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Research Paper

Biochemical and Physiological Properties of a Novel Series of Long-Acting Insulin Analogs Obtained by Acylation with Cholic Acid Derivatives

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Purpose. This study was conducted to assess the suitability of insulin analogs acylated by various cholic acid derivatives for use as basal insulin, and to test the most promising of these, Lys^{B29}(N^{ε}-lithocholyl-γ-Glu) des(B30) human insulin (NN344) in pigs.
Methods. Circular dichroism spectroscopy and size-exclusion chromatography were used to explore the physicochemical properties of the analogs, and affinities for albumin and insulin receptors were determined. After subcutaneous injection in pigs, disappearance half-times were measured, and the plasma profile and glucose-lowering effect in a euglycemic clamp were assessed for NN344.
Results. NN344 showed glucose-lowering activity lasting more than 24 h. Glucose infusion rate was essentially constant from 5 to 19 h after injection. NN344 seemed to be a dodecamer in the presence of zinc ions and phenol. Without phenol, the apparent molecular mass was >5000 kDa. Formation of such a self-assembly at the site of s.c. injection and its subsequent slow decomposition might explain the long duration of action of NN344. A measurable affinity for albumin of the lithocholic acid ligand may also contribute to the prolonged action.
Conclusions. NN344 is a candidate for a neutral soluble basal insulin that might offer people with diabetes a prolonged duration, smooth, and predictable basal insulin supplement.

KEY WORDS: albumin binding; basal insulin therapy; insulin analog; self-assembly; soluble formulation.

INTRODUCTION

The Diabetes Control and Complications Trial (1), showing that near-normal blood glucose control could delay or prevent diabetic complications, drew attention to the need for insulin preparations with action profiles mimicking the situation in people without diabetes. The currently available neutral extended-acting insulin preparations are crystal suspensions (2,3) or amorphous precipitates of insulin (2). These preparations need to be carefully resuspended before s.c. injection, and there is significant variation in serum insulin profiles from one injection to the next (4,5).

We have previously reported a class of extended-acting insulin analogs acylated by fatty acids in position Lys^{B29}. One such analog, Lys^{B29}(N^{ε}-tetradecanoyl) des(B30) human insulin (NN304, insulin detemir), is soluble at neutral pH and displays affinity for human serum albumin (HSA). Its action profile is smooth, and variation between s.c. injections is low (6,7).

The continuing search for even longer duration of action has led to the discovery of a new class of insulin analog, again acylated in Lys^{B29} but this time with cholic acid derivatives. Hexamers of these analogs can self-assemble at neutral pH in the presence of zinc ions and the absence of phenol, resulting in prolonged activity, additional to that conferred by binding to serum albumin.

The most promising of these analogs, Lys^{B29}(N^{ε}-lithocholyl-γ-Glu), des(B30) human insulin (NN344), (Fig. 1) has affinity for HSA but is also capable of forming soluble high-molecular-weight structures by self-assembly of the hexameric complexes it forms with two zinc ions. Preliminary studies showed that a single s.c. injection in pigs gave an action profile that was flat for more than 24 h. Here we describe the physicochemical

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ABBREVIATIONS: CD, circular dichroism; GIR, glucose infusion rate; HIR, human insulin receptor; HSA, human serum albumin; kDa, kilodalton; NN304, Lys^{B29}(N^{ε}-tetradecanoyl) des(B30) human insulin (or insulin detemir); NN344, Lys^{B29}(N^{ε}-lithocholyl-γ-Glu) des(B30) human insulin; NPH, neutral protamine Hagedorn; SEC, size-exclusion chromatography.

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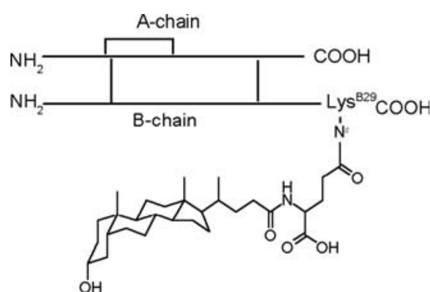

Fig. 1. Schematic representation of des(B30) human insulin acylated at the ε-amino group of the side chain of Lys^{B29}. The A and B chains consist of 21 and 29 amino acids, respectively. The lithocholic acid ligand is bound by an amide linkage to the amino group of glutamic acid and the carboxylic acid side chain is bound by an amide bond to Lys^{B29}. The free α-carboxylic group of the spacer mimics the carboxylic acid of the free lithocholic acid.

characterization of NN344 and its pharmacological properties in a pig model.

