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Insulin Glulisine, a New Rapid-Acting Insulin Analogue, Displays a Rapid Time–Action Profile in Obese Non-Diabetic Subjects

Article

Abstract

Aims/hypothesis: This study compared the pharmacokinetics and pharmacodynamics of insulin glulisine, insulin lispro, and regular human insulin in obese subjects. **Methods:** In this single-dose, randomized, double-blind, crossover euglycaemic clamp study, 18 non-diabetic subjects (mean body mass index [BMI] 34.7 $kg \cdot m^{-2}$) were randomized to receive subcutaneous injections of each insulin (0.3 $U \cdot kg^{-1}$) in pre-determined sequences. **Results:** Insulin glulisine and insulin lispro had more rapid-acting profiles than regular human insulin. Fractional glucose infusion rate (GIR)-area under curves (AUC) of the GIR curve and maximum GIR were greater for insulin glulisine and insulin lispro versus regular human insulin. Total glucose disposal was slightly greater with insulin glulisine than with regular human insulin, and was comparable to insulin lispro, although it decreased with increasing insulin resistance (HOMA index) with all insulins. Time to 20% (early glucose disposal) and 80% (bulk of activity) of total GIR-AUC were shorter for insulin glulisine and insulin lispro versus regular human insulin. This was corroborated by more rapid and shorter residing pharmacokinetic profiles of insulin glulisine and insulin lispro versus regular human insulin, evidenced by shorter times to 20% of total INS-AUC, INS-C_{max} (INS-t_{max}), and mean residence time. Moreover, time to 20% of total GIR-AUC demonstrated a less rapid-acting profile for insulin lispro versus insulin glulisine, which was consistent with the slightly less rapid pharmacokinetic profile of insulin lispro. There was no significant correlation between BMI or subcutaneous fat thickness and pharmacokinetic or pharmacodynamic profiles for insulin glulisine, unlike insulin lispro and regular human insulin. **Conclusions/interpretation:** Insulin glulisine and insulin lispro demonstrated substantially more rapid time–action profiles than regular human insulin in obese non-diabetic subjects, which prevailed with insulin glulisine irrespective of BMI and subcutaneous fat thickness.

Key words
Obesity · insulin analogues · pharmacokinetics · pharmacodynamics

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Abbreviations

sc	subcutaneous
RHI	regular human insulin
MRI	magnetic resonance imaging
PK	pharmacokinetic
PD	pharmacodynamic
MRT	mean residence time
GIR	glucose infusion rate
BMI	body mass index
RIA	radioimmunoassay
LLOQ	lower limit of quantification
EKG	electrocardiogram

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Introduction

Timely prandial (bolus) insulin substitution for the intensified (DCCTRG, 1993; UKPDS, 1998) and convenient treatment of diabetes can be achieved with rapid-acting human insulin analogues (Zinman, 1989). The more rapid onset and shorter duration of action of these insulin analogues, compared with regular human insulin (RHI), have been established predominantly in studies of lean, non-obese subjects (von Mach et al., 2002; ter Braak et al., 1996; Home et al., 1999; Heinemann et al., 1998). However, an increasing majority of patients with Type 2 diabetes are overweight and present with a substantial thickness in subcutaneous (sc) fat layer at the preferred abdominal injection site. Therefore, an investigation into pharmacokinetics and pharmacodynamics in obese subjects became mandatory for any new insulin analogue since rapid bioavailability predominantly is based on both the rate of monomerization of the injected insulin complexes and the rate of absorption. Either step may be significantly altered or even delayed with increasing subcutaneous fat layer to the extent of no discrimination between insulins. In order to fully exploit the advantages of rapid-acting insulin analogues, in combination with basal insulin, as part of various basal and bolus insulin regimens in these patients, the rapid-acting profile of insulin analogues must be maintained irrespective of body weight and abdominal fat.

Insulin glulisine (3B–Lys-29B–Glu-insulin) is a new insulin analogue designed to provide the same total glucodynamic effect as human insulin, but to act over a shorter period of time when given sc (Becker et al., 2003; Frick et al., 2003). Insulin glulisine is like human insulin, except for the replacement of asparagine with lysine at position 3, and of lysine by glutamic acid at position 29 on the B-chain of the human insulin molecule. Insulin glulisine is formulated without additional zinc, unlike other rapid-acting insulin analogues (Kroon, 2003). These minor alterations favour formation of monomers and dimers upon dissolution, which are key to the rapid absorption from sc tissue. Insulin glulisine has already been shown in *in vitro* studies to have low mitogenic potential, with an *in vivo* growth-promoting activity identical to that of RHI (Rakatzi et al., 2003; Hennige et al., 2005).

This study explored the concentration–time and time–action profiles of insulin glulisine in non-diabetic obese subjects, compared with insulin lispro and RHI, using the manual euglycaemic clamp technique; and investigated the corresponding dependence of these profiles on body composition and thickness of the sc fat layer at the injection area. Magnetic resonance imaging (MRI) was employed to estimate sc fat layer as it offers a reliable non-invasive, non-radiological measure of regional and total adipose tissue distribution (Thomas et al., 1998), and compares favourably with other simpler techniques (Hayes et al., 1998).

Material and Methods

Study design

The study followed a single-centre, randomized, double-blind, three-way crossover design, comprising five trial periods (0 [screening], 1, 2, and 3 [treatment], and 4 [follow-up]). Study Period 1 occurred no more than 28 days after Period 0, and Period 4 occurred no more than 14 days after Period 3. There were washout periods of at least 7 days between the treatment periods. During the treatment periods subjects underwent the euglycaemic clamp procedure.

Study population

Male and female, obese, otherwise healthy, non-diabetic subjects were enrolled. Subjects were non-smokers, eligible for MRI, tested negative for human insulin antibodies, and were without medical conditions or requirement for regular use of treatment for concomitant diseases likely to interfere with the conduct of the study.

Treatment assignment

Subjects were randomized (1 : 1:1) to receive single, sc injections of 0.3 $U \cdot kg^{-1}$ of either insulin glulisine (A; Aventis Pharma, Germany), insulin lispro (B; Eli Lilly, USA), or RHI (C; Aventis Pharma, Germany), in one of three treatment sequences, (ABC, BCA, CAB) into the periumbilical abdomen.

Body weight was determined on the morning of Day 1 of each trial period and was used to calculate the amount of insulin to be administered for a dose of 0.3 $U \cdot kg^{-1}$. This insulin dose remained the same for each trial period unless body weight changed by more than 2 kg relative to body weight at the first dose.

Study protocol

At the screening visit, each subject provided written informed consent and the appropriate evaluations and safety checks were performed. On the day before each of the clamp days (study periods 1 – 3), subjects had a standard carbohydrate-rich supper and then fasted (apart from water or ice chips) from 10.00 PM until the end of the euglycaemic clamp procedure, which lasted up to 10 hours.

