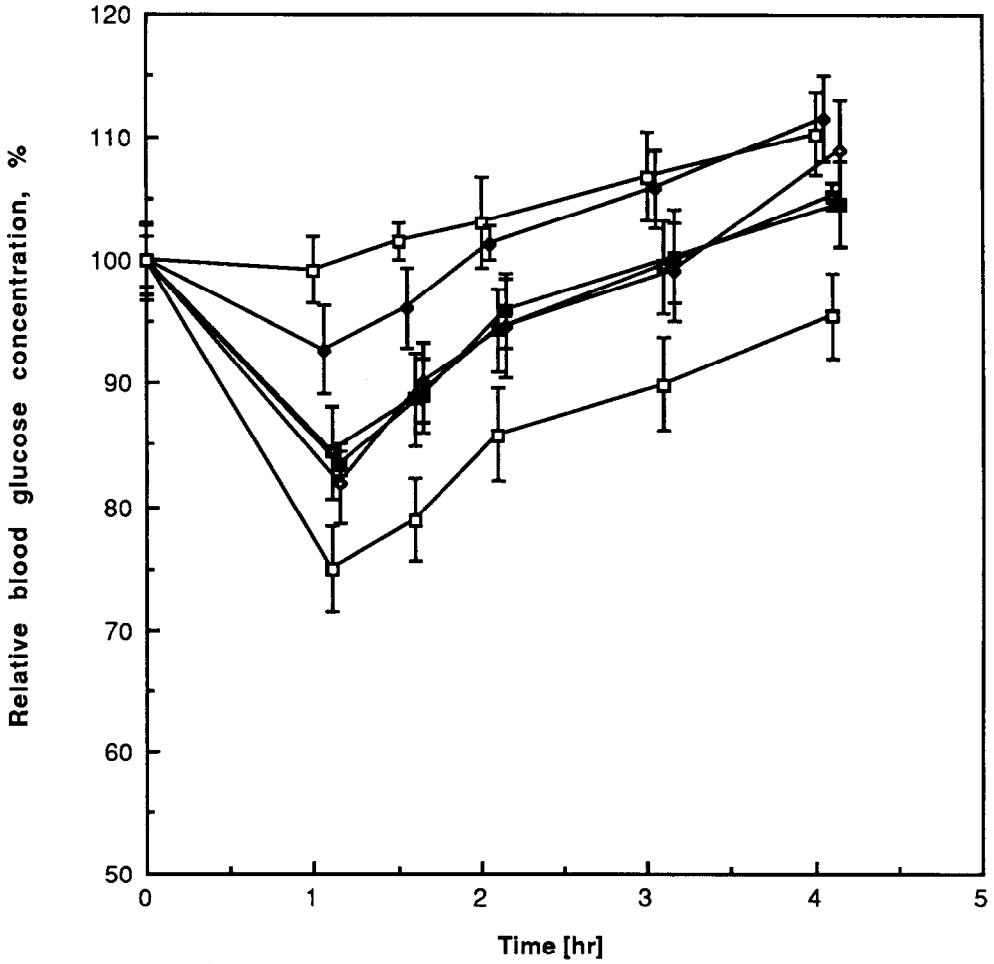

Fig. 1. Effects of cholic acid and its analogues on uptake of insulin. Each point was expressed as mean ± S.D. of five animals (□——□) Saline; (◆——◆) insulin alone; (■——■) bile acids + insulin; (◇——◆) deoxycholic acid + insulin; (◘——◘) glycocholic acid + insulin; (□——□) cholic acid + insulin.

HPLC (20 μl). Each experiment was repeated three times with two injections.