MATERIALS AND METHODS

Preparation, Formulation, and Labeling of Insulin Analogs

Insulin analogs were prepared by acylations of the ε-amino group of Lys^{B29} of biosynthetic des(B30) human insulin. Tetradecanoic acid and a series of cholic acid derivatives were coupled by peptide bonds as described previously (8). Formulations for pharmacokinetic and pharmacodynamic experiments contained 0.60 mmol L^{-1} of insulin, 2–3 Zn^{2+} per hexamer, 1.5% glycerol, and 0.3% phenol. In addition, formulated samples of insulins for pharmacokinetic experiments were labeled either by $^{65}Zn^{2+}$ ions for hexamer formation or by $^{125}I_2$ iodination of Tyr^{A14} (9). To investigate the effects of zinc on the disappearance of the analog NN344, a formulation was prepared without zinc ions (containing 0.3 mmol L^{-1} HSA for stabilization). NPH insulin was labeled by Tyr^{A14}(^{125}I human insulin).

Assessment of Zinc Ion Complexes of Insulin Analogs by Circular Dichroism Spectroscopy

To assess the allosteric interconversion of zinc hexamers, circular dichroism (CD) spectra were recorded in the near-UV (250–350 nm) and far-UV spectra (180–260 nm) at 20°C, using a Jasco J-715 spectropolarimeter calibrated with (+)10-camphorsulfonic acid. The data are expressed as Δε (L mol^{-1} cm^{-1}) normalized to the molar concentration of protein and peptide bond, respectively. Insulin analogs were dissolved at a concentration of 0.6 mmol L^{-1} in Tris–ClO_4^- buffer, 10 mmol L^{-1}, at pH 8.0. Phenol concentration was varied from 0 to 30 mmol L^{-1}, and the CD spectra were repeated in the same buffer containing 100 mmol L^{-1} NaCl.

Assessment of Zinc Ion Complexes of Insulin Analogs by Size-Exclusion Chromatography

Size-exclusion chromatography (SEC) was used to evaluate the ability of insulin and insulin analogs to form hexameric assemblies, in the presence of two zinc ions per hexamer. Samples (200 μL) of human insulin, or insulin analog, in a formulation containing two zinc ions per six insulin monomers and 30 mmol L^{-1} phenol, were subjected to SEC at 37°C using a 1 × 30 cm column of Superose® 6 HR (Superose 6® HR 10/30; Amersham Biosciences, Hilleroed, Denmark) at a flow of 0.25 mL min^{-1} with an eluent comprising NaCl (140 mmol L^{-1}), NaN_3 (3 mmol L^{-1}), and TRIS (10 mmol L^{-1}, pH 7.4). The effluent was monitored continuously by the absorbance at 276 nm. The apparent molecular mass of insulin in the peaks was estimated from a standard curve employing Blue Dextran, Thyroglobulin, Ferritin, Aldolase, HSA, Co(III)insulin hexamer (10), Ovalbumin, Ribonuclease, and X2 (monomeric Asp^{B9}, Glu^{B27} human insulin) (11). The chromatography was repeated with phenol added to the elution buffer to final concentrations of 2, 4, and 8 mmol L^{-1}.

Binding Studies and Insulin Assay

Binding of acylated insulin analogs to HSA was determined as previously described (12). Human fatty-acid-free serum albumin was immobilized to divinylsulfone-activated Sepharose 6B MiniLeak (Kem-En-Tec, Copenhagen, Denmark), to a concentration of 0.2 mmol L^{-1} suction dried gel. The immobilized HSA was suspended and diluted to cover the range 0–10 μmol L^{-1} in 100 mmol L^{-1} Tris buffer, pH 7.4, containing 0.025% Triton X-100 to prevent nonspecific adhesion. After incubation for 2 h at 23°C, free and albumin-bound analog were separated by centrifugation. Plots of bound/free analog vs. albumin concentration were linear and the association constant K_a was estimated from the slope of the plot.

The affinity of the various insulin analogs for the human insulin receptor (HIR) was determined by a microtitre plate antibody capture assay essentially as described in the literature (13). Microtitre plates were coated with affinity-purified goat antimouse IgG antibody (Pierce, Rockford, IL, USA) (50 μL $well^{-1}$ of 20 μg mL^{-1} solution in Tris-buffered saline: TRIS 0.15 mmol L^{-1}, pH 7.5, NaCl 100 mmol L^{-1}). Plates were incubated overnight at 4°C before blocking with 200 μL Superblock (Pierce), and then washed twice with assay buffer. Subsequently, a suitable dilution of a receptor-specific monoclonal antibody F12 (made in-house) was added. The assay buffer for this and all subsequent dilutions was HEPES (100 mmol L^{-1}, pH 8.0). Plates were incubated for 1 h, then washed three times with assay buffer. Human insulin receptor was added in a suitable dilution and the plates were incubated overnight at 4°C, then washed three times with assay buffer. Binding experiments were performed in a volume of 150 μL assay buffer, using 8–10 pmol L^{-1} of Tyr^{A14} ^{125}I insulin tracer, and varying the concentrations of insulin and insulin analogs. After 36 h at 4°C, unbound ligand was removed by washing three times with cold assay buffer, and the tracer bound in each well was counted in a gamma counter. The binding data were fitted using the nonlinear regression algorithm in the GraphPad Prism 2.01 (GraphPad Software, San Diego, CA, USA).