Treatment days

For each treatment day, arterialized venous blood samples were taken using a cannula inserted into a forearm dorsal vein to determine blood glucose, serum insulin, and C-peptide levels. A second cannula was inserted in the contralateral forearm for infusion of glucose to establish euglycaemia.

At approximately 08.00 AM on each dosing day, 0.3 $U \cdot kg^{-1}$ of either insulin glulisine, insulin lispro, or RHI was injected into the periumbilical abdominal sc layer using a standard skin-fold technique. The injection time was defined as time zero, after which blood samples were taken every 5 minutes for 5 hours and then every 10 minutes until the end of the clamp procedure for the measurement of blood glucose. Glucose infusion was to commence when blood glucose fell 10% below baseline value as determined from four measurements at – 60, – 30, – 15, and 0 minutes prior to injection.

Serum insulin and C-peptide levels were determined every 10 minutes until 90 minutes post-administration, every 30 minutes until 180 minutes post-administration, every 60 minutes until 360 minutes post-administration, and then at 2-hour intervals until clamp end. Blood samples for haematological analysis were taken 1 hour before, and 24 hours after, administration of the study insulin.

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Blinding procedures

A pharmacist at the study site, who was otherwise not associated with the study, prepared the syringes with the appropriate study medication, witnessed by a second person who also was otherwise not associated with the study. The syringes were labeled with the subject number and the appropriate trial period. The medical doctors or nurses who administered the study insulin and all nurses who adjusted the glucose infusion were also blinded to the study medication.

Pharmacokinetic assessments

The pharmacokinetic (PK) variables analyzed were area under the curve (INS-AUC, trapezoidal method) between time zero and clamp end (INS-$AUC_{0-clamp\ end}$), and the fractional AUC between time zero and 2 hours (INS-AUC_{0-2h}); maximum insulin concentration (INS-C_{max}); time to C_{max} (INS-t_{max}); times to 20% and 80% of INS-AUC (INS-$t_{20\%-AUC}$; INS-$t_{80\%-AUC}$); and mean residence time (INS-MRT).

Pharmacodynamic assessments

A number of pharmacodynamic (PD) variables were analyzed: area under the glucose infusion rate (GIR) time curve between time zero and clamp end (GIR-$AUC_{0-clamp\ end}$); GIR-AUC between time zero and 1 hour (GIR-AUC_{0-1h}); and AUC between time zero and 2 hours (GIR-AUC_{0-2h}). Times to fractions of total GIR-AUC, such as 20% (GIR-$t_{20\%-AUC}$) and 80% (GIR-$t_{80\%-AUC}$) of GIR-$AUC_{0-clamp\ end}$, were analyzed to assess early glucose disposal and the duration of the bulk of the activity, respectively. In addition, the maximum GIR (GIR_{max}) and time to GIR_{max} (GIR-t_{max}) were calculated from the 3-point running means smoothed GIR.

Anthropometric assessments

Body weight and height measured at screening were used to calculate body mass index (BMI). Skin thickness (abdominal sc fat layer) was measured by sagittal scans at the level of the umbilical injection site by nuclear MRI. A homeostasis model (HOMA) was used to assess insulin resistance (Matthews et al., 1985; Stern et al., 2005).

Study assays

Serum concentrations of insulin glulisine were measured with a radioimmunoassay (RIA) specific for insulin glulisine (lower limit of quantification [LLOQ] 2.0 $\mu U \cdot mL^{-1}$). Serum concentrations of RHI and insulin lispro were determined with an insulin RIA (LLOQ 4.3 $\mu U \cdot mL^{-1}$, for both insulins). Serum concentrations of C-peptide were also measured using an RIA (LLOQ 0.07 $nmol \cdot L^{-1}$). Corrections for endogenous insulin after administration of insulin lispro or RHI were performed according to the equation:

$$Insulin_{EXOG} = Insulin_{OBS} - (F \cdot C\text{-}peptide_{OBS})$$

$Insulin_{EXOG}$ = absolute value for the exogenous serum insulin concentration; $Insulin_{OBS}$ = each value of immunoreactive serum insulin measurements; F = mean serum insulin/serum C-peptide concentration at – 90, – 30, and 0 minutes.

Safety assessments

Adverse events, noted as reported by the subjects, or upon examination by investigator, were any unfavourable and unintended signs, symptoms, syndromes, or illnesses that developed or worsened during the period of observation. Subjects were examined for changes in clinical chemistry, haematology, body temperature, physical condition, blood pressure, radial pulse rate, standard 12-lead electrocardiogram (EKG) readings, lung function, and injection-site reactions.

Statistics

Standard statistical equivalence inferences (analysis of variance [ANOVA] on ln-transformed INS-AUCs, INS-C_{max}, or un-transformed GIR-AUCs, GIR_{max}, MRT, or non-parametric techniques for INS-t_{max}, GIR-t_{max}, INS-$t_{20\%-AUC}$, INS-$t_{80\%-AUC}$, GIR-$t_{20\%-AUC}$, and GIR-$t_{80\%-AUC}$) on 80 – 125% confidence ranges were applied with calculation of 95% confidence intervals (CIs) of pair-wise ratios of mean treatment responses for the various PD and PK parameters. Based on previous experience with insulin glulisine, an estimated total sample size of 18 subjects would provide 80% power to demonstrate equivalence for early insulin exposure and glucodynamic responses, INS-AUC_{0-2h}, INS-C_{max}, or GIR_{max}.

Results

Subjects

Ten male (M) and eight female (F) obese, but otherwise healthy, subjects without diabetes, were enrolled and completed the study according to the protocol. Subjects were non-smokers, eligible for magnetic resonance imaging (MRI), tested negative for human insulin antibodies, and were without medical conditions or the requirement for regular use of treatment for concomitant diseases likely to interfere with the conduct of the study.

There were no major protocol deviations. Minor protocol deviations occurred concerning the deviation from scheduled blood-sampling times, but none were considered to affect the validity of the study results. Nine subjects (4 F/5 M) were allocated to each BMI group (Group I: 30.0 – 34.9 $kg \cdot m^{-2}$; Group II: 35.0 – 40.0 $kg \cdot m^{-2}$). Further details are given in Table **1**.

Performance of the clamp

The mean baseline blood glucose concentrations, calculated from the four glucose values before study drug administration, were similar for all clamp days (insulin glulisine: 87 $mg \cdot dL^{-1}$ [range 76 – 99 $mg \cdot dL^{-1}$], insulin lispro: 86 $mg \cdot dL^{-1}$ [range 77 – 95 $mg \cdot dL^{-1}$], RHI: 86 $mg \cdot dL^{-1}$ [range 73 – 96 $mg \cdot dL^{-1}$]).

Glucose infusion had to be started on average 25 min (range 15 – 50 min) after injection of insulin glulisine, 35/40 min (range 20 – 55 min) after insulin lispro, and 45 min (range 25 – 215 min) after RHI. Glucose levels were clamped on average 11.0 (midrange 4.8; 17.3) $mg \cdot dL^{-1}$ above baseline after insulin glulisine and insulin lispro and slightly lower at 4.7 (midrange – 0.9; 11.8) $mg \cdot dL^{-1}$ after RHI from 90 – 390 min, the effective clamp end. Apart from a slightly earlier drop in glucose concentration after insulin glulisine (nadir 30 min vs. 40 min after insulin lispro), individual differences in changes in glucose concentrations from baseline were the same for insulins glulisine and lispro over the course of the clamp.