Inhibition of leucine-aminopeptidase activity

Dermal tissues were excised from the freshly killed rats and the underlying muscles and fat were carefully removed. The tissues were blotted dry on tissue paper, weighed and placed in a glass tube to be homogenized in a 10-fold volume of cold saline at 10000 rpm and 4–8°C. The homogenates were centrifuged at 7500 × *g* for 15 min at 4°C and the supernatants were stored at −18°C prior to analysis. The leucine-aminopeptidase activity of the supernatant was determined using the Golbard method (Takennaka and Takahashi, 1962; Hirai et al., 1981b; Mishima et al., 1987). A suitable predefined volume (20–250 μl) of the supernatant from the tissue homogenates was added to 0.5 ml of a 1.37 mM solution of L-leucine-β-naphthylamide hydrochloride; 0.1 M phosphate buffer at pH 7.0 was then added to bring the volume to 1.2 ml. The mixture was incubated at 37°C for 30 min and the enzymic reaction was quenched by adding 2 ml of 0.2 N HCl; 2 ml of a 4% *p*-dimethylaminobenzaldehyde ethanolic solution was added to produce a yellow colour. After 10 min, the

absorbance of the mixture was read at 465 nm. Sodium cholate and its analogues, fatty acid salts and hyaluronidase solutions were prepared by dissolving in physiological saline at various concentrations. The inhibition of cutaneous leucine-aminopeptidase activity by each of those agents

TABLE 2

Summary of effects of additives on insulin absorption

Reference	Compound/(Animal)	Tissue	Results
(I) Cholic acid and its analogues			
Hirai et al. (1981a)	Sodium taurocholate Sodium cholate Sodium deoxycholate Sodium glycocholate Sodium chenodeoxycholate (Rats)	Nasal	The decrease in plasma glucose level by different bile salts was as follows: sodium taurocholate, 56.7; sodium cholate, 53.1; sodium deoxycholate, 55.7; sodium glycocholate, 53.1; sodium chenodeoxycholate, 49.4%.
Hirai et al. (1981b)	Same as above (Rats)	Nasal	The inhibitory effect of bile acid salts on the leucine aminopeptidase activity in the nasal mucosa was found to be: sodium taurocholate, 87.4; sodium cholate, 84.9; sodium glycocholate, 87.2%.
Ziv et al. (1981)	Sodium deoxycholate Sodium cholate (Rats)	Intes-tinal	Plasma glucose was lowered by 50% 1 h after administration of 12 U soluble insulin mixed with 1–10 mg/ml DOC, or 2–20 mg/ml sodium cholate.
Moses et al. (1983)	Sodium deoxycholate (Diabetes)	Nasal	With administration of an insulin-deoxycholate 1% (w/v) aerosol, plasma glucose began to fall by 10 min, reaching 54% of control levels by 30 min. Nasal insulin absorption was approx. 10% as efficient as intravenous insulin.
Mishima et al. (1987)	Sodium caprylate Sodium caprate Sodium laurate Sodium glycocholate (Rats and Rabbits)	Nasal	The inhibition of aminopeptidase activity by these enhancers was found to be: sodium caprylate, 70 (rat homogenate) and 88 (rabbit homogenate); sodium caprate, 74 (rat homogenate) and 89 (rabbit homogenate); sodium laurate, 98 (rat homogenate) and 87 (rabbit homogenate); sodium glycocholate, 79 (rat homogenate) and 86% (rabbit homogenate).

(continued)

TABLE 2 (continued)

Reference	Compound/(Animal)	Tissue	Results
Aungst et al. (1988a)	Sodium glycocholate (Rats)	Nasal	5% of sodium glycocholate was found to significantly increase insulin efficacy by each route. The rank order was nasal > rectal > buccal > sublingual. Nasal and rectal insulin were roughly half as effective as i.m. insulin
(II) Aprotinin			
Berger et al. (1980)	(Normal subjects)	Subcutaneous	More rapid insulin entry into the circulation. Maximum glucose level-decreasing effect occurs at about 1 h instead of two hours after injection.
Williams et al. (1983)	(Diabetic subjects)	Subcutaneous	Local hyperaemia produced by aprotinin providing a possible mechanism for increase in insulin bioavailability.
Linde and Gunnarsson (1985)	(Diabetic subjects)	Subcutaneous	Injected soluble insulin more rapidly absorbed in Type 1 diabetics.
Ziv et al. (1987)	(Rats)	Intestinal	Dose response shown in effect of aprotinin on glucose level. When administered together with aprotinin and cholate, insulin was absorbed more effectively by up to 200 times relative to insulin on its own.
Owens et al. (1988)	(Normal subjects)	Intestinal	A significantly higher insulin level compared with insulin alone, leading to an acceleration of the early phase of absorption from subcutaneous tissue.
Aungst et al. (1988b)	(Rats)	Rectal Nasal Buccal	Ineffective in increasing insulin efficacy either alone or in combination with enhancer.
Deurloo et al., 1989	(Rats and Rabbits)	Nasal	Coadministration with aprotinin did not statistically significantly increase insulin bioavailability in rats and/or rabbits.

was determined by comparing the test solution against 0.1 M phosphate buffer at pH 7.0.