The concentration of insulin in plasma samples from pigs was determined by an insulin ELISA assay (DAKO K6219; Dako Ltd., Cambridgeshire, UK). The concentration of NN344 in plasma samples was detected by a dissociation-enhanced lanthanide fluorescent sandwich immunoassay, using a monoclonal antibody against human insulin for

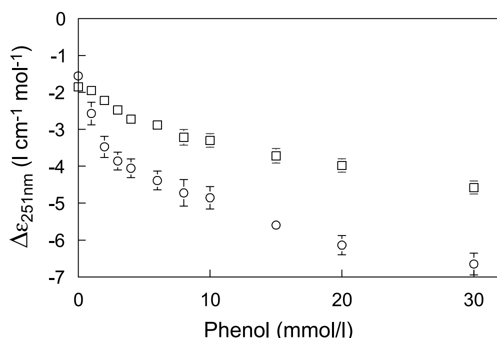

Fig. 2. Circular dichroism spectra at 251 nm of NN344 (□) and human insulin (○) with increasing phenol concentration. Concentration of insulin or analog was 0.6 mmol L^{-1} containing 2 Zn^{2+} per hexamer in TRIS/ClO_4^- 10 mmol L^{-1}, pH 8.0. The *bars* represent SEM.

capture and, for detection, a monoclonal antibody directed against the lithocholic acid side chain and labeled by the fluorescent isotope of europium. Recovery of NN344 from plasma, and linearity in dilutions of plasma, were satisfactory. The lower limit of quantification was 32 pmol L^{-1}. No cross-reactivity was observed against pig insulin.

Studies in Pigs

The principles of laboratory animal care were followed. Specific pathogen-free LYYD, nondiabetic female pigs, crossbreed of Danish Landrace, Yorkshire, and Duroc (Holmenlund, Haarloev, Denmark) were used in the conscious state for pharmacokinetic and pharmacodynamic studies after being fasted overnight. The pigs were 4–5 months of age and weighed 70–95 kg.

Disappearance of Labeled Insulin from Injection Site in Pigs

Preparations of insulin analogs labeled in position Tyr^{A14} by ^{125}I, or as hexamers by $^{65}Zn^{2+}$, were injected s.c. in pigs as previously described (14). The disappearance of the radioactive label from the site of s.c. injection was monitored using external gamma counting (15). The method was modified to allow the disappearance of radioactivity from a subcutaneous depot to be measured continuously over several days using a cordless portable device (Scancys Laboratorieteknik, Vaerloese, Denmark). The counts were accumulated at intervals of 1 min, and the values corrected for background radioactivity. An insulin dose of 60 nmol (equal to 10 units of human insulin) was used, and in each experiment each pig received separate depots of both the test analog and the reference, insulin detemir.

Euglycemic Glucose Clamp

Each pig was clamped at its fasting blood glucose level for 24 h after s.c. injection. The dose was divided between three identical depots in the NN344 clamp experiment, and two in the NPH experiments, to administer a clinically relevant dose and to counteract the variation in absorption observed with NPH (5). A total of 432 nmol NN344 (corresponding to 72 units of human insulin) was administered to each of eight pigs (fasting blood glucose level, 4.3 ± 0.3 mmol L^{-1}). Two experiments were conducted by administration of either 148 or 216 nmol of NPH to seven and five pigs, respectively (fasting blood glucose level, 4.4 ± 0.3 and 4.4 ± 0.1 mmol L^{-1}). In another experiment, four pigs received 210 nmol s.c. of insulin analog Lys^{B29}(N^{ε}-hyocholyl) des(B30) human insulin in two depots (fasting blood glucose 4.8 ± 0.5 mmol L^{-1}). All pigs were kept euglycemic at their individual fasting glucose levels for 24 h by a variable rate intravenous infusion of a 1.11 mol L^{-1} glucose solution. The infusion was given through a catheter inserted in the jugular vein using a Braun Infusumat Secura pump (Braun, Melsungen, Germany). Depending on changes in plasma glucose concentrations observed during frequent plasma glucose monitoring, the necessary adjustments of the glucose infusion were made empirically. Blood samples were collected in heparinized glass tubes every 15 min, plasma was separated, and glucose was determined within 1.5 min of blood sampling with a YSI glucose analyzer (glucose oxidase method) (Yellow Springs Instrument, Yellow Springs, OH, USA). During the experiment, the pigs were free to move in their pens. The observed GIR profiles were extended by exponential extrapolation, and $AUC_{0-\infty}$ was calculated by standard pharmacokinetic methods (16). The relative potency of NN344 was calculated by standard methods for parallel line bioassay for the relation between $AUC_{0-\infty}$ and the logarithm of the insulin dose (17).