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Table **1** Demographic data

Parameter	*Age (years)*	*Body weight (kg)*	*BMI ($kg \cdot m^{-2}$)*	*Skin thickness (mm)*	*FPG ($mg \cdot dL^{-1}$)*	*f-Insulin ($\mu U \cdot mL^{-1}$)*	*HOMA-Index*	*IR*
Mean (range)	29 (19; 47)	107 (84; 140)	34.7 (30; 40)	36.9 (18; 59)	86.1 (77; 94)	20.7 (8; 41)	4.4 (1.9; 9.6)	11 of 18
Group I/II (mean)	28; 31	97; 117	32; 37	30; 44	85; 87	17; 24	3.6; 5.2	4 of 9; 7 of 9

BMI (body mass index [$kg \cdot m^{-2}$]) = body weight divided by height^2; skin thickness (mm) = subcutaneous fat layer (measured by MRI); FPG (fasting plasma glucose; $mg \cdot dL^{-1}$); f-Insulin (fasting serum insulin concentration; $\mu IU \cdot mL^{-1}$); IR = insulin resistant (HOMA-Index > 4.65 or HOMA-IR > 3.6 and BMI > 27.5 $kg \cdot m^{-2}$); HOMA-Index (homeostasis model assessment = f-Insulin [$\mu IU \cdot mL^{-1}$] multiplied by FPG [$mmol \cdot L^{-1}$] divided by 22.5 [$L^2 \cdot \mu U^{-1} \cdot mol^{-1}$])

Table **2** Pharmacodynamic results

Pharmacodynamic variable	*Insulin glulisine*	*Insulin lispro*	*Regular human insulin*	*Insulin glulisine/ insulin lispro*	*Insulin glulisine/ regular human insulin*	*Insulin lispro/regular human insulin*
	Arithmetic mean (n = 18)			*Point estimate (95% CI)**		
GIR-AUC_{0-1h} ($mg \cdot kg^{-1}$)	101	60	29	1.70 (1.2; 2.7)	3.53 (2.1; 9.9)	2.08 (1.3; 4.4)
GIR-AUC_{0-2h} ($mg \cdot kg^{-1}$)	427	354	197	1.21 (1.0; 1.5)	2.17 (1.8; 2.7)	1.80 (1.4; 2.4)
GIR-$AUC_{0-clamp\ end}$ ($mg \cdot kg^{-1}$)	1700	1625	1448	1.05 (1.0; 1.2)	1.17 (1.1; 1.3)	1.12 (1.0; 1.3)
GIR_{max}† ($mg \cdot min^{-1} \cdot kg^{-1}$)	6	6	5	1.02 (0.9; 1.1)	1.30 (1.2; 1.4)	1.27 (1.1; 1.4)
	Median (n = 18)			**Point estimate (95% CI)‡**		
GIR-t_{max} (min)	100	138	233	– 8 (– 43; 28)	– 81 (– 123; – 26)	– 65 (– 107; – 33)

* Point estimates and 95% confidence interval (CI) for the ratio of treatment means, according to Fieller's Theorem, based on untransformed data; † determined from "smoothed" glucose infusion rate (GIR) profiles. ‡ Point estimates and 95% CI for the median of differences from non-parametric data analysis

The mean maximum suppression of serum C-peptide relative to baseline was similar at 56% (range 33 – 78%) after administration of RHI, and 47% after administration of insulin lispro (range 31 – 64%). However, it was slightly larger at 54% (range 33 – 78%) with RHI, compared with 40% with insulin lispro (range 0 – 62%), at the end of the clamp period. It was not necessary to determine C-peptide levels for insulin glulisine because of the specific nature of the assay used.

Pharmacodynamics

Insulin glulisine and insulin lispro both had more rapid-acting profiles than RHI as assessed by greater fractional GIR-AUCs ($p < 0.05$ at 2 hours for insulin glulisine or insulin lispro vs. RHI) and GIR_{max}. Also, the time to 80% of GIR-$AUC_{0-clamp\ end}$, GIR-$t_{80\%-AUC}$, representing duration of the bulk of action, was shorter and similar for both insulin glulisine and insulin lispro compared with RHI (Table **2**, Fig. **1 A** and **C**). Insulin lispro displayed nearly equivalent total glucose disposal (GIR-$AUC_{0-clamp\ end}$) to insulin glulisine, which was slightly less with RHI ($p < 0.05$ vs. insulin glulisine).

Moreover, insulin lispro had a somewhat delayed action profile compared with insulin glulisine, as displayed by smaller fractional GIR-AUCs and longer time to 20% of GIR-$AUC_{0-clamp\ end}$, GIR-$t_{20\%-AUC}$ ($p = 0.025$ at 2 hours), suggesting a less intense onset of activity for insulin lispro compared with insulin glulisine.

While overall glucose disposal was not significantly dependent on skin thickness or BMI, in this obese population there was a significant, negative correlation for GIR-AUC_{0-1h}, GIR-AUC_{0-2h}, GIR-$AUC_{0-clamp\ end}$, and GIR_{max} with all insulins to insulin resistance according to HOMA index classification (Pearson correlation coefficients range from – 0.50 [95% CI – 0.78; – 0.22] to – 0.74 [95% CI – 0.88; – 0.60]). However, the positive correlation for insulin lispro and RHI between skin thickness or BMI and GIR-t_{max}, indicating some shift in the action profiles with increasing skin thickness (sc fat layer) was not seen with insulin glulisine (Table **3**).

Pharmacokinetics

The predominant focus of this analysis was qualitative aspects of the PK profiles. The total exposure (INS-$AUC_{0-clamp\ end}$) was similar for insulin lispro and RHI, and was larger after insulin glulisine administration (Table **4**, Fig. **1 B** and **D**), reflecting quantitative limitations when comparing results obtained with different RIAs.

Nevertheless, insulin glulisine and insulin lispro had more rapid and shorter residing PK profiles than RHI as evidenced by shorter times to 20% of INS-$AUC_{0-clamp\ end}$ (INS-$t_{20\%-AUC}$), to INS-C_{max} (INS-t_{max}), and shorter MRT (at higher fractional INS-AUCs and INS-C_{max}) as would be expected for any rapid-acting insulin compared with RHI. Reflecting the PD profile, the PK profile of insulin lispro was less rapid than that of insulin glulisine, as demonstrated by longer INS-$t_{20\%-AUC}$, INS-t_{max}, and more extended MRT (Table **5**).

