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The effect of absorption enhancers on the initial degradation kinetics of insulin by α-chymotrypsin

Mahasen A. Radwan [a,*], Hassan Y. Aboul-Enein [b]

[a] *Department of Clinical Pharmacy, College of Pharmacy, Science and Medical Studies, Department for Women Students, King Saud University, P.O. Box 22452, Riyadh 11495, Saudi Arabia*

[b] *Department of Biological and Medical Research, Laboratory of Pharmaceutical Analysis, (MBC-03), King Faisal Specialist Hospital and Research Centre, P.O. Box 3354, Riyadh 11211, Saudi Arabia*

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Abstract

The goal of this investigation was to establish a fast method to screen various insulin absorption enhancers by following their effect on the initial kinetics of insulin incubated with α-chymotrypsin at 37°C. A simple, sensitive and reproducible reversed phase high performance liquid chromatography (HPLC) method has been developed to carry out this goal. Linear responses ($r > 0.999$) were observed over the range of 0.4–4 U/ml for insulin. There was no significant difference ($P < 0.05$) between inter- and intra-day studies for insulin. The mean relative standard deviations (RSD%) of the results of within-day precision and accuracy of insulin were $< 12\%$. The assay was sensitive to detect the existence of any metabolite due to the addition of any absorption enhancers, even if it was not seen with insulin alone. Three metabolites (A–C) were detected only when insulin was incubated with α-chymotrypsin at 37°C. Metabolite D was observed when either glycocholic acid (0.5, 1%) or taurochenodeoxycholate (0.5, 1%) was incubated with insulin in the absence of α-chymotrypsin at 37°C. The compounds that significantly increased insulin $T_{50\%}$ were glycyrrhizic acid (0.5%) > deoxycholic acid (1%) > deoxycholic acid (0.5%) > glycyrrhizic acid (1%) > cholic acid (0.5, 1%). Capric acid (0.5%), hydroxypropyl-α-cyclodextrin (0.5, 1%) and dimethyl-α-cyclodextrin (0.5, 1, 5%) did not significantly affect insulin $T_{50\%}$. The bile salts increased insulin $T_{50\%}$ in this order: deoxycholate > cholate > glycocholate > taurocholate > taurodeoxycholate > taurochenodeoxycholate > glycodeoxycholate. The results obtained would support the feasibility of utilizing such method for screening any compound incorporated in insulin formulation. These compounds should be used in the minimum possible concentration to avoid or minimize insulin degradation.

Keywords: Insulin stability; α-Chymotrypsin; HPLC; Absorption enhancers; Bile salts; Cyclodextrins; Glycyrrhizic acid

1. Introduction

Bioactive proteins and peptides are a rapidly growing class of therapeutic agents and, even though there are only a few currently marketed,

* Corresponding author. Tel./fax: +966-1-4616991.
E-mail address: mradwan@ksu.edu.sa (M.A. Radwan).

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there are hundreds in clinical testing. Most of these, however, can be given parenterally because their oral bioavailability is generally very low. They are extensively degraded by proteolytic enzymes in the gastrointestinal (GI) tract and are impermeable to the intestinal mucosa due to their hydrophilic characteristics and large molecular size (Lee and Yamamoto, 1990). Moreover, injections are given frequently because their in vivo half-lives are generally no more than several hours (Lee, 1995).

Various approaches have been proposed and demonstrated, such as incorporation of protease inhibitors (Yamamoto et al., 1994a,b; Bai and Chang, 1996), absorption enhancers (Muranishi, 1990; Scott-Moncrieff et al., 1994; Shao et al., 1994; Yamamoto et al., 1994a,b), chemical modification (Hashizume et al., 1992) and dosage