RESULTS

Self-Assembly of Insulin Analogs

An increase in phenol concentration from 0 to 30 mmol L^{-1} was associated with a change in the CD spectrum of human insulin at 251 nm, from −1.5 to −6.5 Δε (L mol^{-1} cm^{-1}) (Fig. 2), reflecting a change in conformational state from T_6 through T_3R_3 to R_6. The spectrum for NN344 gave a smaller response, from −1.8 to −4.5 Δε (L mol^{-1} cm^{-1}) (Fig. 2). Chloride ions produced only a weak effect with NN344 (data not shown).

The propensity of zinc ion complexes of insulin analogs to self-assemble was studied by SEC. Profiles obtained for

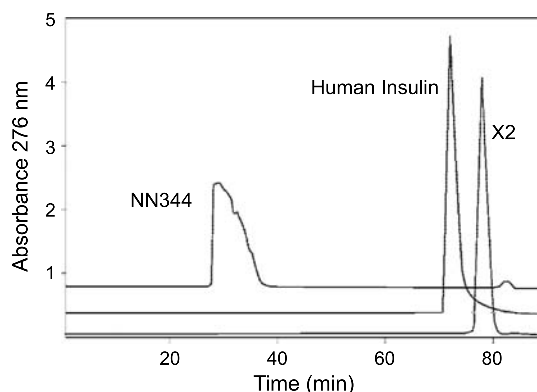

Fig. 3. Size-exclusion chromatography profiles of NN344, human insulin, and the monomeric insulin analog X2 (B9Asp,B27Glu) human insulin, all three formulated at neutral pH in the presence of zinc ions and phenol, giving apparent molecular mass of >5000, 20, and 6 kDa, respectively. Further details are given in Materials and Methods.

Table I. Physicochemical and Pharmacological Characteristics of Insulin Analogs Acylated by Cholic Acid Derivatives

Analog[a]	Disappearance (h) Zn^{2+} + phenol[b]	Apparent molecular mass (kDa)		Binding (relative affinity)	
		Zn^{2+c}	Zn^{2+} + phenol[d]	To HSA[e]	To HIR[f]
Lys^{B29}(N^{ε}-lithocholyl) des(B30) HI	>34.0	>5000	3	0.38	0.33
Lys^{B29}(N^{ε}-lithocholyl-β-Asp) des(B30) HI	26.8	>5000	46	0.23	0.08
Lys^{B29}(N^{ε}-hyocholyl) des(B30) HI	25.8	>5000	27	0.05	0.43
Lys^{B29}(N^{ε}-norlithocholyl) des(B30) HI	23.0	44	6	0.44	0.35
Lys^{B29}(N^{ε}-lithocholyl-γ-Glu) des(B30) HI (NN344)	22.8	>5000	74	0.33	0.13
Lys^{B29}(N^{ε}-ursodeoxycholyl) des(B30) HI	14.0	26	25	0.07	0.37
Lys^{B29}(N^{ε}-lithocholyl-α-Glu) des(B30) HI	11.8	46	38	0.30	0.11
Lys^{B29}(N^{ε}-cholyl) des(B30) HI	10.2	18	23	0.01	0.39
Lys^{B29}(N^{ε}-3-β-hydroxy-cholanoyl) des(B30) HI	6.5	30	24	0.05	0.48
Insulin detemir (Lys^{B29}(N^{ε}-tetradecanoyl) des(B30) HI	14.3	45	28	1.00	0.28
Human insulin	2.0	20	25	–	1.00

