NB1-C16-Insulin: Site-Specific Synthesis, Purification, and Biological Activity

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Purpose. To develop a simple and efficient method for the synthesis and purification of NB1-lipid-modified-insulin without the use of protecting agents.

Methods. Bovine insulin was allowed to react with cis-9-hexadecenal in an aqueous-organic medium in the presence of $NaBH_3CN$ at room temperature overnight. HPLC and ESI LC/MS coupled with dithiothreitol and trypsin treatment were employed for product identification and optimization. The product was purified by a differential C18 solid-phase extraction. The biological effects of the modified insulin were evaluated by receptor binding assay and hypoglycemic effect measurement.

Results. NB1-cis-9-hexadecenyl insulin was synthesized by a one-step reductive alkylation in sodium salicylate and isopropanol solution in high yield (80%). The site selectivity and yield of the reaction were found to be affected by pH, medium, and insulin-to-aldehyde ratio. After solid phase extraction, the purity was found to be approximately 98%. This derivative showed a Kd to the insulin receptor of 5.72×10^{-9} M and a significantly slower glucose lowering rate than insulin.

Conclusions. NB1-hexadecenyl insulin was synthesized by reductive alkylation without the use of protective agents in high yield. NB1-hexadecenyl insulin retained significant binding affinity to insulin receptor and showed a pronounced hypoglycemic effect.

KEY WORDS: lipid modified insulin; site-specific synthesis; hypoglycemic effect.

INTRODUCTION

Lipid modification of insulin has been intensively investigated by pharmaceutical industries (1–2). Lipid-modified insulins can improve insulin stability in the small intestinal fluid (3), facilitate absorption of orally administered insulin (4), and endow membrane anchor on insulin. Additionally, by virtue of its sustained-release property via binding to tissue and circulating albumin, lipid-modified insulins dramatically prolong the circulation time and elevate concentrations of insulin in circulation (1–2,5). Insulin contains three amino acid moieties: A1Gly, B1Phe, and B29Lys; all of which are potentially useful for modification. A structure-activity study showed modification at B1Phe or B29Lys did not cause a significant loss in activity (6). However, synthesis of this site specific modified insulin invariably involved the use of protecting agents (7–8), which in turn required tedious separation and purification. Consequently, not only the preparation method was lengthy but the yield was also low. The aim of this present study is to simplify the method of preparation of lipidized-insulin. This method utilized reductive alkylation (9) of bovine insulin with hydrophobic aldehyde and sodium cyanoborohydride without the use of protecting agents (10). In order to derive an optimal condition, the effects of reaction medium composition, pH and insulin-to-aldehyde ratio on selectivity of the three amino groups were studied. The structure of the lipid-modified insulin was confirmed by electrospray liquid chromatography/mass spectrometry (ESI LC/MS) coupled to the enzymatic and chemical cleavages. Additionally, a simple C-18 solid-phase extraction procedure was developed for purification of the product in preparative scale. Furthermore, the biological activity of this insulin-analog was evaluated by the *in vitro* competitive insulin receptor binding assay and by the *in vivo* hypoglycemic test.

MATERIALS AND METHODS

Materials

Bovine insulin, dithiothreitol, trypsin, cis-9-hexadecenal, sodium cyanoborohydride, and streptozotocin were purchased from Sigma (St. Louis, MO). Sodium salicylate was obtained from Aldrich (Milwaukee, WI). ^{125}I-(A14)-insulin was purchased from LinCo. Research Inc, (St. Charles, MO). All chemicals used were of analytical grade and used as received.

Synthesis of NB1-cis-9-hexadecenyl Insulin

The synthetic approach of reductive alkylation of insulin by Young was adopted (10). Five mg of insulin were dissolved in 1.5 M, pH6.8 sodium salicylate in 54% isopropanol containing 1.25 mg of cis-9-hexadecenal and 110 μl of 50 mM $NaBH_3CN$. The reaction was carried out in a 10 × 12 mm glass tube at room temperature in a metabolic shaker (Dubnoff, Chicago, Il) with agitation overnight (60 rpm). The reaction was stopped by dialyzing the reaction mixture against distilled water (MW cutoff was 3500) at 4°C. Twenty μl of the dialyzed reaction mixture was removed in order to evaluate the extent of modification by reversed phase HPLC as described in the later section. Another 5 μl of the same mixture was also removed for ESI LC/MS analysis.