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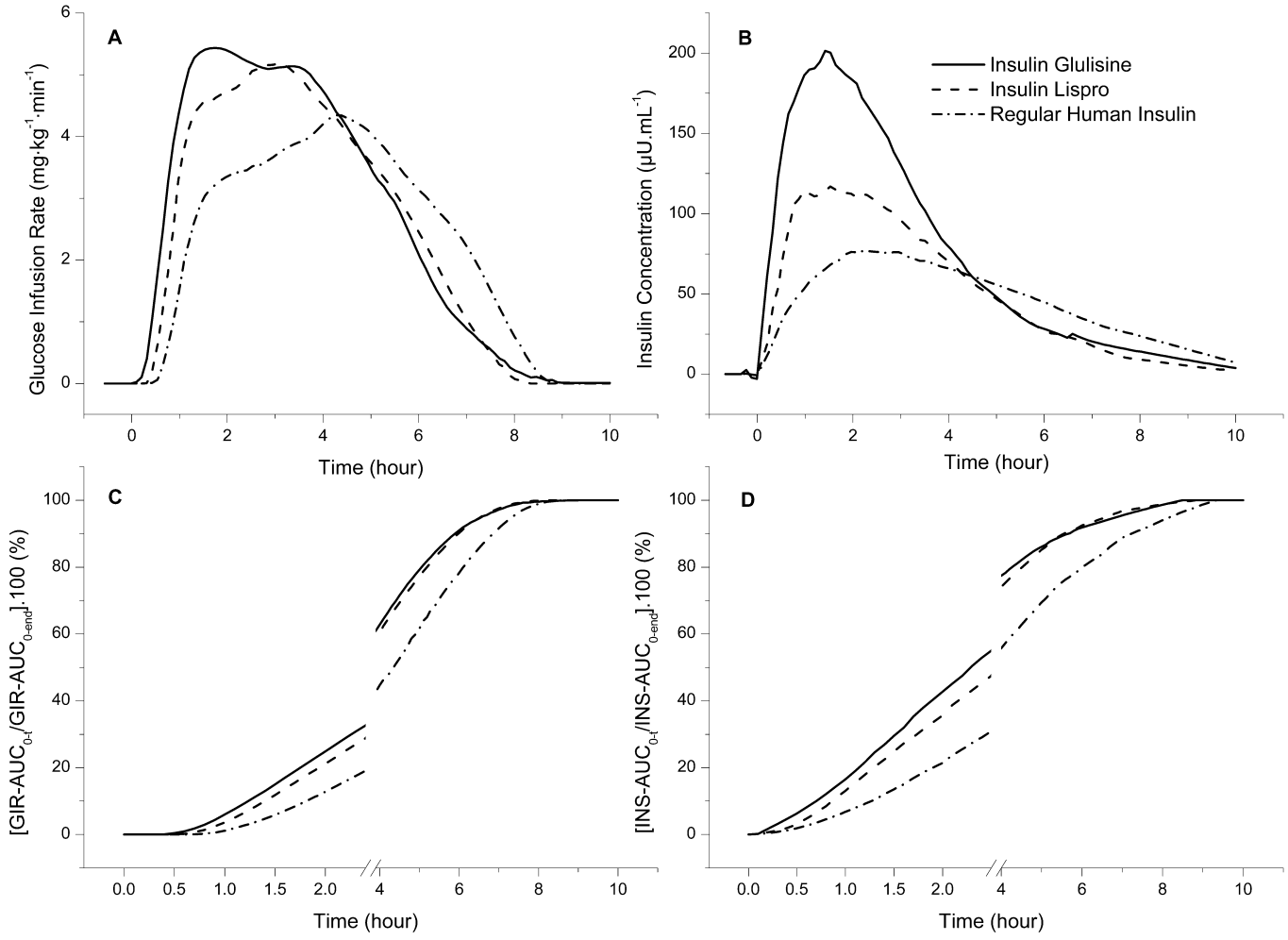

Fig. **1 A** to **D** Pharmacodynamic and pharmacokinetic profiles following subcutaneous injection of 0.3 $U \cdot kg^{-1}$ of insulin glulisine, insulin lispro, or regular human insulin in the abdominal area. Panel **A** Average glucose infusion rates ($mg \cdot kg^{-1} \cdot min^{-1}$); Panel **B** Average insulin concentrations ($\mu U \cdot mL^{-1}$); Panel **C** Cumulative glucose disposal (%), and Panel **D** Cumulative exogenous insulin exposure (%).

Table **3** Correlation of skin thickness and body mass index with time to maximum activity

		Insulin glulisine		*Insulin lispro*		*Regular human insulin*	
Parameter	***Anthropometric measure***	***Pearson****	***95% CI†***	***Pearson****	***95% CI†***	***Pearson****	***95% CI†***
t_{max} *(min)*	body mass index ($kg \cdot m^{-2}$)	0.13	– 0.33, 0.59	0.42	0.02, 0.82	0.46	0.08, 0.84
	skin thickness‡ (mm)	0.29	– 0.14, 0.71	0.67	0.46, 0.87	0.61	0.39, 0.83

* Pearson's correlation coefficient; †95% confidence interval (CI). ‡Arithmetic mean of the three periumbilical MRI measures at 0°, 45°, and 180°

The point estimates and confidence intervals demonstrate the treatment differences in the rapid-acting properties of the three insulins, particularly the between-treatment differences in INS-$t_{20\%-AUC}$ and INS-$t_{80\%-AUC}$ (Fig. **2**). INS-AUC_{0-2h}, INS-$AUC_{0-clamp\ end}$, and INS-C_{max} were not significantly ($p > 0.05$; data not shown) correlated to insulin resistance classified by HOMA.

Safety

Adverse events

No serious adverse events were reported during the study. None of the 28 adverse events reported (10 subjects) were considered related to the study medication, and all adverse events ceased without sequelae. Headache (a common side effect of clamp studies) was the most frequently reported adverse event and was equally distributed between treatment groups (seven, five, and six for insulin glulisine, insulin lispro, and RHI, respectively). This was followed by mild to moderate nausea (one subject each for insulin glulisine and RHI), and moderate nausea and vomiting (one subject for insulin lispro).