[a] Acylated $N^{\varepsilon B29}$-des(B30) human insulin.
[b] Time of 50% disappearance after s.c. injection in pigs of 0.6 mmol L^{-1} ^{125}I-labeled insulin analog formulated in 1.5% glycerol, 30 mmol L^{-1} phenol, and two zinc ions per hexamer. Insulin detemir was used as an internal standard in all experiments.
[c] The maximum molecular mass observed after SEC analysis of 200 μL 0.6 mmol L^{-1} insulin analog formulated in 1.5% glycerol, 30 mmol L^{-1} phenol, and two zinc ions per hexamer at 37°C. The column was eluted by 140 mmol L^{-1} NaCl, 3 mmol L^{-1} NaN_3, and 10 mmol L^{-1} TRIS, pH 7.4, at a flow of 0.25 mL min^{-1}.
[d] SEC analysis as in footnote c after addition to the elution buffer of phenol to 8 mmol L^{-1}.
[e] Affinity for the HSA relative to Lys^{B29} (N^{ε}-tetradecanoyl) des-(B30) human insulin (insulin detemir) (2.4×10^5 L mol^{-1} at room temperature).
[f] Affinity for the human insulin receptor relative to human insulin (4.3×10^9 L mol^{-1}).

N344, human insulin, and a monomeric insulin analog are shown in Fig. 3, and results are summarized in Table I. The analogs NN344 and Lys^{B29}(N^{ε}-lithocholyl) des(B30) human insulin each eluted in a single peak in the exclusion volume corresponding to a molecular mass >5000 kDa, followed by a long narrow tail and a small peak in the range of 30 to 6 kDa. However, Lys^{B29}(N^{ε}-lithocholyl) des(B30) human insulin was found to form a precipitate when stored for 2 weeks in a formulation containing zinc ions and phenol. More complex chromatographic profiles were obtained with Lys^{B29}(N^{ε}-lithocholyl-β-Asp) des(B30) human insulin and Lys^{B29}(N^{ε}-hyocholyl) des(B30) human insulin. Each gave rise to two peaks of similar size, the high-molecular-weight peak displaying an apparent molecular mass of >5000 kDa and the low-molecular-weight peak appearing just outside the separation volume of the column. For all the other insulin analogs carrying a ligand derived from cholic acid, the profile showed a single peak in the range of 46 to 18 kDa (Table I). Human insulin and insulin detemir standards each displayed a single peak, at 20 and 45 kDa, respectively.

Addition of 2, 4, and 8 mmol L^{-1} phenol to the eluent changed the chromatographic profiles of most of the analogs to a single peak eluting at an apparent molecular mass of about 30 kDa. Exceptions were NN344, which seemed to have a molecular mass of 74 kDa, and Lys^{B29}(N^{ε}-lithocholyl) des(B30) human insulin and Lys^{B29}(N^{ε}-norlithocholyl) des(B30) human insulin, which both appeared as multiple peaks with molecular mass close to 6 kDa (Table I).

Binding Studies

Results of the binding studies are summarized in Table I. Affinity for HSA is expressed relative to that found for the fatty acid acylated analog, insulin detemir. The affinities of insulin analogs acylated by cholic acid derivatives ranged from 0.01 for Lys^{B29}(N^{ε}-cholyl) des(B30) human insulin to 0.44 for Lys^{B29}(N^{ε}-norlithocholyl) des(B30) human insulin, with NN344 having an affinity one-third that of insulin detemir.

Binding to the human insulin receptor gave values ranging from 0.08 to 0.48 relative to human insulin, comparable to that found for insulin detemir (0.28).

Disappearance of Radioactivity After s.c. Injection of Labeled Analogs

The time to 50% disappearance ($T_{50\%}$) of ^{125}I labeled analogs obtained in pigs presented in Table I are corrected for between-assay variation employing insulin detemir as an internal standard (9). The 50% disappearance increased with increasing apparent molecular mass of the zinc complex, as determined without phenol in the elution buffer (Table I, rank correlation coefficient 0.82, $p < 0.01$). Four analogs with apparent molecular mass >5000 kDa gave a $T_{50\%}$ in the range 22.8 to >34.0 h. Four analogs with apparent molecular mass ranging from 46 to 18 kDa gave a $T_{50\%}$ ranging from 14 to 6.5 h. One analog, Lys^{B29}(N^{ε}-norlithocholyl) des(B30) human insulin, did not follow this pattern, having an apparent molecular mass of 44 kDa but with a $T_{50\%}$ as long as 23 h.