Effect of Medium Composition on Reaction

The site selectivity of the three amino acids (A1Gly, B1Phe and B29Lys) and the extent of reaction were explored by carrying out the reaction in different reaction media. In addition to sodium salicylate and isopropanol as described above, several reaction media evaluated were: 1) pH 7.0, 0.1 M NaH_2PO_4 in 33% (v/v) isopropanol; 2) DMSO/water, 2/1 (v/v); 3) pyridine/water, 1:0.25 (v/v); and 4) pH 8.5, 0.5 M $NaHCO_3$ in 50% isopropanol.

Effect of pH

Using 1.5 M sodium salicylate in 54% isopropanol, the reductive alkylation was carried out at pH values of 6.8, 7.8, and 10. Attempts were also made to carry out the reaction at pH 6.0 and 6.8 in 0.1 M NaH_2PO_4 in 33% isopropanol. This pH range was previously found to be effective for sodium

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cyanoborohydride reduction (11). After dialysis, 20 μl of the reaction mixture was injected onto the HPLC for evaluation of the extent of reaction.

Effect of Insulin-to-Reagent Ratio

Five mg of insulin in 1 ml of 1.5 M sodium salicylate solution was allowed to react with different amounts of cis-9 hexadecenal (0.75, 1, 1.25, 1.5, 2 and 3 mg) while keeping the molar ratio of aldehyde-to-$NaBH_3CN$ constant. The volumes of isopropanol used in each reaction tube were 1.2, 1.2, 1.2, 1.2, 1.5, and 2.0 ml, respectively. The increase in volume of isopropanol in the later two reactions was necessary in order to solubilize the increased amount of hydrophobic aldehyde. After dialysis, 20 μl of the reaction mixture was injected onto the HPLC for evaluation of the extent of reaction using the conditions as described below. The extent of reaction was evaluated by comparing the peak areas of the products to those of unreacted insulin.

Reversed-Phase HPLC

HPLC analysis was conducted on a BioRad HRLC system using a Polypropyl A column (5 μm, 200 mm × 4.6 mm, PolyLC Inc, Columbia, MD) and UV detection at 220 nm. A linear gradient was employed starting with 90% solvent A (10% acetonitrile in 0.025M, pH 2.5 H_3PO_4) to 50% solvent B (50% acetonitrile in 0.025M pH 2.5 H_3PO_4) in 25 min at a flow rate of 1 ml/min.

Purification Using Differential C18 Solid-Phase Extraction

One g of C18 reversed-phase resin (Varian, Harbor City, CA) was packed into a 0.8 × 4 cm Polyprep column (BioRad, Hercules, CA) followed by the washing of the column with 10 ml each of methanol and distilled water. After diluting the reaction mixture with 5 ml of distilled water, the reaction mixture was loaded onto the column using centrifugation (110 g, 4 min). The loading was repeated 6 times. UV absorbance at 280 nm was used to monitor the elution. Salts were first removed by washing the column with distilled water followed by centrifugation. Then, the components were sequentially eluted by 0.1% TFA with increasing methanol content. One and half ml of 0.1% TFA in 60% methanol was added to the column to elute the first component using centrifugation at 200 g for 3 min. The elution was repeated until the absorbance of the eluant was negligible. These eluants were pooled as Fraction I. The second and the third components were eluted by 0.1% TFA in 80% methanol and 0.1% TFA in 100% methanol, respectively, in the same manner as the first component. All the eluants of 0.1% TFA in 80% methanol were pooled as Fraction II and all the eluants of 0.1% TFA in 100% methanol were pooled as Fraction III. An aliquot from each fraction was analyzed by reversed phase HPLC for the tentative identity and purity determination. Methanol and TFA in these fractions were removed under vacuum, followed by lyophilization. The dry products from Fractions I, II and III were stored in −20°C for future use.