Other safety assessments

Observed decreases in haemoglobin concentration, haematocrit, erythrocyte count and platelets were deemed to be clinically irrelevant as they were attributed to the significant blood loss dur-

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Table **4** Pharmacokinetic results

Pharmacokinetic variable	Insulin glulisine	Insulin lispro	Regular human insulin	Insulin glulisine/ insulin lispro	Insulin glulisine/ regular human insulin	Insulin lispro/regular human insulin
	Geometric mean (n = 18)			Point estimate (95% CI)*		
INS-AUC_{0-2h} ($\mu IU \cdot min^{-1} \cdot mL^{-1}$)	18439	10940	5509	1.69 (1.4, 2.0)	3.35 (2.9, 3.9)	1.99 (1.7, 2.3)
INS-$AUC_{0-clamp\ end}$ ($\mu IU \cdot min^{-1} \cdot mL^{-1}$)	43319	30011	26132	1.44 (1.3, 1.6)	1.66 (1.5, 1.8)	1.15 (1.1, 1.2)
INS-C_{max} ($\mu IU \cdot mL^{-1}$)	203	133	77	1.53 (1.3, 1.8)	2.65 (2.3, 3.0)	1.73 (1.5, 2.0)
MRT (min)	149	166	229	0.90 (0.8, 1.0)	0.65 (0.6, 0.7)	0.72 (0.7, 0.8)
	Median (n = 18)			Point estimate (95% CI)†		
INS-t_{max} (min)	76	99	144	– 10 (– 20, 1)†	– 65 (– 79, – 53)†	– 55 (– 71, – 40)†

* Point estimates and 95% confidence interval (CI) for the ratio of treatment means, based on *ln*-transformed data. † Point estimates and 95% CI for the median of differences from non-parametric data analysis

Table **5** Correlation of insulin resistance with glucose disposal

Parameter	Insulin glulisine		Insulin lispro		Regular human insulin	
	Pearson*	95% CI†	Pearson*	95% CI†	Pearson*	95% CI†
GIR-AUC_{0-1h} ($mg \cdot kg^{-1}$)	– 0.64	– 0.89; – 0.40	– 0.51	– 0.76, – 0.26	– 0.50	– 0.78; – 0.22
GIR-AUC_{0-2h} ($mg \cdot kg^{-1}$)	– 0.68	– 0.89; – 0.47	– 0.62	– 0.79, – 0.44	– 0.67	– 0.84; – 0.49
GIR-AUC_{0-end} ($mg \cdot kg^{-1}$)	– 0.62	– 0.77; – 0.48	– 0.52	– 0.68, – 0.36	– 0.74	– 0.88; – 0.60
GIR_{max} ($mg \cdot kg^{-1} \cdot min^{-1}$)	– 0.65	– 0.87; – 0.43	– 0.60	– 0.78, – 0.41	– 0.73	– 0.89; – 0.56

* Pearson's correlation coefficient; † 95% confidence interval (CI)

ing the study. None of the observed changes in platelets, fasting blood glucose, total protein and EKGs were considered to be clinically relevant.

Discussion

The results of this study demonstrate that insulin glulisine has a rapid and short time–action profile in obese, non-diabetic subjects, as has insulin lispro. All fractional GIR-AUCs, as well as GIR_{max}, were greater, and GIR-t_{max} occurred earlier with insulin glulisine and insulin lispro, than with RHI. Notwithstanding that GIR-AUC_{0-1h}, GIR-AUC_{0-2h}, GIR-$AUC_{0-clamp\ end}$, and GIR_{max} of all insulins decreased with increasing insulin resistance, insulin glulisine provided slightly greater total glucose disposal (based on GIR-$AUC_{0-clamp\ end}$) than RHI, while it was not significantly less after insulin lispro. Also, GIR-$t_{20\%-AUC}$ (reflecting early glucose disposal) and the GIR-$t_{80\%-AUC}$ (reflecting the duration of the bulk of activity), were shorter for both insulin analogues, confirming that about the same glucose disposal as with RHI was achieved in less time. Overall however, insulin lispro displayed a less rapid-acting profile than insulin glulisine in this obese population, as evidenced by significantly smaller GIR-AUC_{0-1h} and GIR-AUC_{0-2h}, and a longer GIR-$t_{20\%-AUC}$. The difference in the time–action profiles of the two analogues was observed within the 2 hours post-administration, when insulin glulisine maintained its rapid action. This is an important period, particularly in Type 2 diabetes, in light of proposals that early postprandial hyperglycaemia may be an important epidemiological predictor of cardiovascular mortality (Pfeifer et al, 1981; DECODE study Group, 2001).

The rapid glucodynamic effects of both insulin glulisine and insulin lispro are corroborated by the more rapid and shorter residing PK profiles as compared to RHI. This is demonstrated by earlier INS-t_{max}, shorter times to 20% and 80% of total INS-AUC, shorter MRT at higher fractional INS-AUCs as well as higher INS-C_{max}, which is in line with the rapid PK profile in non-obese healthy volunteers (Becker et al., 2003; Frick et al., 2003). Moreover, even with the quantitative limitations incurred by the use of different RIAs (an assay specific for insulin glulisine and an assay for human insulin as well as for insulin lispro), insulin lispro displayed significantly less early exposure than insulin glulisine. This difference in assays has, however, already been accounted for in large part, by standardization of the PK curves. While it is acknowledged that different assays employed for evaluation of serum insulin levels may still impact on the PK data quantitatively, this in no way impacts on the qualitative results.

It is important to concede that human error introduced into the glucodynamic results by the use of a manual clamp, rather than using the Biostator technique, may have resulted in variability early in the clamp, that could have contributed to the GIR differences observed between the treatments. These misgivings are tenuous as both techniques have been shown to give effective glucose clamping; moreover, the manual method does not re-

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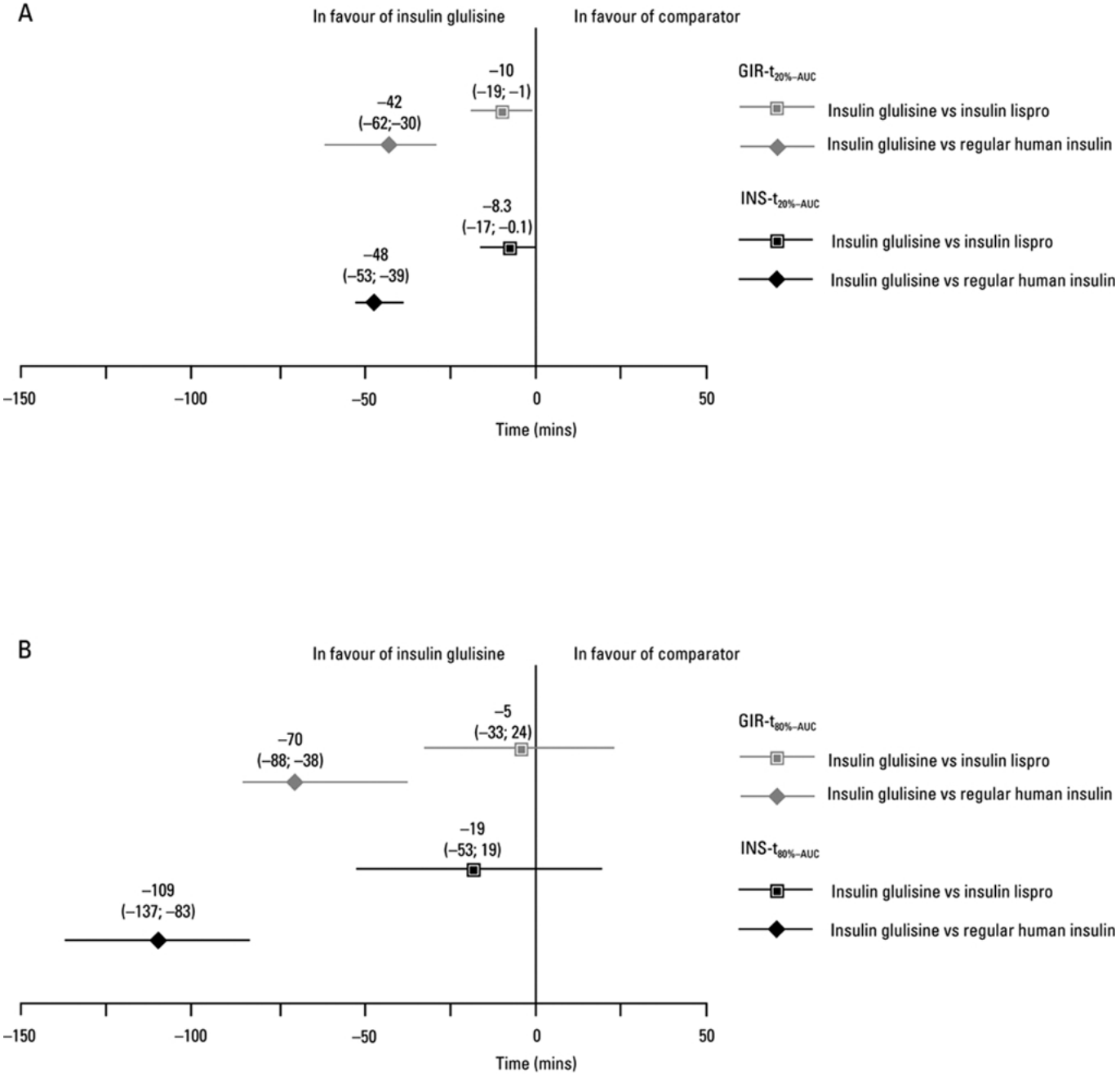