High-performance liquid chromatography (HPLC) method

The HPLC system used for assaying sodium cholate consisted of an LKB series liquid chromatograph (LKB Ltd, Bromma, Sweden) equipped with an HPLC pump (Model 2150), a UV variable-wavelength monitor (Model 2151), a sample loop injector with a maximum injection volume of 20 μl (Model 7125), and an ODS C-18 column (Spherisorb ODS II, Jones Chromatography, Hengoed, U.K.). Absorbance measurement was

carried out at 210 nm with a sensitivity of 0.04–0.016 AUFS. The pre-filtered mobile phase (0.45 μm), consisting of acetonitrile : water : phosphoric acid (60 : 40 : 2) with a pH of 2.36, was delivered at a flow rate of 1 ml/min. The internal standard solution was prepared by adding 36 mg of butyl-*p*-hydroxybenzoate to a 1000 ml volumetric flask and making up to volume with the mobile phase.

Statistical analyses

In evaluating the effects of insulin with and without additives on plasma glucose levels, the area under the curve was calculated after adjustment for baseline drift using saline as a control treatment. Therefore, for each treatment a negative area under the curve (AUC) was obtained as glucose levels decreased after insulin treatment. This summary measure (AUC) was used in all the statistical analyses. This method is recommended for designs with repeated measures such as the one in the present study (Yates, 1982). The AUC with different treatments were subjected to Analysis of Variance and in cases where a significant effect was observed, the means were compared using Neuman-Keul's method (Winer, 1971).

The Kwikstat and Minitab packages were employed for linear regression and analysis of variance.