Structural Elucidation by Electrospray LC/Mass Spectrometry

The mass spectrometric analysis of the synthetic mixture and each of the purified products (I, II or III) and the DTT/trypsin treated samples were conducted on a PE Sciex API 300 LC-MS instrument. For analysis of the reaction mixture, a loop injection (without a column) was used. The mobile phase used was 50% (v/v) acetonitrile containing 0.1% TFA and the flow rate was 200 μl/min with a 1:20 (source:waste) split before entrance to the mass spectrometer. The sites of modification were determined by comparing the differences in mass of the corresponding fragments of insulin derivatives with the fragments of the unmodified insulin after they had been treated with DTT and trypsin. DTT treatment reduces the three disulfide bonds to sulfhydryl groups and separates insulin or insulin derivatives into A chain and B chain. Subsequent treatment with trypsin cleaves the B chain into 3 fragments: B1Phe-B22Arg, B23Gly-B30Ala and B23Gly-B29Lys, while keeps A chain intact. Thus, the modification on B1Phe, B29Lys or A1Gly can be determined if a mass increase of 222 is observed on fragment B_{1-22}, B_{23-30} or A chain, respectively. For the mass analysis of the intact molecule using ESI LC/MS, each sample was first dissolved in 100 mM NH_4HCO_3 at a concentration of approximately 2 mg/ml. Five μl of sample was then injected directly into the LC/MS for mass determination. For site modification determination, samples were first treated with DTT and trypsin. Approximately 0.5 mg each of the purified protein products (from Fractions I, II or III) was dissolved in 250 μl of 5 mM DTT in 100 mM NH_4HCO_3 solution, which was incubated in a 50°C water bath for 30 min followed by an addition of 5–10 μg of trypsin. The resultant solution was then incubated at 37°C for 3–4 hrs. Five μl of each of the treated samples was then separated on a reversed-phase HPLC column (Deltabond C8, 5 μm, 2 mm × 150 mm). A linear gradient starting with 90% solvent A (10% acetonitrile in water with 0.1% TFA) to 90% solvent B (70% acetonitrile in water with 0.09% TFA) in 40 min was used. The flow rate was 0.2 ml/min, with approximately 5 μl/min delivered into the mass spectrometer by a post column split. For all the mass analysis, ionspray and orifice voltages were set at 450 and 50 e.v., respectively, and the ring voltage was set at 400 e.v. The mass scanning rate was approximately 4.5 second/scan from m/z 500 to 2000 m/z.

Determination of Biological Activity

In Vitro *Insulin Receptor Binding Assay*

The biological activity of the lipid-modified insulin was determined using a competitive insulin receptor binding assay modified by Crettaz (12). HepG2 human hepatoma cell line (ATCC, Rockville, MD) was selected as a insulin receptor source and ^{125}I-(A14)-insulin as the radioligand. About 6 × 10^5 HepG2 cells were inoculated to each of the 33 mm cell culture dishes and allowed to attach to the dishes overnight. After the cells were washed twice with cell binding media (MEM with 0.1% bovine serum and 20 mM Hepes, pH 7.8), the cells were incubated in 1 ml binding medium containing 10 pmole of ^{125}I-Insulin (1757 mCi/μmole) and varying amounts of cold insulin or its lipid derivatives at 4°C for 2 hrs. In order to remove the unbound ^{125}I-insulin, each dish was quickly washed twice with 5 ml chilled Hank's media. The cells in each dish were then washed with 1 ml of 0.01% sodium dodecyl sulfate and an aliquot of 100 μl was transferred into a 12 × 77 mm plastic tube for γ-radiation counting. The binding displacement

curve and the Scatchard plot were constructed and the binding dissociation constant and maximum binding site were calculated from the Scatchard plot.

Induction of Diabetes

All animal studies were conducted under the guidelines of the Ohio State University Animal Review Board. The induction of diabetes in the rat was accomplished by a single i.v. injection of streptozotocin sodium citrate solution at a dose of 70 mg/kg (13). The onset of hyperglycemia usually occurred after one week. Following an overnight fast, glucose levels were then examined by Accu Chek® Advantage™ glucose meter (Boehringer Mannheim, Indianapolis, IN) using 15 μl of blood drawn from the tail vein. Only those rats with fasting glucose level >300 mg/dl were used for the hypoglycemic experiments.

In Vivo *Hypoglycemic Assay*

The hypoglycemic effect of NB1-cis-9-hexadecenyl insulin was evaluated in 6 normal rats and 6 streptozotocin-diabetic rats according to the following design. Male SD rats with an average weight of 180 g were used for all of the experiments. On the first day, three normal rats were first given i.v. insulin at 2.5 nmole/kg through the tail vein. Another 3 rats were given 2.5 nmole/kg of NB1-C16-insulin. After 24 hrs, the first group of rats was given NB1-C16-insulin at the same dose, while the second group the same dose of insulin. The same experiments were repeated using streptozotocin-diabetic rats. All of the rats were fasted overnight prior to the experiment. The drug solution was prepared in 0.025 M sodium salicylate from the dry powder in the day of the experiment. Prior to initiation of all of these experiments, a control sham run on the same rats was performed using 0.025 M sodium salicylate as the drug substitute. Blood glucose levels were measured periodically by Accu Chek® Advantage™ Glucose meter as before. After obtaining the glucose-time profile, glucose nadir and rate of glucose fall of this lipidized-insulin were compared with those of native insulin.