Fig. **2A** and **B** Point estimates and 95% CI for GIR-$t_{20\%–AUC}$ and INS-$t_{20\%–AUC}$ for insulin glulisine versus insulin lispro and regular human insulin (**A**) and GIR-$t_{80\%–AUC}$ and INS-$t_{80\%–AUC}$ for insulin glulisine versus insulin lispro and regular human insulin (**B**).

quire complex machines and has also been demonstrated to show less variability in the GIR compared with the Biostator (Ponchner et al., 1984). In spite of this, the more rapid drop in glucose concentrations and the correspondingly earlier start of glucose infusion provide evidence for a genuine more rapid absorption and action of insulin glulisine (Heise et al., 2005, preliminary data[1]). In addition, the time concentration profiles, which are independent of clamp variability, corroborate the glucodynamic data.

Finally, since the investigators adjusting the clamp were blinded to the study medication being administered, they would have no pre-formed treatment expectations. Consequently, there is no justification to suggest over- or under-compensation of the manual clamp in a treatment-specific manner that could explain the between-treatment differences in PD profiles.

The reasons for the differentiation in absorption between insulin glulisine and insulin lispro require additional explorations. It is known from insulin lispro that added zinc in the commercialized formulation promotes self-association into stabilized hexamers (Bakaysa et al, 1996) and ensures practical shelf life, but also causes some delay in absorption and action as compared to ma-

[1] Preliminary data by Heise et al. (2005) employing a BIOSTATOR supported clamp technique are affirmative.

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terial without added zinc used in developmental studies (Howey et al., 1994). Although the action profiles of insulin lispro and insulin glulisine, which is formulated without added zinc, are superimposable in lean subjects (Becker et al., 2003), suggesting total equivalence in dynamics of bioavailability (i.e. dissociation into monomers and absorption), either step may be differentially influenced with increasing sc fat layer. Whether absence of added zinc in the insulin glulisine formulation is the pivotal clue to the differentiation in absorption in obese subjects remains speculative.

While additional *in vitro* and clinical investigations into this phenomenon may be warranted, there may already be practical clinical implications. The lack of a significant effect of sc adiposity on the absorption rate of insulin glulisine could add to more reliable and, therefore, superior prandial glycaemic control when injected immediately prior to, or soon after, meals in obese patients, who represent the vast majority of patients with Type 2 diabetes.

With regards to consistency of activity in obese subjects, both insulin lispro and RHI displayed some dependence of absorption and action on thickness of the sc fat layer, as evidenced by a significant shift in time to maximum activity. The lack of a relevant correlation between anthropometric parameters within the BMI range studied, and PK or PD profiles for insulin glulisine, demonstrates that this analogue consistently maintains its rapid-acting properties, irrespective of increased thickness in the sc fat layer that is associated with obesity.

Adipose tissue accumulates in two main sites, sc and intra-abdominal, manifesting during puberty (Slyper, 1998). While increased visceral fat is a feature of Type 2 diabetes, and resistance to insulin action with compensatory hyperinsulinaemia are the hallmarks of obesity, it is the sc fat layer that predominantly correlates with a delay in absorption and, hence, onset of activity of RHI (Vora et al., 1993). Published studies support this concept that visceral fat determines the overall glucose disposal efficacy, while the sc fat layer determines absorption characteristics. Glucose disappearance rate has been negatively correlated with visceral fat, but not with sc fat in 21 Type 2 patients (Gautier et al., 1998), while sc fat layer thickness has been negatively correlated with human insulin concentrations, regardless of the concentration of injected RHI, in 50 healthy subjects (Sindelka et al., 1994). In a comparison of the absorption of radiolabelled human insulin in 10 obese and 10 non-obese Type 2 patients, overall slower absorption was reported in patients with Type 2 diabetes compared with previously published data from patients with Type 1 diabetes; this, however, was not differentiated by BMI or fat layer depth (Clauson and Linde, 1995).

As observed in the present study, this is different with rapid-acting insulin analogues, and with insulin glulisine in particular, where there is no significant shift in the PK and glucodynamic profiles within the BMI range studied and compatible with non-obese healthy subjects (Becker et al., 2003). However, attenuation of total glucose disposal remains a feature of insulin resistance associated with obesity and regardless of the insulin employed.

In conclusion, insulin glulisine and insulin lispro both have a more rapid time–action profile than RHI in obese non-diabetic subjects. In addition, insulin glulisine has a more consistent rapid-acting profile across a range of BMI and skin thickness, while insulin lispro appears to be less rapid-acting with increasing BMI and skin thickness. To fully understand the clinical ramifications of these findings, trials that compare the glucose-lowering efficacy of insulin glulisine to that of RHI and insulin lispro in obese patients with Type 2 diabetes will need to be undertaken.