In an experiment to investigate the effect of zinc ions, disappearance profiles were obtained for zinc and phenol formulations of NN344 and insulin detemir that had been labeled either with ^{65}Zn or with ^{125}I (Fig. 4). The $T_{50\%}$ of ^{65}Zn-labeled NN344 was 14.3 h, very different to the 2.5 h obtained for insulin detemir (NN304), which is close to the 2.1 h obtained with a $^{65}ZnCl_2$-labeled formulation containing no insulin analog (Fig. 4). For NN344, $T_{50\%}$ for the ^{125}I-labeled analog was 18.8 h, whereas for insulin detemir (NN304) $T_{50\%}$ was 9.7 h for the analog (Fig. 4). When

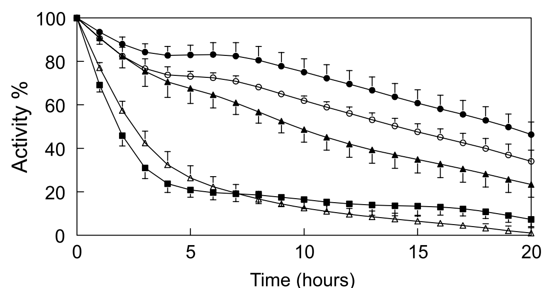

Fig. 4. Disappearance of radioactivity from the skin of pigs ($T_{50\%}$, $n = 5$) after s.c. injection of a 2 zinc per hexamer and phenol formulated sample of either NN344 or NN304 (insulin detemir) labeled by ^{125}I or $^{65}Zn^{2+}$. Insulin analog ^{125}I-NN344/Zn^{2+} (●), $T_{50\%}$ = 18.8 h; NN344/$^{65}Zn^{2+}$ (○), $T_{50\%}$ = 14.3 h; ^{125}I-NN304/Zn^{2+} (▲), $T_{50\%}$ = 9.7 h; NN304/$^{65}Zn^{2+}$ (△), $T_{50\%}$ = 2.5 h; and a $^{65}ZnCl_2$ control (■), $T_{50\%}$ = 2.1 h, $n = 6$. The bars represent SEM.

NN344 was injected in the form of a formulation containing 0.3 mmol L^{-1} HSA and no zinc ions, the $T_{50\%}$ was only 7 h.

Values of $T_{50\%}$ obtained for the nine analogs acylated by cholic acid derivatives did not correlate ($r = 0.54$, $p > 0.05$) with their affinity for HSA (Table I).

Euglycemic Glucose Clamp

Figure 5 shows the plasma profiles, after subcutaneous injection, of NN344 or NPH insulin. The prolonged action of NN344 is reflected in a continued concentration of analog in the plasma of pigs for a long time after the s.c. injection (Fig. 5). In glucose clamp experiments, NN344 displayed an almost constant GIR (4.8 ± 0.6 mg kg^{-1} min^{-1}) from 5 to 19 h after s.c. injection (Fig. 6). To estimate the potency of the analog, the area under the GIR curve was extrapolated to infinity and compared to the areas from two similar experiments using NPH. The estimated $AUC_{0-\infty}$ indicated a potency of the analog of 118% relative to NPH with a lower 95% limit of 66% (no upper limit could be determined). The time to maximum glucose infusion rate (T_{max}) was 10.8 ± 1.7 h for NN344, compared with 3.4 ± 0.4 and 3.1 ± 0.2 h for NPH at the two dosages used in the clamp experiments (Table II). The maximum infusion rate for NN344 was 5.8 ± 0.6 mg kg^{-1} min^{-1} after the s.c. administration of 5.1 nmol

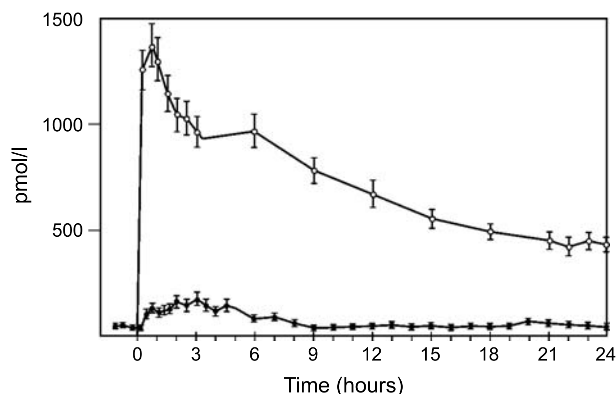

Fig. 5. Plasma profiles after s.c. injection in pigs of 148 nmol NPH insulin (■, $n = 7$) or 432 nmol of NN344 (○, $n = 8$). The *bars* represent SEM.