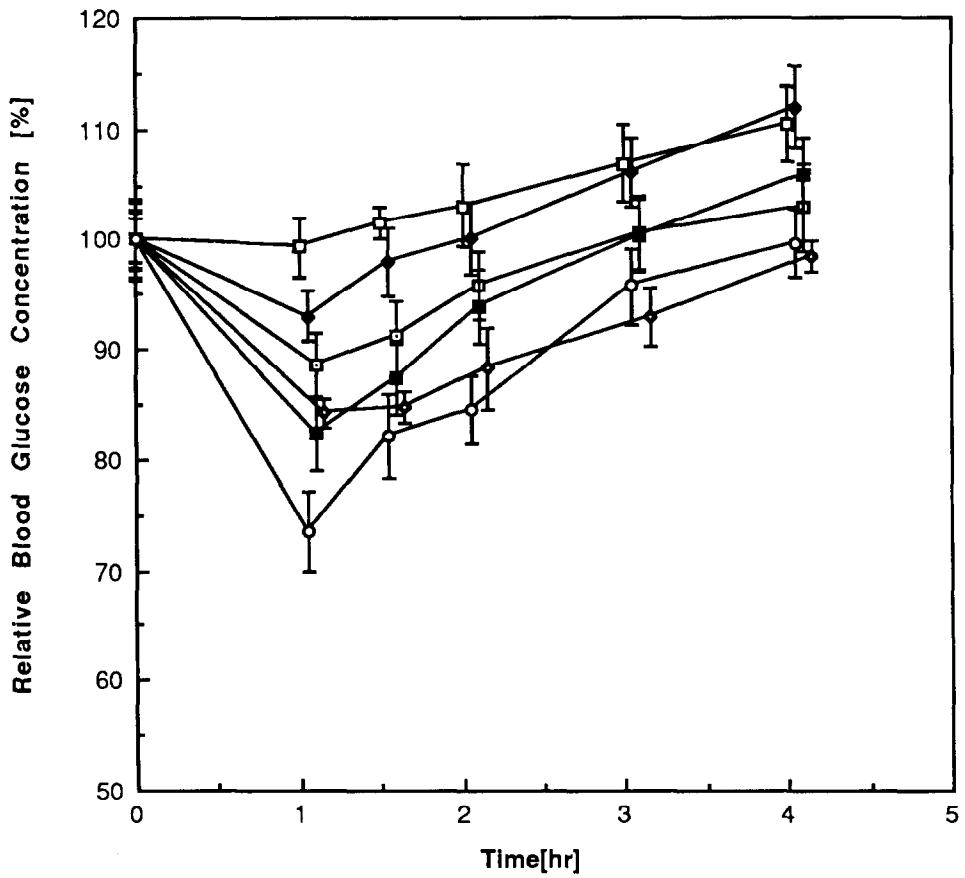

Fig. 2. Enhancement of insulin absorption by aprotinin. Each point was expressed as mean ± S.D. of five animals. (□——□) saline; (◆——◆) insulin alone; (⊡——⊡) aprotinin + insulin; (◇——◇) aprotinin + hyaluronidase + insulin; (■——■) aprotinin + cholic acid + insulin; (○——○) cholic acid + insulin.

Results and Discussion

In the present study, plasma glucose level was used as an index of the amount of insulin absorbed into the systemic circulation. This circumvents the need for a specific immunoassay for insulin. Given that insulin is clinically used to control blood glucose level, it can be argued that glucose is a better index for insulin bioavailability in therapeutic equivalence testing.

A number of specific assay methods have recently been developed using enzymes such as glucose oxidase, hexokinase and glucose dehydrogenase for determining glucose in biological fluids. In the present study, the method using glucose oxidase was chosen (Burrin and Price, 1985). The relationship between absorbance (y) and glucose concentration (x) could be described by the equation ($y = -0.0131 + 0.0589x$, $r^2 = 0.9996$). Using this method, the mean glucose concentration of five rats was found to be 77.69 ± 2.3 mg/dl, a result which is in agreement with those reported by Ziv et al. (1987) (80 ± 8.2 mg/dl) and Bar-on et al. (1981) (78.2 ± 1.8 mg/dl).

Effect of sodium cholate and its analogues on the bioavailability of insulin

Sodium cholate and its analogues have been reported to be more effective enhancers for insulin absorption when administered via a number of absorption routes (see Table 2) but most notably by the nasal route (Hirai et al., 1981; Ziv et al., 1981; 1987; Moses et al., 1983; Mishima et al., 1987; Aungst et al., 1988a). In this study, sodium cholate is found, on a weight basis, to be more effective in the enhancement of insulin absorption following subcutaneous injection than bile acids, sodium deoxycholate or glycocholate although all

TABLE 3

Results of multiple-range tests comparing the effects of insulin with and without additives [a]

(A) Effect of sodium cholate and its analogues

Population	Treatment [a]				
	1	5	4	3	2
1		———			
2	———				
3					———

[a] Treatment 1, insulin only; treatment 2, insulin plus sodium cholate; treatment 3, insulin plus sodium deoxycholate; treatment 4, insulin plus sodium glycocholate; treatment 5, insulin plus oxidised bile acids.

(B) Effect of aprotinin, hyaluronidase and sodium cholate

Population	Treatment [a]				
	1	2	4	3	5
1		———			
2	———				
3					———

[a] Treatment 1, insulin only; treatment 2, insulin plus aprotinin; treatment 3, insulin plus aprotinin and hyaluronidase; treatment 4, insulin plus aprotinin and sodium cholate; treatment 5, insulin plus sodium cholate.

(C) Effect of different concentration of hyaluronidase

Population	Treatment [a]				
	1	4	2	5	3
1	———				

[a] Treatment 1, insulin only; treatment 2, insulin plus hyaluronidase, 560 U; treatment 3, insulin plus hyaluronidase, 280 U; treatment 4, insulin plus hyaluronidase, 210 U; treatment 5, insulin plus hyaluronidase, 140 U.