RESULTS

Synthesis of the Lipid-Modified-Insulin

The mass spectrum of the insulin reaction mixture (pH 6.8 sodium salicylate) as analyzed by loop injection LC/MS is shown in Fig. 1. There were a major ion at m/z 5956 which is consistent to the mass of mono cis-9-hexadecenyl (C16) insulin and two minor ions at m/z 5734 and at m/z 6179, corresponding to the masses of the unreacted insulin, and dihexadecenyl (di-C16) insulin, respectively. Fig. 2A shows the product profile of the reductive alkylation mixure of insulin as analyzed by HPLC. As shown, there were three major peaks α, β, and γ with retention times of 4, 13, and 19 min, respectively. Based on the molecular weights of the reaction products (Fig. 1) and their retention times on reversed phase HPLC (Fig. 2) in comparison with the insulin standard, the tentative identities of peaks α, β, and γ were assigned as the unreacted insulin, mono-C16 insulin, and di-C16 insulin, respectively.

Purification of C-16 Insulin with Differential C18 Solid-Phase Extraction

Using differential C18 solid-phase extraction, the reaction mixture (pH 6.8 sodium salicylate) was separated into three fractions (Fig. 3). Each fraction was analyzed by HPLC. Fraction I as eluted by 0.1% TFA in 60% methanol contained mainly Peak α (insulin). Fraction II as eluted by 0.1% TFA in 80% methanol was composed of only Peak β (mono-C16-insulin). Fraction III as eluted by 0.1% TFA in 100% methanol consisted of primarily Peak γ (di-C16 insulin). HPLC analysis of the reconstituted solution for the lyophilized powder of Fraction II showed a purity of 98% of mono-C16-insulin.

Structural Elucidation by ESI LC/MS

The mass spectral data for the purified fractions following the DTT or DTT/trypsin treatment are shown in Table I. The masses of Peak α (m/z 5733), Peak β (m/z 5857), and Peak γ (m/z 6178) were the same as the previously tentative assignments. After treatment with DTT only, a mass increase of 444 on the B chain was observed on the product of di-C16-insulin (Fraction III or Peak γ). No mass increase of 222 on A chain was observed for this dialkylated product. This indicated that B1Phe and B29Lys in the dialkylated insulin had been modified. After treatment with DTT and trypsin, a mass increase of 222 on fragment B_{1-22} was observed for the tentatively designated mono-C16-insulin (Peak β or Fraction II). No mass increase of 222 on A chain or on fragment B_{23-30} was observed. Thus, on this basis, the modification site of this mono-C16-insulin was determined solely on B1Phe.

Effect of Reaction Media on Synthesis

The results indicated there was a profound influence of the reaction media on the solubility, yield, and selectivity. Solubility of insulin was found to be rather poor in sodium phosphate buffer below pH 6.8 with and without isopropanol. Addition of sodium salicylate appeared to assist the dissolution of insulin. In pH 7.0 sodium phosphate buffer while insulin was initially soluble, significant precipitation of insulin occurred during the course of the reaction, resulting in a significant amount of unreacted insulin. In DMSO or pyridine with water, although there was no precipitation problem, substantial amount of unreacted insulin remained. In pH 8.5 sodium bicarbonate/isopropranol, nearly 90% of the product was di-C16-insulin. The highest yield for the intended product (76%) was obatined in pH 6.8 sodium salicylate in isopropanol.

Effect of pH on Synthesis

The effect of pH on the site selectivity and reaction rate were evaluated in the reaction media of sodium salicylate/isopropanol at pH values of 6.8, 7.8, and 10. It was found the product profile was not changed significantly for the first two pH values evaluated (Figs. 2A and 2B). At pH 10, the HPLC analysis gave 46.8% of the unreacted insulin (Peak α) and an overlapping multiple peak (Peak β region) (Fig. 2C), indicating that at a higher pH, the reaction rate was slower and the reaction selectivity was suppressed. An attempt of lowering the pH to 6.8 using sodium phosphate buffer and isopropanol was not successful because of severe precipitation of insulin under this condition.