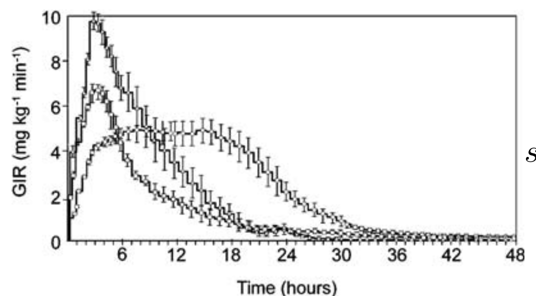

Fig. 6. Glucose infusion rate profiles obtained in euglycaemic glucose clamp experiments in pigs after s.c. injection. The *bars* represent SEM. The glucose clamp levels were equal to fasting glucose levels. Eight pigs received NN344 432 nmol per animal (○), glucose level 4.3 ± 0.3 mmol L^{-1}. Seven pigs received NPH insulin 148 nmol per animal (□), glucose level 4.4 ± 0.3 mmol L^{-1}. Five pigs received NPH insulin 216 nmol per animal (△), glucose level 4.4 ± 0.1 mmol L^{-1}.

kg^{-1}. In contrast, the maximum infusion rate obtained with NPH was 7.1 ± 0.3 and 9.9 ± 0.4 mg kg^{-1} min^{-1} after the administration of 1.8 and 3.3 nmol kg^{-1}, respectively. A similar experiment obtained employing Lys^{B29}(N^{ε}-hyocholyl) des(B30) human insulin resulted in a biphasic profile with a sharp peak 3–4 h after s.c. injection, followed by a long narrow tail for 24 h (data not shown).

DISCUSSION

Earlier investigations of fatty acid acylated insulin analogs, which include insulin detemir, revealed that disappearance $T_{50\%}$ was strongly correlated ($r = 0.97$) with affinity for HSA, and it was concluded that the affinity for HSA was related to the prolonged action of these analogs (6). In the present study, des(B30) human insulin analogs acylated in position $N^{\varepsilon B29}$ by derivatives of cholic acid were found to display a wide range of affinities for HSA, but when the relationship to $T_{50\%}$ was analyzed, the weak correlation ($r = 0.54$, $p > 0.05$) indicated that serum albumin affinity was not making a significant contribution to the long disappearance times observed with these analogs. Affinity of these cholic-acid-derived analogs for HSA was reduced compared to the free ligands (18).Thus, Lys^{B29}(N^{ε}-lithocholyl) des(B30) human insulin, Lys^{B29}(N^{ε}-cholyl) des(B30) human insulin, and Lys^{B29}(N^{ε}-ursodeoxycholyl) des(B30) human insulin displayed 46%, 6%, and 8% of the affinity for HSA shown by their respective free ligands (18).

The insulin analogs NN344, Lys^{B29}(N^{ε}-lithocholyl) des(B30) human insulin, Lys^{B29}(N^{ε}-hyocholyl) des(B30) human insulin, and Lys^{B29}(N^{ε}-lithocholyl-β-Asp) des(B30) human insulin all displayed a $T_{50\%}$ longer than 20 h and an apparent molecular mass >5000 kDa determined in the SEC analytical system developed to mimic the conditions at the site of s.c. injection. Only insulin analogs NN344 and Lys^{B29}(N^{ε}-lithocholyl) des(B30) human insulin eluted as a single major peak when subjected to SEC analysis. However, the $N^{\varepsilon B29}$(N-lithocholyl) derivative precipitated when stored in a formulation containing zinc ions and phenol (19). The analogs Lys^{B29}(N^{ε}-lithocholyl-β-Asp) des(B30) human insulin and Lys^{B29}(N^{ε}-hyocholyl) des(B30) human insulin each displayed both a high- and a low-molecular-weight peak of

Table II. Euglycaemic Glucose Clamps After Single Subcutaneous Injection of NPH or NN344 in Pigs

	Dose (nmol kg^{-1})	No. of pigs (*n*)	GIR_{max} (mg kg^{-1} min^{-1})	T_{max} (h)	Total glucose infusion $AUC_{0-\infty}$ (g kg^{-1})
NPH	1.80	7	7.1 ± 0.3	3.4 ± 0.4	3.8 ± 0.3
NPH	3.26	5	9.9 ± 0.4	3.1 ± 0.2	5.4 ± 0.6
NN344	5.07	8	5.8 ± 0.6	10.8 ± 1.7*	7.0 ± 0.7

*$p < 0.0001$ relative to both NPH experiments.
By extrapolation of the dose-response effect of $AUC_{0-\infty}$ with NPH insulin the relative potency of NN344 is found to be 118% with lower 95% limit of 66% (no upper limit can be determined).

roughly equal size. The latter of these two analogs displayed a biphasic activity profile when subjected to the euglycemic glucose clamp in pigs. Analogs displaying low apparent molecular mass in the SEC analysis (46 to 18 kDa) also showed low $T_{50\%}$ after s.c. injection (14 to 6.5 h). One analog did not follow this pattern. Lys^{B29}(N^{ε}-norlithocholyl) des(B30) human insulin displayed an apparent molecular mass of 43 kDa and a $T_{50\%}$ of 23 h. The reason for this is unclear.