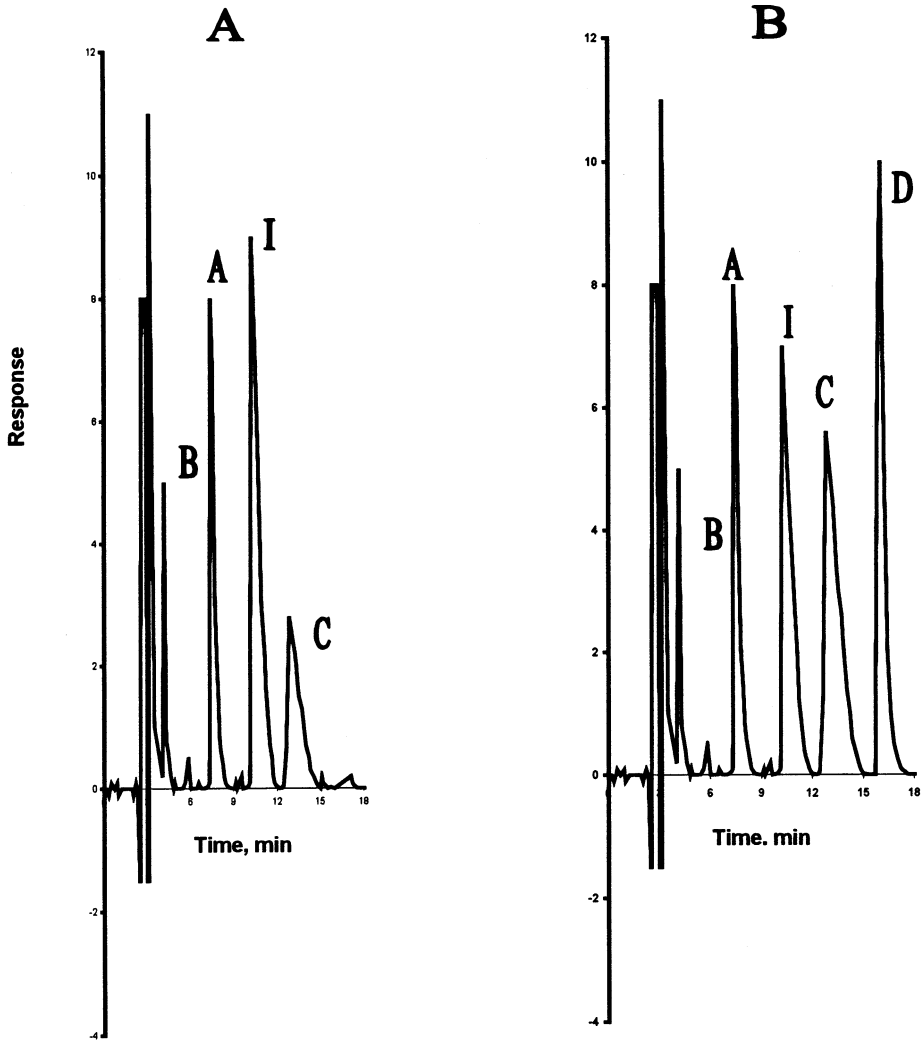

Fig. 1. (A) Representative chromatograms of insulin and its metabolites after 5 min of incubation with α-chymotrypsin at 37°C. Peaks I and A–C refer to insulin and its metabolites, respectively. (B) The effect of glycocholic acid on insulin instability in the presence of α-chymotrypsin after 2 min. Peaks I and A–D refer to insulin and its metabolites, respectively.