Effect of Insulin-to-Aldehyde Ratio on the Yield of NB1-C16-Insulin

The percentages of mono-C16 linked insulin produced with various insulin-to-aldehyde molar ratios between 1:3.6 to

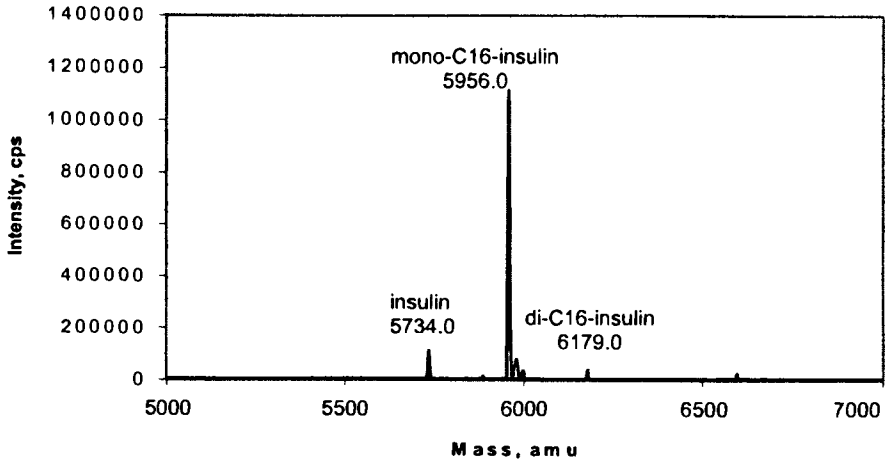

Fig. 1. Mass spectrum of the reductive alkylation mixture of insulin with cis-9- hexadecenal and sodium cyanoborohydride in sodium salicylate/isopropanol.

1:14.5 in pH 6.8 sodium salicylate/isopropanol solvent system are shown in Table II. As shown, the insulin-to-aldehyde ratio had an important effect on the yield of the mono-C16-insulin. At insulin-to-aldehyde ratio of 1:4.8, almost no di-C16-insulin was generated. When the insulin-to-aldehyde ratio was changed to 1:6, the yield of mono-C16-insulin was the highest (81.7%), and the di-C16-insulin was 12.5%.

Competitive Insulin Receptor Binding Assay

The binding displacement curves and the Scatchard plots of native bovine insulin and NB1-C16-insulin are shown in Fig. 4. As shown, both insulin and NB1-C16-insulin curves merged at a common point, indicating they have the same maximum binding sites. The insulin line showed a steeper slope, indicating the insulin has a lower dissociation constant. NB1-C16-insulin gave a dissociation constant Kd = 5.72×10^{-9} M, which is approximately 3-fold higher than that of unmodified bovine insulin (Kd = 1.87×10^{-9} M). Thus, the modified insulin still possesses significant binding strength to the insulin receptor.

In Vivo Hypoglycemic Effect

The results of the *in vivo* hypoglycemic effect of the mono-C16 insulin on normal and diabetic SD rats are shown in Fig. 5. Compared to the unmodified insulin, the alkylated insulin did not show differences in the glucose nadir in both normal and diabetic rats ($p > 0.05$). However, significant differences in times-to-nadir and rates of glucose fall were observed ($p < 0.01$) in both normal and diabetic rats. The difference was more pronounced in diabetic rats. Additionally, in diabetic rats, intravenous injection of alkylated insulin induced a slower rate of glucose fall (0.24 ± 0.038%/min) that lasted for 300 min, while native insulin caused a more rapid fall (1.125 ± 0.057%/min) which only lasted for 60 min. The duration of glucose fall is a better indicator for duration of hypoglycemic effect in the present model. The decreased blood glucose level did not return to the initial level without any exogenous glucose input, since glucagon response is impaired in streptozotocin-diabetic rats (15).

DISCUSSION

Insulin has a limited solubility in aqueous solution at neutral pH and tends to aggregate (14). The reaction medium for insulin alkylation should first be a good solvent for insulin. Most additives that can improve insulin solubility and minimize insulin aggregation are amino acids and Tris buffer that will interfere with insulin reductive alkylation. On the other hand, the solubility of insulin can be increased at higher pH, such as in $NaHCO_3$ buffer. However, this condition was found to retard the selectivity on B1Phe and gave a major product of NA1, NB29 dialkylated insulin (data no shown). Organic solvents such as DMSO and pyridine are good solvents for insulin, but they failed to produce adequate selectivity on any of the three amino groups in insulin.