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original article

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ORIGINAL ARTICLE

Comparative pharmacodynamic and pharmacokinetic characteristics of subcutaneous insulin glulisine and insulin aspart prior to a standard meal in obese subjects with type 2 diabetes

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Aims: A multinational, randomized, double-blind, two-way crossover trial to compare the pharmacokinetic and pharmacodynamic properties of bolus, subcutaneously administered insulin glulisine (glulisine) and insulin aspart (aspart) in insulin-naïve, obese subjects with type 2 diabetes.
Methods: Thirty subjects [9/21 females/males; mean ± SD age: 60.7 ± 7.7 years; body mass index (BMI): 33.5 ± 3.3 kg/m^2; duration of diabetes: 6.8 ± 4.6 years; HbA1c: 7.1 ± 0.8%] were included in the analysis. They fasted overnight and then received a 0.2 U/kg subcutaneous dose of glulisine or aspart 2 min before starting a standardized test meal, 7 days apart, according to a randomization schedule. Blood samples were taken every 15 min, starting 20 min before the meal and ending 6 h postprandially.
Results: The area under the absolute glucose concentration–time curve between 0 and 1 h after insulin injection and maximal glucose concentration was significantly lower with glulisine than with aspart ($p = 0.0455$ and 0.0337, respectively). However, for the total study period, plasma glucose concentration was similar for glulisine and aspart. Peak insulin concentration was significantly higher for glulisine than for insulin aspart ($p < 0.0001$). Hypoglycaemic events (≤70 mg/dl with or without symptoms) occurred in 13 and 16 subjects treated with glulisine and aspart, respectively, but there were no cases of severe hypoglycaemia requiring intervention.
Conclusions: Glulisine was associated with lower glucose levels during the first hour after a standard meal; the remaining glucose profiles were otherwise equivalent, with higher insulin levels observed throughout the study period.
Keywords: insulin analogues, insulin aspart, insulin glulisine, insulin therapy, obesity, obesity therapy, pharmacodynamics, pharmacokinetics, type 2 diabetes

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Introduction

The ultimate goal of therapy in type 2 diabetes (T2DM) is to achieve near-normoglycaemia [1]. The Global Task Force on Glycaemic Control recommended HbA1c levels of less than 6.5% as a good target for certain people with T2DM [2], although it also stated that HbA1c and blood glucose targets should be individualized, taking into account factors such as age, existing complications, risk of future complications, diabetes duration and risk of hypoglycaemia. Type 2 diabetes is generally characterized by the presence of relative insulin deficiency, including postprandial insulin deficiency [3], in the presence of insulin resistance. Therefore, an important facet of T2DM treatment is to support and/or supplement the insulin deficit to replicate as closely as possible the normal insulin secretory pattern, including an early response to a nutrient challenge. The time–action profile of subcutaneously injected regular human insulin (RHI) provides a slow onset of action, with a peak effect at 3 h after dosing and a relatively prolonged duration of action beyond 8 h [4]. This requires the insulin to be administered up to 1 h premeal in an attempt to accommodate these deficiencies.

In response to these limitations of RHI, three rapid-acting insulin analogues have been introduced: insulin aspart (aspart), insulin glulisine (glulisine) and insulin lispro (lispro). These analogues all have a rapid onset of action (within 30–60 min) and a peak action within 2 h to allow for appropriate control of postprandial glucose (PPG) fluctuations when given within 5 min preprandially [5]. Glulisine differs from RHI by the replacement of asparagine by lysine at position B3 and lysine by glutamic acid at B29 [6]. The modifications in glulisine allow it to exist as more stable dimers and monomers at pharmaceutical

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concentrations, allowing glulisine to be suspended in a zinc-free buffer, unlike RHI and other rapid-acting insulin analogues [6]. Lispro differs in that the lysine and proline residues at the C-terminal end of the B chain are reversed, which prevents the formation of insulin dimers and hexamers. Aspart differs in that the amino acid residue at position B28 is substituted with aspartic acid, which increases charge repulsion to inhibit the formation of hexamers [6].

Glulisine has been shown to have a more rapid onset of action and a shorter duration of action compared with RHI in obese subjects without diabetes [7]. In addition, glulisine was shown to have a faster onset of action in obese subjects without diabetes [8] and faster absorption with higher postprandial insulin levels in people with T2DM compared with lispro [9]. Similar findings have also been reported in healthy individuals [10] and individuals with type 1 diabetes (T1DM) [11,12]. A recent study in healthy individuals has also shown a more rapid onset of action for glulisine compared with aspart [13].

To date, however, no study has directly compared the pharmacokinetic (PK) and pharmacodynamic (PD) properties of glulisine with those of aspart in people with T2DM. Therefore, the aim of this study was to conduct such a study in obese subjects with T2DM with the comparative insulins given immediately before a standardized test meal.

Materials and Methods

This was a multinational, randomized, double-blind, two-way crossover trial comparing the PK and PD characteristics of glulisine with those of aspart.

Study Population

Obese [body mass index (BMI) 30–40 kg/m^2] males or females aged 18–70 years with T2DM for at least 1 year, treated with oral hypoglycaemic agents (OHAs) for at least 6 months and with HbA1c levels of less than 8.5% were eligible for this study. Subjects were excluded if they had T1DM or were currently using insulin. Further exclusion criteria were pregnancy or breastfeeding, taking medications known to influence insulin sensitivity (e.g. corticosteroids), a history of acute metabolic complications in the past 3 months, recurrent severe hypoglycaemia or hypoglycaemia unawareness, impaired renal or hepatic function and any history of drug or alcohol abuse.

All subjects provided written informed consent and the study was approved by an independent ethics committee at each of the three study sites (Perugia, Italy; Nantes, France and Cardiff, UK).

Study Design and Treatment

Subjects attended a screening visit, performed 1–2 weeks before the first study day, to confirm eligibility. At this visit, baseline characteristics, vital signs and laboratory tests (haematology, clinical chemistry, C-peptide level, HbA1c level and urinalysis) were evaluated after a 12-h fast. On the first study day, the subjects arrived at the respective research centres at approximately 8 a.m., after fasting and omitting their OHAs for 12 h before the visit. In accordance with the randomization scheme, subjects received a 0.2 U/kg dose of either glulisine or aspart subcutaneously within 2 min before starting a standardized meal (692 kcal: 54% carbohydrate, 17% protein and 28% lipid), which they had to finish within 30 min. After a 7-day washout period, the same procedure was repeated using the alternative insulin preparation.

Blood samples were collected at −20 and −10 min and immediately before the meal (0 min), every 10 min for the first 2 h after the meal and then every 15 min for the remaining 4-h period of the study. Plasma glucose, insulin, C-peptide (Invitron, Monmouth, UK) and non-esterified fatty acid (NEFA; Wako NEFA-C kit, Wako Chemicals, Neuss, Germany) levels were determined using validated techniques. Aspart (Capio Diagnostics AS, Copenhagen, Denmark) and glulisine (Linco Research, Missouri, USA) concentrations were determined using analogue-specific assay kits at a central laboratory. All adverse events and episodes of hypoglycaemia were recorded.

Outcome Measures

The primary objective of this study was to assess the PD effect of glulisine compared with aspart on PPG excursions during the first hour after a standard meal, as measured by the area under the glucose concentration–time curve (AUC) between 0 and 1 h after insulin injection ($AUC_{0-1\,h}$). Secondary objectives included assessment of the PD effects of these insulins on PPG excursions up to 6 h after a standard meal ($AUC_{0-6\,h}$) and assessment of the postprandial insulin excursion after a standard meal in each treatment group. Other objectives were to evaluate C-peptide and NEFA levels in each treatment group.