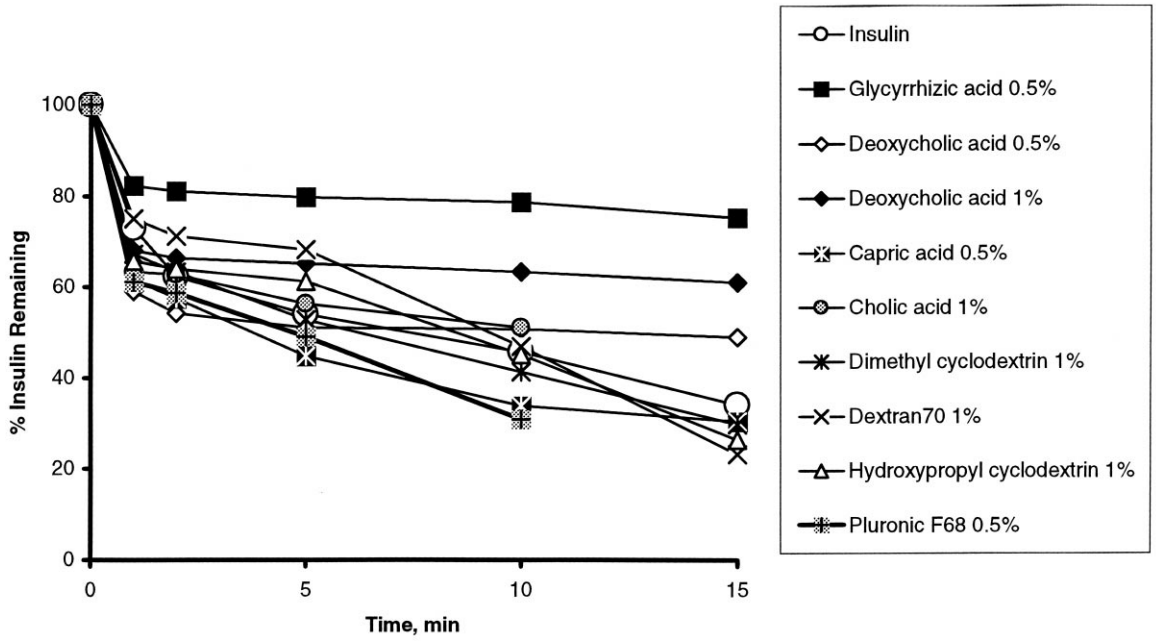

Fig. 2. The percent of insulin remaining versus time after incubation with α-chymotrypsin at 37°C in the absence or presence of some representative compounds that increased or did not affect insulin stability.

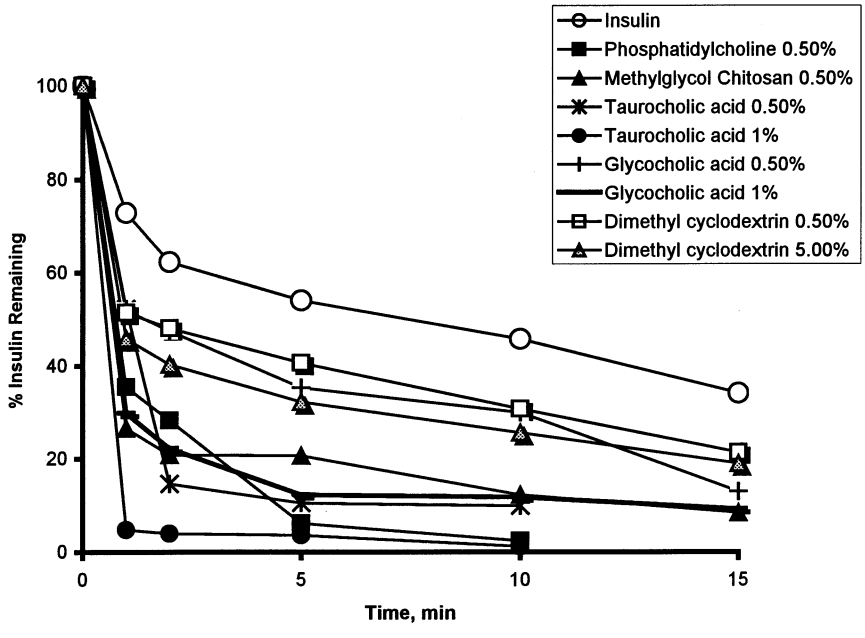

Fig. 3. The percent of insulin remaining versus time after incubation with α-chymotrypsin at 37°C in the absence or presence of some representative compounds that decreased insulin stability.

forms (Hosny et al., 1997; Suzuki et al., 1998) to overcome the problems encountered with the delivery of peptides and proteins via the GI tract.

Insulin has been chosen as a model polypeptide in this study for two reasons. First, this peptide is among the most widely studied for possible absorption through alternative routes. Second, improved insulin delivery could significantly influence diabetes treatment. Insulin is composed of 51 amino acids in two peptide chains linked by two disulfide bonds. Chemical deterioration of insulin during storage of pharmaceutical preparations is mainly due to two categories of chemical reactions, hydrolysis and intermolecular transformation reactions (Brange and Langkjoer, 1993).