[a] Treatment groups joined together by lines do not differ significantly in their mean values; the groups (1–5) are arranged in increasing numerical magnitude.

of those substances improved the absorption of subcutaneous insulin. Typical examples of the change in plasma glucose level after subcutaneous administration of the insulin solution containing 0.35% of the additives are shown in Fig. 1. The insulin solution (0.1 IU/kg) without surfactant produced slight hypoglycaemia throughout the experimental period, whereas the addition of sodium cholate significantly decreased the plasma glucose level. The enhancement of insulin absorption by sodium cholate was more pronounced than that produced by its analogues ($P < 0.05$, Table 3a). At the time of injection, glucose concentration was 76.20 ± 2.3 mg/dl (4.23 ± 0.13 mM).

Enhancement of absorption of insulin by aprotinin

Aprotinin, a bovine pancreatic kallikrein inhibitor, consisting of a single-chain polypeptide of 58 amino acids has a molecular weight of 6500 (Kassell et al., 1965). It has been used to inhibit plasmin, trypsin, chymotrypsin and various intracellular proteases (Trautschold et al., 1967). It has also been reported that the degradation of insulin by adipose tissue can be inhibited by this compound (Offord et al., 1979).

Aprotinin has been used in attempts to improve the absorption of insulin (Table 2) (Berger et al., 1980; Freidenberg et al., 1981; Williams et al., 1983; Dandona et al., 1985; Linde and Gunnarson, 1985; Owens et al., 1988). In the present study, insulin absorption was found to be increased by aprotinin. As shown in Fig. 2, the drop in blood glucose was found to be higher after subcutaneous injection of insulin with aprotinin than when the hormone was administered alone. The insulin-enhancing effect of aprotinin was not increased further by hyaluronidase or by sodium cholate. Interestingly the enhancement produced by sodium cholate on its own was more pronounced than when combined with aprotinin (Table 3b). A possible explanation is that aprotinin competes with insulin with respect to the sodium cholate so that more of the insulin is available free for attack by proteases. When hyaluronidase was studied on its own as a possible enhancer of insulin absorption, it was found that increasing hyaluronidase concentration produced little further reduction in glucose levels (Table 3c). The

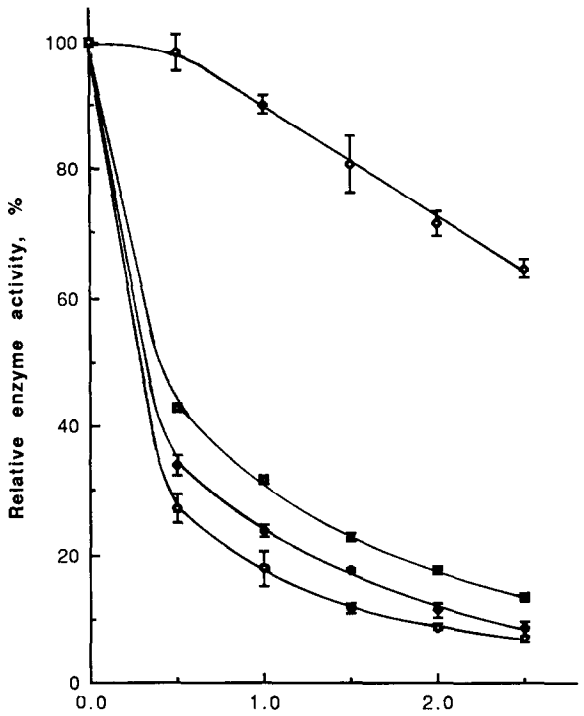

Fig. 3. Effects of sodium cholate and its analogues on the activity of leucine aminopeptidase on the rat dermal tissue. Each point was expressed as mean ± S.D. of three experiments. (◆) Sodium cholate; (■) sodium glycocholate; (○) sodium deoxycholate; (◇) bile acids.

results reported here were consistent with those reported by Berger et al. (1980), Linde and Gunnarson (1985), and Owens et al. (1988) in their work with normal subjects and insulin-dependent diabetics. They concluded that aprotinin increased the absorption rate of subcutaneously injected insulin by inhibition of degradation of exogenous insulin at the injection site.