The reactivity of the three amino groups in insulin toward alkylation is a reflection of their nucleophilicities (11). Thus, pH, the association state of insulin, and polarity of the solvent have an important effect in controlling the reactivity of these three amino groups (16). B29Lys with a relatively high pKa value of 11.2 (17) is less reactive to an electrophile than AlGly and B1Phe. Thus, the relatively reactivity of the α-amino and ϵ-amino groups can be controlled by pH adjustment. The effect of pH on the site selectivity and reaction rate was therefore evaluated in 1.5 M sodium salicylate in 54% isopropanol at pH values of 6.8, 7.8 and 10. It was found the product profile was not significantly changed at the first two pH values evaluated. At pH 10, the HPLC analysis gave 46.8% of the unreacted insulin (Peak α), and an overlapping multiple peak (Peak β or C16 insulin region) with retention time around 15 min, indicating that at a higher pH, the reaction rate was slower and the reaction selectivity was suppressed.

The selectivity of AlGly and B1Phe could be further achieved by solvent selection to control the accessibility of B1 instead of pH adjustment. Because the pKa values of AlGly (8.0) and B1Phe (6.7) are close (17), the precipitation problem does not permit the lowering of the pH value of the reaction medium to much below 6.8 which is close to the isoelectric point of insulin (4.5–6.5) (18). It has been shown native insulin assumes a stable hexamer formation (T6) in the presence of Zn(II) and B1Phe is buried in an hydrophobic pocket in the

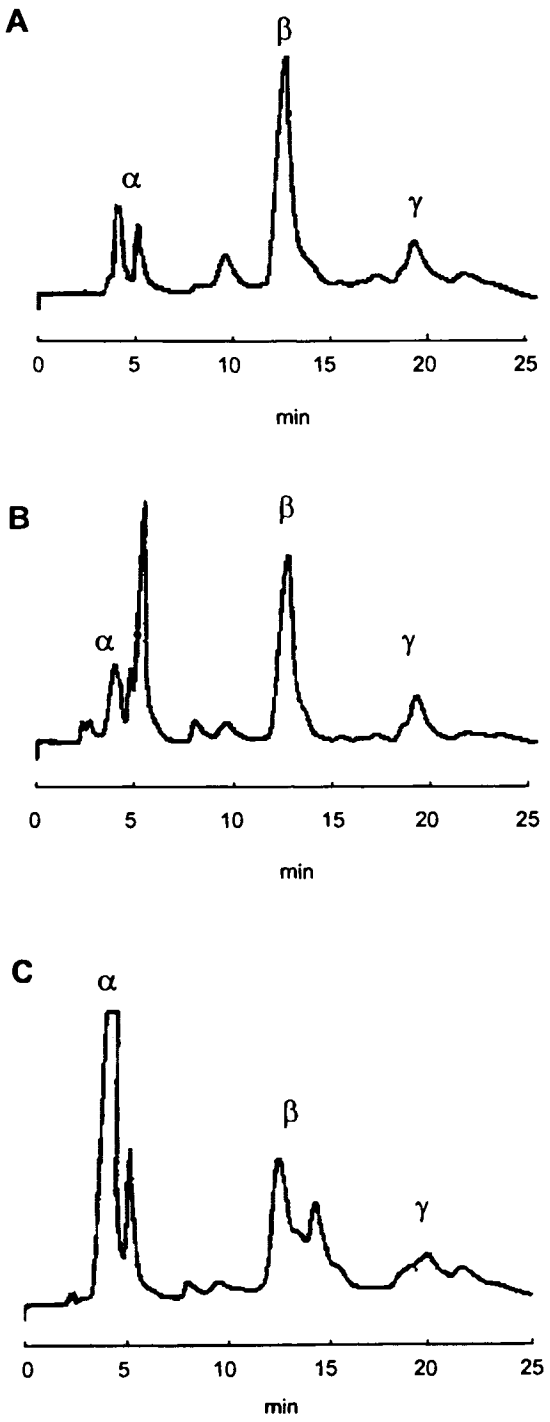

Fig. 2. HPLC chromatograms of the reductive alkylation mixture of insulin with cis-9-hexadecenal and sodium cyanoborohydride in sodium salicylate/isopropanol at different pH values: (A). pH 6.8, (B). pH 7.8, and (C). pH 10.