Trypsin and α-chymotrypsin are the major proteolytic enzymes secreted by the pancreas into the intestinal lumen. Both are known to cleave various bonds within the insulin molecule (Ginsburg and Schachman, 1960; Young and Carpenter 1961). Schilling and Mitra (1991) have reported the degradation of insulin by trysin and α-chymotrypsin using a gradient HPLC assay. At a molar ratio of 172:1 (insulin:enzyme), chymotrypsin caused near-total loss of insulin within 40 min, while very little insulin was degraded by trypsin. α-Chymotrypsin has been found to be the primary proteolytic enzyme responsible for initial cleavage and unfolding of insulin globular structure, exposing the molecule to subsequent attack

Table 1
The effect of some compounds on the initial kinetics of insulin incubated with α-chymotrypsin at 37°C[a]

Compound (%)	% Degraded within 1 min	Degradation $T_{50\%}$ (min)	T_{max}, min, of metabolite	
			A	C
Insulin	27.1	13.6 (1.6)	10	≥15
Dimethyl cyclodextrin				
0.5	48.6	12.1 (1.0)	10	NA
1	32.8	12.8 (1.2)	15	15
5	54.2	12.7 (0.9)	15	≥15
Hydroxypropyl cyclodextrin				
0.5	27.8	11.3 (1.0)	≥15	NA
1	34.3	11.7 (1.5)	10	NA
Dextran 70				
1	25	10.3 (1.1)	≥15	NA
5	35.5	10.5 (0.8)	≥15	NA
Pluronic F68				
1	38.9	9.0 (0.7)	15	NA
5	43.0	5.0 (0.2)	≥15	NA
Glycyrrhizic acid				
0.5	17.7	92.2 (8.0)	≥15	10
1	20	43.5 (6.0)	2	10
Capric acid				
0.5	38.3	13.6 (1.3)	5	5
1	99	–	2	2
Methylglycol chitosan				
0.5	73.4	11.0 (1.1)	2	NA
1	77.8	10.0 (0.8)	2	NA
L-α-phosphatidylcholine didecanoylC10:0				
0.5	64.6	4.9 (0.6)	1	NA

[a] NA, not applied since metabolite C was not detected.

Table 2
The effect of bile salts incubated with insulin in the presence of α-chymotrypsin at 37°C on the initial kinetics of insulin

Bile salt (%)	% Degraded within 1 min	Degradation $T_{50\%}$ (min)	T_{max}, min. of metabolite	
			A	C
Cholic acid				
0.5	41.3	22.1 (2)	10	5
1	36.7	22.2 (1.8)	5	2
Deoxycholic acid				
0.5	41	52.3 (3)	≥15	1
1	32	74.1 (6)	≥15	5
Taurocholic acid[a]				
0.5	47.8	8.0 (0.8)	2	NA
1	95.3	6.4 (0.8)	1	NA
Taurochenodeoxycholic acid[b]				
0.5	84.1	1.3 (0.1)	2	1
1	97.3	*	1	1
Taurodeoxycholic acid				
0.5	51.9	9.3 (1.0)	5	1
1	51.1	2.3 (0.2)	5	2
Glycocholic acid[c]				
0.5	48.1	10.0 (0.7)	≥15	2
1	70.2	11.9 (0.5)	≥15	5
Glycodeoxycholic acid 1				
0.5	80.7	*	2	2
1	98.8	*	1	1

[a] A fifth metabolite was detected at zero time, metabolite E, with retention time 6 min which reached its T_{max} at 1 min.
[b] A fourth metabolite was detected at zero time, metabolite D, with retention time 16 min which reached its T_{max} at 1, 0 min, for 0.5, 1%, respectively.
[c] A fourth metabolite was detected at zero time, metabolite D, with retention time 16 min which reached its T_{max} at 10 min.
* Less than 1% of insulin was left after 2 min.

by brush boarder and eterocytic enzymes (Schilling and Mitra, 1991). Therefore, insulin must be protected from the pancreatic digestive enzymes to maintain the greatest amount of intact, biologically active, insulin to be available for absorption.