NN344 is unique in that it is stable formulated as a hexameric zinc complex in the presence of 30 mmol L^{-1} phenol, and as a high-molecular-weight structure composed of insulin zinc hexamers in the absence of phenol. The disappearance $T_{50\%}$ of NN344 depends on the presence of zinc. When NN344 was injected s.c. in pigs in a formulation containing 0.3 mmol L^{-1} HSA and no zinc ions, $T_{50\%}$ disappearance was only 7 h, whereas a zinc and phenol formulation resulted in a $T_{50\%}$ of 18.8 h for the analog and 14.3 h for the zinc ions. The slow disappearance of zinc from the injection site suggests a slow dissociation of the NN344 zinc hexamer. In contrast, $T_{50\%}$ values for insulin detemir were 9.7 h for the analog and 2.5 h for the zinc ions, respectively, the latter being close to the 2.1 h obtained after s.c. injection of $^{65}ZnCl_2$. Thus, it seems that NN344 in solution is stabilized by zinc ions and that this stabilization contributes to its long disappearance $T_{50\%}$.

SEC analysis of NN344 after addition of phenol to the elution buffer resulted in a decrease in apparent molecular mass from >5,000 kDa to 74 kDa, corresponding to an insulin zinc dodecamer.

Within insulin hexamers, the monomers may be present in a T or an R form. The conformation of the zinc hexameric complexes of human insulin and insulin detemir changes from T_6 through (20,21) T_3R_3 to R_6 when the phenol concentration is increased from 0 to 30 mmol L^{-1} at neutral pH (20). The CD measurements conducted in the present study suggest that the same conformational changes occur with NN344. However, the present study does not reveal whether it is the removal of phenol or the associated conformational changes of the hexamers that causes the formation of the high-molecular-weight complex. In the case of human insulin, chloride ions are known to promote formation of T_3R_3 and, in the presence of phenol, of the R_6 insulin zinc hexamer (22), but they seemed to have only a weak effect on NN344 when analyzed by CD. Attempt to study the NN344 hexamer contacts in the crystalline state was unsuccessful in contrast to Lys^{B29}(N^{ε}-lithocholyl) des(B30) human insulin (19). This structure revealed interhexamer contacts mediated by the lithocholic acid ligand that binds to pocket in a neighboring hexamer in close proximity to a symmetrically contact formed by the lithocholic acid ligand of a neighboring hexamer (19). The unique contacts observed with Lys^{B29}(N^{ε}-lithocholyl) des(B30) human insulin might be related to the ability of NN344 to self-assemble as well as other insulin analogs acylated by derivatives of cholic acid (Table I).

The flat action profile from 5 to 19 h revealed by the glucose clamp study is highly attractive for intensified basal-bolus therapy in diabetic patients. Previous work with another neutral soluble acylated insulin analog, insulin detemir, has demonstrated that the absorption varies significantly less than that of NPH insulin (6) and insulin glargine (7).

The improved kinetics have been explained by the distribution of the analog in a larger volume in subcutaneous tissue than that of an NPH depot, deposited near the tip of the needle. The superiority of neutral soluble preparations with respect to tissue damage, as compared with NPH insulin, was first shown by histological examination at the site of injection of insulin detemir (6). This has since been confirmed in a similar experiment using NN344 (Erik Hasselager, personal communication). The nature of the NN344 plasma profile and the nearly constant GIR for 14 h after a single s.c. injection in pigs is in contrast to the peak obtained in the experiments with NPH insulin.

Extrapolation of the NN344 activity profile shows that activity is present for 36 h after a single s.c. injection and that the analog is equipotent to NPH (118%). The reduced affinity of NN344 for the insulin receptor does not affect the potency *in vivo* (23). However, the reduced affinity provides a minor contribution to the prolonged duration of NN344; this result was confirmed in a study where Lys^{B29}(N^{ε}-7-deoxocholyl) human insulin was injected i.v. to rats (23,24).

We conclude that formulations of NN344 with 2–3 zinc ions per 6 insulin molecules at neutral pH in the presence of phenol display an apparent molecular mass of 74 kDa, corresponding to a dodecamer. This value increases to more than 5000 kDa when the phenol is omitted. The mechanism leading to prolonged action of NN344 when injected as a neutral formulation containing zinc and phenol is likely to be the very rapid absorption of phenol followed by self-assembly of NN344, forming a soluble and high-molecular-weight network of hexamers, each stabilized by two zinc ions. The slow decomposition of the network results in a slow release of the analog from the site of s.c. injection into the blood stream. Moreover, the affinity of NN344 for serum albumin makes a minor contribution to the extended action, both at the site of injection and in the circulation.

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