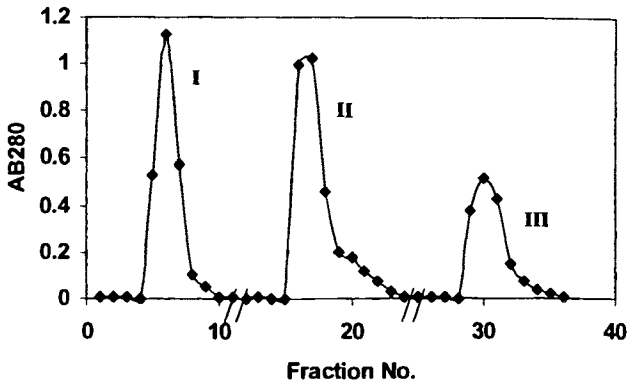

Fig. 3. The elution profile of the reductive alkylation reaction mixture of insulin from C18 solid phase column; Fraction I was eluted by 0.1% TFA in 60% methanol; Fraction II was eluted by 0.1% TFA in 80% methanol; Fraction III was eluted by 0.1% TFA in 100% methanol. The dimension of C18 column was 0.4 × 3.5 cm. Fraction size was 1.5 ml. See text for details.

dimer-dimer interface (19). Therefore, the accessibility of B1 will greatly enhanced when insulin hexamer is dissociated into dimer or monomer (16). It was also reported that in the presence of phenolic compounds, insulin undergoes a more stable hexamer transition (T6 → R6), which exposes B1 from the buried pocket to hexamer surface (20–21). Therefore, The accessibility of B1 may also be increased in a solvent system that does not dissociate insulin hexamers while facilitating the transformation of the insulin T6 to R6. It was reported that sodium salicylate, a phenolic compound, is able to dissociate a number of self-associated compounds including insulin and dramatically increases insulin solubility (22). Experimentally, we found that 1.5 M sodium salicylate, pH 6.8 was very effective in dissolving insulin and preventing precipitation of insulin during the reaction, whereas using a reaction mixture of 0.1 M sodium phosphate/isopropanol at pH 6.8 or 7.0 caused severe precipitation. Most importantly, the selectivity at the B1Phe site was greatly enhanced. Whether this B1Phe selectivity in sodium salicylate/isopropanol was due to the insulin hexamer dissociation, or T6 to R6 conformation transition remains to be confirmed by conformational study (23), which is beyond the scope of the present investigation. This high B1Phe selectivity allowed us to produce B1-lipid modified insulin in high yield without the use of protecting agents.

Two properties, charge and hydrophobicity, can be used to separate insulin and its lipid derivatives. A cation exchange separation method usually requires a two-step dialysis. First, the reaction mixture must be dialyzed to remove the salts that will interfere with the cation exchange process. After separation, the product needs to be dialyzed again to remove 7M urea that is required to unfold and expose the charge sites of insulin or its lipid derivatives during the cation exchange process. The addition of a C16 chain significantly increases the hydrophobicity of insulin. Separation based on this difference in hydrophobicity was thus explored. Young (10) employed hydrophobic interaction chromatography (HIC) to separate insulin and its lipid modified derivatives. However, insulin was detected in two peaks, suggesting separation of insulin in different association states. The second peak of insulin in HIC overlapped with the lipidized insulin product. It was possible that the column employed was not sufficiently hydrophobic. Therefore, a stronger hydrophobic phase, C18 solid-phase resin was selected, which has led to a simple, successful separation. In the current method, no dialysis was needed. With the use of centrifuge, the elution was facilitated, solvent consumption was reduced and processing capacity was increased.

Compared to the previously reported procedures (7–8), the current preparation procedure is much more efficient with

Table I. Mass Spectrometric Analysis of Insulin and Its Derivatives by ES LC/MS

	Peak α		Peak β		Peak γ	
	Observed	Calculated (insulin)	Observed	Calculated (NB 1 hexadecenyl insulin)	Observed	Calculated (NB 1,29 di-hexadecenyl insulin)
Mass of intact molecule	5733.0	5734.6	5857.0	5957.0	6178.0	6177.6
Mass fragments (DTT treatment)	not determined		not determined		3843.0	3843.9 (DiC16-B)
					4672.0	4671.3 (Dimer of A)
Mass fragments (DTT/trypsin treatment)	4671.2	4671.3 (Dimer of A)	4671.6	4671.3 (Dimer of A)		
	2487.6	2487.9 (B_{1-22})	5419.6	5418.7 (Dimer of C16-B_{1-22})	not determined	
	930.0	930.0 (B_{23-30})				
	859.6	859.0 (B_{23-29})	860.1	859.0 (B_{23-29})		

only one step of synthesis without the use of protecting agent, one step of purification, and final lyophilization. The entire preparation is extremely simple and only takes 1 day. Additionally, there was no isolation and purification of the reaction intermediates needed. The overall yield of the method was therefore significantly higher and was in the range of 50%.

LC/MS coupled with chemical and enzymatic digestion is a powerful tool in protein structural analysis (8). This method is more direct and efficient relative to the amino acid analysis. A similar approach using matrix-assisted laser desorption (MALDI) time-of-flight (TOF) mass spectrometry has been used for structural elucidation of insulin derivative (8).