Coadministration of insulin with different enzyme inhibitors, either specific or non-specific for trypsin or chymotrypsin, had shown potential for enhancing the absorption of insulin (Shao et al., 1994; Sugiyama et al., 1997; Morimoto et al., 1998; Donnelly et al., 1998; Martin et al., 1998; Sakai et al., 1998a, 1999; Kotze et al., 1999; Schipper et al., 1999). Bile salts were demonstrated to increase insulin bioavailability (Sugiyama et al., 1997; Donnelly et al., 1998; Morimoto et al., 1998; Sakai et al., 1998a, 1999) through a combination of several postulated mechanisms, such as the alteration of biological membrane integrity, inhibition of protease's activity and dissociation of molecular aggregates through micellar solubilization (Gordon et al., 1985; Donovan et al., 1990). Li et al. (1992) investigated the dissociation of insulin oligomers by bile salt, sodium glycocholate, micelles and its effect on α-chymotrypsin-mediated proteolytic degradation. In the presence of sodium glycocholate, the rate of degradation of both zinc and sodium insulin increased in an asymptotic manner. The effects of α-cyclodextrin derivatives on α-chymotryptic degradation was followed (Shao et al., 1994). No inhibitory effect on α-chymotryptic activity was observed by either cyclodextrins at 10% concentration. Sakai et al. (1997)

reported that capric and deoxycholate enhanced the transepithelial transport of three model compounds in Caco-2 cell monolayers, while glycyrrhizic acid was found to have no effect on the transport of any of these compounds.

Capric acid showed significant promoting action on corneal penetration, but not on conjunctival penetration. Taurocholic acid had a significant effect on conjunctival penetration but not on corneal penetration of β-blockers, atenolol, carteolol, tilisolol, timolol and befunolol, of various lipophilicities (Sasaki et al., 1995).

Morishita et al. (1993) showed that sodium caprate significantly promoted the hypoglycemic effect of insulin at all sites and their intensity increased towards the distal regions of the intestine. On the other hand, sodium glycocholate improved only colonic insulin efficacy. These results suggest that the ileum seems to be the most useful region in the small intestine for insulin absorption; however, insulin must be protected from proteolysis to enhance its absorption. In addition, the insulin efficacy could be increased by absorption promoters more effectively in the colon than in the small intestine (Morishita et al., 1993).

Previous studies have addressed only the effect of cyclodextrins or sodium glycocholate (Li et al., 1992; Shao et al., 1994) on the stability of insulin, but not the effect of other compounds, as absorption enhancers, on insulin stability. Therefore, the present study was carried out to establish a simple, sensitive and rapid method to screen various bile salts and other chemicals that have potential as absorption enhancers, or incorporated in insulin formulations, on the initial kinetics of insulin in the presence of α-chymotrypsin, a model enzyme with high proteolytic activity (Schilling and Mitra, 1991). Several HPLC methods have been applied for insulin (Benzi et al., 1990; Hashizume et al., 1992; Yamamoto et al., 1994a; Iwanaga et al., 1999). However, these often consume more organic solvents or apply gradient elution with longer run time, resulting in many interfering peaks when tested in our laboratory. To carry on this investigation, the present study also describes a sensitive, specific and rapid sample preparation assay for determining insulin in