Our present results contrast with those reported by Ziv et al. (1987) in their insulin transport work using intestinal tissue as a model. Their results showed that the rate of insulin absorption across the intestinal wall was increased to a lesser extent with sodium cholate than with aprotinin and much less than with aprotinin and sodium cholate combined. The reason for the difference between those results and the ones reported in this study may be due to the different proteolytic enzymes present in

the intestine and in subcutaneous tissues. Indeed, the proteolytic activity of skin homogenates is much lower than that of intestinal tissues (Zhou and Li Wan Po, 1990). More recently, some contradictory results have also been reported with respect to the nasal absorption of insulin and calcitonin (Hanson et al., 1986; Aungst and Rogers, 1988b; Deurloo et al., 1989). When the effects of laureth-9, sodium salicylate, Na_2EDTA and aprotinin on insulin absorption via the rectal, nasal and buccal tissues were examined by Aungst and Rogers (1988b), aprotinin was found to be ineffective, either alone or in combination with laureth-9. The coadministration of sodium taurodihydrofusidate with aprotinin also failed to significantly increase nasal insulin bioavailability in rabbits (Deurloo et al., 1989). Hanson and coworkers (1986) examined the effects of several protease inhibitors, including bile salts, fatty acid derivatives, aprotinin, kallikrein inhibitor, RG-1, bestatin, fusidic acid, chemostatin, benzamidine, chymotrypsin inhibitor, trypsin inhibitor III-0 and leupeptin on intranasal delivery of calcitonin, and found that aprotinin in vitro did not inhibit the proteolytic activity of nasal extracts. In vivo the inhibitor did not enhance the drop in serum calcium observed. In our study, aprotinin produced a significant increase in the effect of insulin on plasma glucose, after subcutaneous administration. Clearly, further studies are required to better understand the effects of aprotinin on the absorption of insulin. However, with the available evidence, the appropriate conclusion is that aprotinin enhances insulin availability after subcutaneous injection but not when administered by other routes. With those routes, the barriers to systemic availability are largely diffusional in nature, whereas with subcutaneous administration peptidase attack assumes a proportionately greater role.

Mechanism of enhancement of absorption of insulin by cholic acid

Figs 1 and 2 show the relative efficacy of several agents in promoting subcutaneous insulin absorption. Sodium cholate was the most effective among the compounds tested. To investigate the enhancement of insulin absorption by sodium cholate, a number of other experiments were carried out as follows.

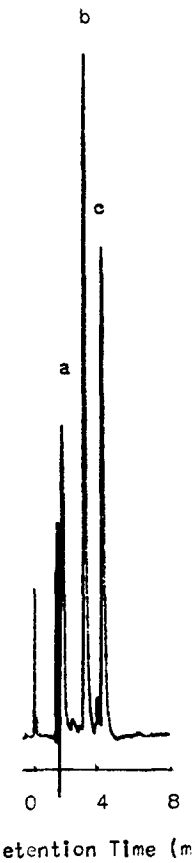

Fig. 4. Typical high performance liquid chromatograph of cholic acid. Components: a, solvent front; b, sodium cholate; c, butylparaben.

Inhibition of leucine-aminopeptidase activity by sodium cholate

The additives used for increasing the availability of insulin may do so by inhibiting tissue peptidase activity in addition to increasing permeability. Indeed, Hirai et al. (1981) showed that leucine aminopeptidase activity was inhibited by bile acid salts. The inhibitory effect of this enzyme by cholate was found to be more than 80% under the conditions used. In a more recent study, sodium glycocholate was also shown to cause significant inhibition of leucine aminopeptidase both in rats and rabbit nasal mucosae (Mishima et al., 1987). In the present study, as shown in Fig. 3, the