This NB1-lipid-modified insulin maintains significant biological activity both *in vitro* and *in vivo*. Comparing to the unmodified insulin, this insulin analog shows a 3-fold decrease in binding constant and no difference in glucose nadir. Its biological activity was comparable to NB1-palmitoyl insulin that was prepared by different methods (2,7–8), which further confirmed that modification of B1Phe retains the biological activity of insulin. The alkylated insulin also showed slower and extended glucose lowering rate in diabetic rats, indicating that this insulin analog may be a good candidate for the soluble

Table II. Percentage of Unreacted Insulin, Mono C16 Insulin and Di C16 Insulin in the Sodium Salicylate/Isopropanol with Different Insulin-to-Aldehyde Ratio

Insulin:aldehyde (mole/mole)	Peak α (insulin)	Peak β (mono-C16 insulin)	Peak γ (di-C16 insulin)
1:3.6	93.4	6.6	0
1:4.8	41.3	58.7	0
1:6	5.7	81.7	12.5
1:7.2	4.9	77.8	17.2
1:9.6	3	57.4	39
1:14.5	4	43.7	52.2

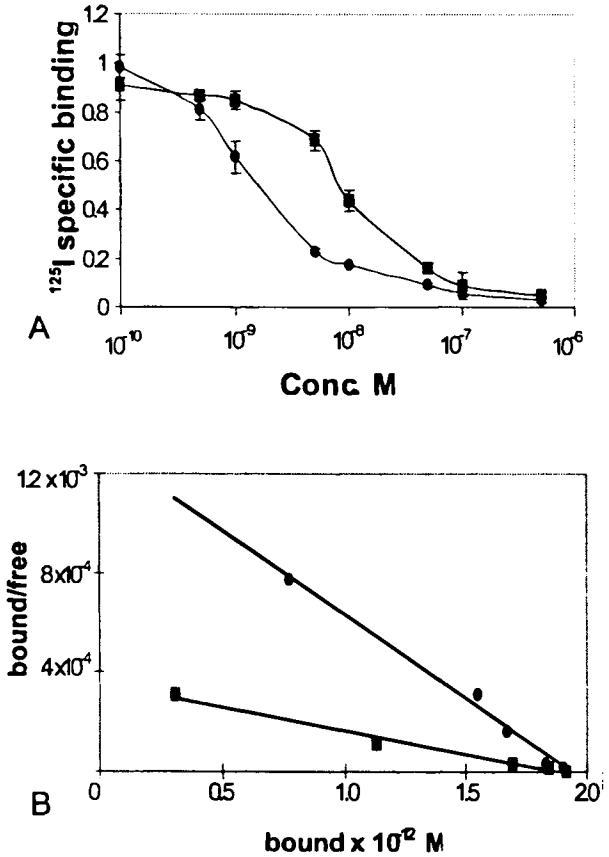

Fig. 4. Comparison of binding property of NB1-C16-insulin and unmodified insulin by competitive insulin receptor binding assay: (A). The displacement curve, (B). The Scatchard plot.

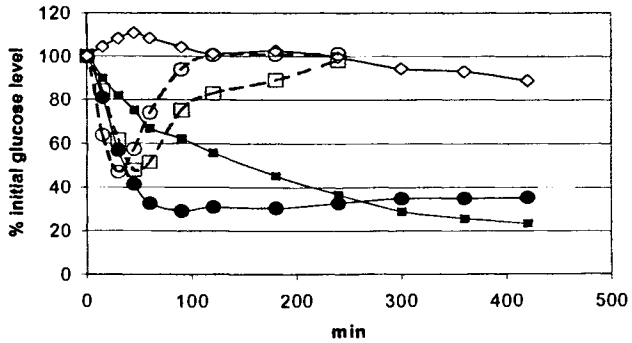

Fig. 5. Comparison of hypoglycemic effects of NB1-C16-insulin and insulin on normal SD and strepzotocin-diabetic rats following i.v. administration of 2.5 nmole/kg of: (○) insulin in normal rats, (●) insulin in diabetic rats, (□) NB1-C16-insulin in normal rats (■) NB1-C16-insulin in diabetic rats, and (◇) control in diabetic rats. Each point represents the mean ± S.D. of 6 experiments.

and long-acting insulin formulation. Additionally, it can facilitate its incorporation onto the surface of SUV liposome (27), serving as a targeting ligand for human hepatocellular carcinoma (28).

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