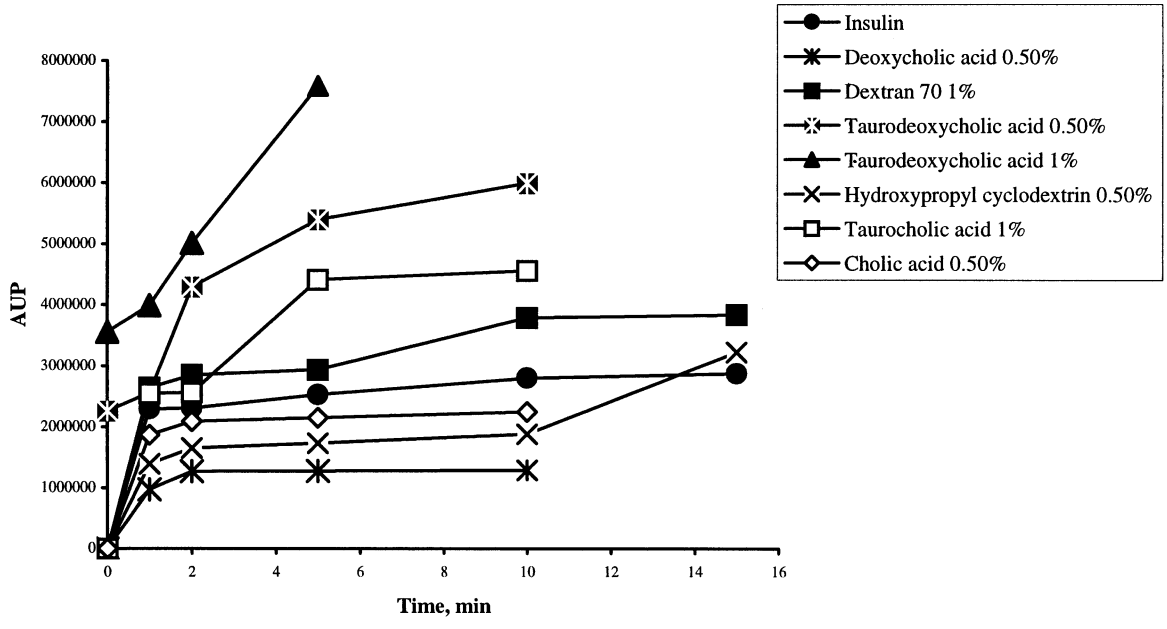

Fig. 4. The profile of HPLC AUP of insulin metabolite A versus time in the presence of α-chymotrypsin without and with some representative compounds.

solutions with no interference from its metabolites in the presence of α-chymotrypsin and several compounds, which could be utilized as absorption enhancers for insulin.

2. Materials and methods

2.1. Chemicals

Crystalline bovine insulin (28.4 U/mg), dextran 70, hydroxypropyl-α-cyclodextrin, dimethyl-α-cyclodextrin, the sodium salts of glycocholic acid, glycodeoxycholic acid, taurocholic acid, taurodeoxycholic acid, taurochenodeoxycholate, glycyrrhizic acid, capric acid, methylglycol chitosan, sodium salicylate, L-α-phosphatidylcholine didecanoyl C10:0 and α-chymotrypsin were purchased from Sigma, St. Louis, MO. Cholic acid and deoxycholic acid sodium salts were obtained from Serva Feinbiochemica and Co., Heidelberg, Germany. Pluronic F68 Prilled was obtained from Ruger Chemical Co. Inc., Irvington, NJ. All other reagents and chemicals were analytical grade and used as received.

2.2. Instruments

Waters HPLC system was equipped with a Water Lambda Max 461 variable UV absorbance detector (set at 215 nm), and a Waters 710B autosampler. Waters 510 solvent delivery system was used to adjust the flow through a Zorbax C8 column (3.9 × 300 mm) packed with 5 μm spherical particles. Waters automated gradient controller monitored the flow rate. Chromatograms were recorded on a Waters 746 Data Module integrator chart. The HPLC system was operated at ambient temperature. The mobile phase was acetonitrile (31.5%) containing triethylamine (0.15%); trifloroacetic acid was used to adjust the pH to 2.2. Degassing was achieved by filtration through 0.22 μm filter and sonication under vacuum. The flow rate was 1.3 ml/min and the sample run time was 18 min. The injection volume was 50 μl.

2.3. Selection of optimum incubation condition for insulin and α-chymotrypsin

Several preliminary trials were conducted to select the ratio of α-chymotrypsin to insulin, which produces a linear relationship of insulin degradation with time and causes 100% degradation of insulin in ≥ 30 min but < 60 min at 37°C. Incubating insulin with α-chymotrypsin for 1 min resulted in the appearance of two metabolites peaks. One metabolite peak appeared at 7 min (metabolite A) with the highest area under the peak (AUP), and the other metabolite (metabolite B) appeared at 4 min. Within 5 min of incubation, a third metabolite peak started to show at ≈ 13 min (metabolite C). However, after 15 min of incubation, using the optimum condition selected at 37°C, at least ten metabolite peaks showed up. Therefore, 15 min was chosen as the optimum time to follow the initial kinetics of insulin degradation in the presence of α-chymotrypsin. It was noticed that the AUP for metabolite B was not significantly increased after 1 min. Thus, metabolites A and C were selected for the comparisons between the tested compounds.