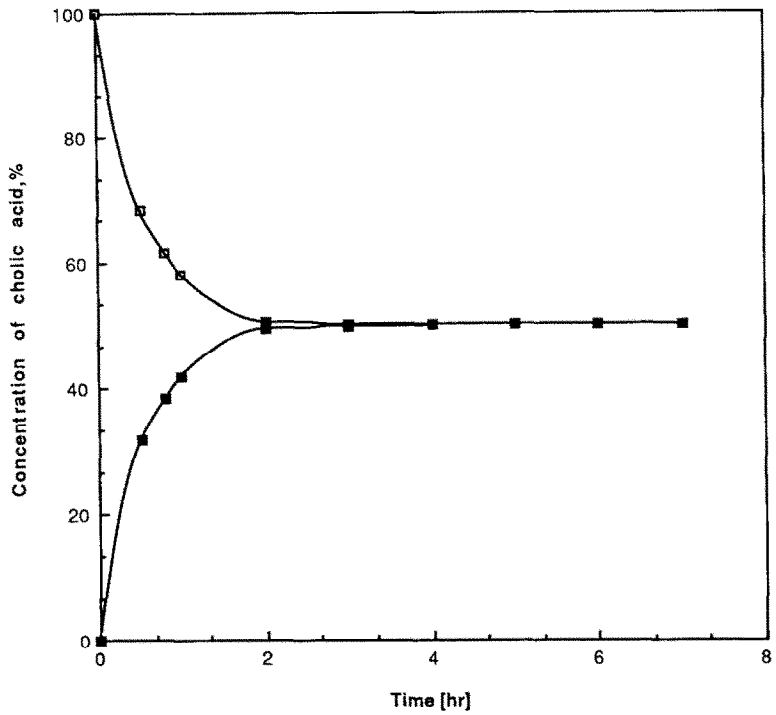

Fig. 5. Measurement of equilibration time of cholic acid. Cholic acid diffusion across a dialysis Teflon membrane with a molecular weight cut-off of 3500 at an initial concentration of 21.4 mM, at 37°C. (⊡) Donor phase concentration; (■) receiver phase concentration.

activity of leucine aminopeptidase decreased with increasing concentrations of the surfactants. These results are in agreement with those of Hirai et al. (1981b).

The binding of insulin to cholic acid

In an early study by Hirai et al. (1981b), investigating the stability of insulin, over 90% of the original amount of insulin remained after a 60 min incubation in the supernatant from nasal mucosal homogenate containing 1% sodium glycocholate. When the experiment was repeated with 1% polyoxyethylene 9-lauryl ether, instead of sodium glycocholate, only 9% of the initial insulin remained. The mechanism by which sodium glycocholate stabilises insulin is unclear although insulin binding of the glycocholate ion may be involved. Numerous studies have shown that albumin, for example, binds to cholic acid (Rudman and Kendall, 1957; Burke et al., 1971; Cowen et al., 1975; Bekett et al., 1981; Roda et al., 1982; Takikawa et al., 1985; 1987).

To investigate this further, dialysis of sodium cholate in the presence and absence of insulin was performed. Sodium cholate was measured by high-performance liquid chromatography (Fig. 4). Fig. 5 shows the equilibration for sodium cholate across a teflon^R dialysis membrane with a molecular weight cut-off of 3500. Equilibration in the presence of insulin shows that binding takes place between cholic acid and insulin. At an initial molar ratio, sodium cholate to insulin, of 100, the bound cholate to insulin ratio at equilibrium was 4.14. When the initial ratio was changed to 20, the equilibrium ratio reduced to about 2.56. It would therefore appear that insulin and cholic acid binding may be contributing to the improved stability of insulin reported by Hirai et al. (1981b).

Conclusion

The present study has shown that with subcutaneous injection, insulin absorption may be enhanced by both surfactant-type compounds, such as cholic acid, and by aminopeptidase inhibitors such as aprotinin. Aprotinin most probably increases insulin absorption by minimising hydrolysis by subcutaneous tissues. Cholic acid binds to insulin and this may protect against hydrolysis too, because this would prevent the formation of enzyme-insulin complex which aligns the catalytic site on the protease. It is also likely that the surfactant properties of cholic acid promote absorption by lowering surface tension and hence improve transfer across the absorption barriers. It would appear that while peptidase inhibition improves availability of subcutaneously administered insulin, this becomes insignificant when an additional absorption barrier is presented to the insulin molecule as is the case with administration by all other non-parenteral routes.

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