2.4. In vitro stability of insulin on the presence of α-chymotrypsin

α-Chymotrypsin solution (75 U/ml) was prepared in 100 mM Tris buffer containing 1 mM $CaCl_2$ (pH 8). Insulin (10 mg) was dissolved in 100 μl of 0.05 M HCl and 1 ml of Tris buffer containing 0.2% TFA acid (pH 2.45). Aliquots of insulin solution, 110 μl, were diluted up to 2 ml with Tris buffer (pH 8). The solution was preequiliberated at 37°C for 15 min. Before the addition of α-chymotrypsin solution, insulin solution was vortexed at high speed for 5 s and a 40 μl sample was withdrawn and diluted with 60 μl of cold Tris buffer solution containing 0.2% TFA acid (pH 2.45) as the zero time sample. Enzyme solution (40 μl) was then added to insulin solution and maintained at 37°C. Samples (40 μl) were withdrawn after vortexing the solution for 5 s at 1, 2, 5, 10 and 15 min after the addition of the enzyme solution. The collected samples were immediately diluted as described above to arrest the reaction.

The samples were vortexed at high speed for 10 s and stored at −30°C until assayed by HPLC. Each experiment was carried out in triplicate.

2.5. In vitro stability of insulin on the presence of α-chymotrypsin and enhancers

The same procedure mentioned above was performed throughout the degradation study but only the tested compound was dissolved or dispersed in the Tris buffer solution before the addition of insulin solution. Linear regression was used to calculate insulin degradation rate constant for all concentration-time profiles, excluding the zero time, in the presence of α-chymotrypsin and each of the tested compounds.

2.6. Drug analysis

The effect of different compounds on insulin stability was conducted by fitting the concentration time data from 1 up to 15 min according to the zero order kinetic equation, $A = A_0 - kt$, where A is the percent of insulin remaining at any time t and A_0 is the amount remaining after 1 min of the addition of any compound in the presence of α-chymotrypsin. The degradation rate constant (k) as percent degraded per unit time, was calculated from the slope of the linear regression analysis of the percent insulin remaining versus time curve. The elimination $T_{50\%}$ was determined from $(0.5\ A_0)/k$.

2.7. Statistical analysis

All results are expressed as mean ± S.D. The RSD% was calculated for all values. The t-test was used to examine the concentration difference at each day, and one-way analysis of variance (ANOVA) was employed to evaluate the reproducibility of the assay. The level of confidence was 95%.

3. Results and discussion

To carry on this investigation, HPLC method has been developed for the determination of insulin initial kinetics in the presence of α-chymotrypsin and absorption enhancers. Linear responses ($r > 0.999$) were observed over the range of 0.4–4 U/ml for insulin. There was no significant difference ($P < 0.05$) between inter- and intra-day studies for insulin. The RSD% of the results of within-day precision and accuracy of insulin were $< 12\%$. The concentration of α-chymotrypsin causing insulin instability at 37°C, but in a reasonable rate within 15 min, was selected. Insulin showed three metabolites peaks, called here A–C metabolites. Fig. 1(A) shows representative chromatograms of insulin and its metabolites after 5 min of incubation with α-chymotrypsin at 37°C. Peaks I and A–C refer to insulin and its metabolites, respectively. The effect of glycocholic acid on insulin instability, in the presence of α-chymotrypsin after 2 min, is shown in Fig. 1(B). A comparison between Fig. 1(A and B) and indicates that insulin and metabolites A–D peaks are free from matrix interference. Metabolite D was detected even without the addition of α-chymotrypsin, at zero time sample, when either glycocholic acid (0.5, 1%) or taurochenodeoxycholate (0.5%) was incubated with insulin. Using the chromatographic conditions described, insulin and its metabolites A–D were well resolved with average retention times of 10.2, 7.3, 4.1, 12.8, 16 min, respectively.

Figs. 2 and 3 represent the percent of insulin remaining versus time after incubation with α-chymotrypsin at 37°C in the absence or presence of some representative compounds. α-Chymotrypsin seemed to degrade insulin in a biphasic fashion. It was noticed that $27.1 \pm 3\%$ insulin degraded within 1 min and $\geq 30\%$ insulin remained at 15 min. The correlation coefficient between the percent insulin remaining and time from 1 to 15 min was > 0.97 with a rapid degradation within the first minute. Therefore, insulin data were fitted to zero order degradation kinetics from 1 to 15 min for insulin alone or in combination with any of the tested compounds. Accordingly, insulin degradation $T_{50\%}$ was 13.6 ± 1.6 min (Table 1). The compounds that significantly increased insulin stability and hence, $T_{50\%}$, were in the order of glycyrrhizic acid (0.5%) > deoxycholic acid (1%) > deoxycholic acid (0.5%) > gly-

cyrrhizic acid (1%) > cholic acid (0.5, 1%) (Tables 1 and 2). Capric acid (0.5%), hydroxypropyl-α-cyclodextrin (0.5, 1%) and dimethyl-α-cyclodextrin (0.5,1, 5%) did not significantly affect insulin $T_{50\%}$. This finding is in agreement with Gordon et al. (1985), who demonstrated that sodium cholate was much less effective than deoxycholate in promoting insulin nasal absorption, although deoxycholate could damage plasma and nuclear membranes. The finding of Shao et al. (1994) that no inhibitory effect on α-chymotryptic activity was observed by the tested cyclodextrins at 10% concentration is supporting the present result.

The cytotoxicities of sodium deoxycholate (0.02–0.1% w/v), sodium caprate (0.1–0.5% w/v) and dipotassium glycyrrhizinate (0.5–2% w/v) were evaluated (Sakai et al., 1998b). It was found that out of these three enhancers, dipotassium glycyrrhizinate was not cytotoxic. High concentrations of sodium caprate and sodium deoxycholate could damage plasma and nuclear membranes.

The percent of insulin remaining versus time after incubation with α-chymotrypsin at 37°C, in the absence or presence of selected compounds that decreased its stability, is shown in Fig. 3. Although dextran 70 and pluronic F68 decreased insulin $T_{50\%}$, these compounds did not significantly affect insulin amount degraded within 1 min. Methylglycol chitosan (0.5, 1%), glycocholic acid (1%) and dimethyl-α-cyclodextrin (0.5, 5%) enhanced the amount of insulin degraded within 1 min, but did not significantly alter insulin $T_{50\%}$. In the meantime, L-α-phosphatidylcholine, taurocholic acid, taurodeoxycholic acid, taurochenodeoxycholate and pluronic F68 (1%) significantly decreased both insulin $T_{50\%}$ and the amount degraded within 1 min.

It was noticed that the incubation of sodium salicylate (1 and 2%) with insulin without α-chymotrypsin at 37°C produced a strange peak at the same insulin retention time with the appearance of metabolite A at zero time. Therefore, the use of sodium salicylate as an absorption enhancer should be reinvestigated.

Among the tested bile salts, taurocholic acid did not cause the formation of insulin metabolite C. Surprisingly, the incubation of taurocholic acid with insulin, in the absence of α-chymotrypsin, lead to the formation of a fifth metabolite E with 6.7 min retention time. Metabolite A was followed for insulin alone or in combination with all the tested compounds because it was detected with all compounds. Fig. 4 shows the profile of HPLC AUP of insulin metabolite A versus time in the presence of α-chymotrypsin without and with some representative compounds. The AUP of metabolite A showed a plateau level for most of the tested compounds, except taurodeoxycholic acid 1% and dextran 70 (1%), which showed a second plateau after 5 min.

Even though metabolites A and B were detected at zero time sample upon the addition of glycyrrhizic acid 1%, the percent degraded after 1 min was not significantly different ($P < 0.05$) than that with 0.5% concentration. The concentration of metabolite C was doubled with increasing glycyrrhizic acid concentration from 0.5 to 1%. Insulin's degradation $T_{50\%}$ s was 92.2 ± 8 and 43.5 ± 6 min after the addition of 0.5 and 1% glycyrrhizic acid, respectively. Therefore, glycyrrhizic acid should be used in concentration $\leq 0.5\%$ to increase insulin stability.

The results obtained would support the feasibility of utilizing such a method in the development of future insulin formulation to screen all the compounds that could affect insulin stability.

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