Statistical Analysis

Pharmacodynamic parameters were derived from the individual glucose concentration profiles and PK parameters from the serum aspart and glulisine concentrations. The AUCs were calculated according to the linear trapezoidal rule [14]. PK analyses were carried out using a non-compartmental approach in order to determine maximum insulin concentration (C_{max}) and time to maximum insulin concentration (T_{max}) parameters from serum insulin concentrations. Also, the incremental AUCs (0–1, 0–2, 0–4 and 0–6 h for PD and PK), maximum glucose concentration (GLU_{max}), maximum incremental glucose excursion (ΔGLU_{max}) and C_{max} were analysed by analysis of variance with subject, treatment, sequence group and period effects. Two-sided 90% confidence intervals (CIs) were calculated for the mean differences or mean ratios. Time to ΔGLU_{max} and time to fraction of total glucose AUC (10 and 20%) and corresponding PK parameters [T_{max} and time to fraction of total insulin AUC (10 and 20%)] were analysed using Wilcoxon's signed rank test and Hodges–Lehmann 90% CIs were calculated for the median difference, as previously described [15]. Superiority testing was carried out at the 5% significance level. For any given variable (except time measurements), glulisine and aspart were considered to be clinically similar if the difference between them was non-significant and if the two-sided 90% CIs for the ratios of the means were within 80–125%.

PK and PD analyses were performed in all subjects who completed the study with no major protocol deviations and who had data considered as evaluable. Safety (hypoglycaemia and adverse events) was assessed for all subjects who were exposed to study treatment.

Results

Subject Disposition

A total of 43 subjects were screened, of whom six were excluded because of having a BMI outside the predefined range (n = 2), an HbA1c level of more than 8.5% (n = 2), age over 70 years (n = 1) or taking prohibited medication (n = 1). Therefore, 37 subjects [mean (± standard deviation) age 60.3 ± 8.3 years, BMI 33.7 ± 3.3 kg/m^2, diabetes duration 7.3 ± 4.9 years, HbA1c 7.1 ± 0.8%] were randomized. Of the 37 subjects randomized, seven were subsequently excluded from the PK and PD analyses: one for premature withdrawal after the first study day (having received aspart) and six for major protocol deviations [two subjects with medical conditions at inclusion who were erroneously included; one each for use of corticosteroids during the study, missing PK/PD values in the first hour after drug administration, unusable PK assessments (very low aspart plasma levels, incompatible with aspart administration) and duration of meal intake longer than 30 min (85 min)]. The latter two subjects were excluded after the database lock, following a recommendation by the Steering Committee. Therefore, 30 subjects were included in the final analysis and the baseline characteristics are represented in Table 1. There were no differences between the subjects included in the final analysis and all randomized subjects (data not shown). The mean doses of glulisine and aspart were 19.5 ± 2.7 and 19.4 ± 2.7 U, respectively.

Pharmacodynamics

Mean blood glucose levels at baseline were 137.4 ± 33.2 and 140.5 ± 32.5 mg/dl for the glulisine and aspart groups, respectively. The plasma glucose concentrations over time are shown in figure 1. Both mean $AUC_{0-1\,h}$ (149 vs. 158 mg·h/dl; p = 0.0455) and mean GLU_{max} (170 vs. 181 mg/dl; p = 0.0337) were significantly lower with glulisine than with aspart. Point estimates (glulisine/aspart) for $AUC_{0-1\,h}$ and GLU_{max} were 94% (90% CI: 90–99) and 94% (90% CI: 90–99), respectively (Table 2). No statistically significant differences were observed with baseline-subtracted data in any of the periods analysed (data not shown).

The AUC ratios for $AUC_{0-1\,h}/AUC_{0-6\,h}$ (p = 0.0334) and $AUC_{0-2\,h}/AUC_{0-6\,h}$ (p = 0.0341) were significantly lower for glulisine than aspart, with point estimates of 95% (90% CI: 92–99) and 96% (90% CI: 94–99), respectively (Table 2). Moreover, taking into account the total study duration (6 h), the overall plasma glucose concentration was similar between groups treated with glulisine and aspart.

Mean C-peptide plasma concentration profiles were similar after glulisine and aspart injections (data not shown), with maximum concentrations of 2.08 and 2.07 pmol/ml, respectively, occurring at 90 min for both insulin analogues.

Mean NEFA concentrations decreased from 0.50 to 0.11 mmol/l at 180 min with glulisine and from 0.51 to 0.11 mmol/l at 120 min with aspart; the NEFA concentrations then increased to 0.32 and 0.31 mmol/l with glulisine and aspart, respectively.

Pharmacokinetics

Table 2 also represents the PK results derived from the insulin concentration profiles illustrated in figure 2a. Peak insulin concentration was significantly higher for glulisine than for aspart (geometric mean of 534 vs. 363 pmol/l; p < 0.0001; figure 2b). Although T_{max} tended to be longer with glulisine (median of 120.0 vs. 93.0 min), this difference was not significant (p = 0.5133). Glulisine was associated with significantly higher AUCs for all four measurement durations (0–1, 0–2, 0–4 and 0–6 h; all: p < 0.0001), with point estimates for mean ratios (glulisine/aspart) ranging from 155% (90% CI: 141–171) for $AUC_{0-6\,h}$ to 197% (90% CI: 157–248) for $AUC_{0-1\,h}$. In terms of AUC ratios, only $AUC_{0-1\,h}/AUC_{0-6\,h}$ was significantly different between the groups, with the value

Table 1. Baseline characteristics of the study subjects.

	Sequence glulisine/aspart (n = 16)	Sequence aspart/glulisine (n = 14)	All (n = 30)
Females/males, n	3/13	6/8	9/21
Age, years*	61.2 ± 7.7	59.7 ± 8.3	60.7 ± 7.7
Weight, kg*	100.4 ± 16.1	94.1 ± 10.7	96.3 ± 14.3
Height, cm*	173.1 ± 8.6	166.3 ± 7.2	169.4 ± 8.7
BMI, kg/m^2*	33.3 ± 3.4	34.0 ± 3.3	33.5 ± 3.3
Diabetes duration, years*	6.3 ± 4.0	7.5 ± 5.3	6.8 ± 4.6
HbA1c, %*	7.0 ± 0.8	7.2 ± 0.8	7.1 ± 0.8
Oral hypoglycaemic agents, n (%)	16 (100)	14 (100)	30 (100)
Biguanides	15 (3.8)	14 (100)	29 (96.7)
Sulphonylureas	5 (31.3)	9 (64.3)	14 (46.7)
Thiazolidinediones	3 (18.8)	3 (21.4)	6 (20.0)
Glinides	1 (6.3)	1 (7.1)	2 (6.7)

BMI, body mass index.
*Data are mean ± standard deviation.

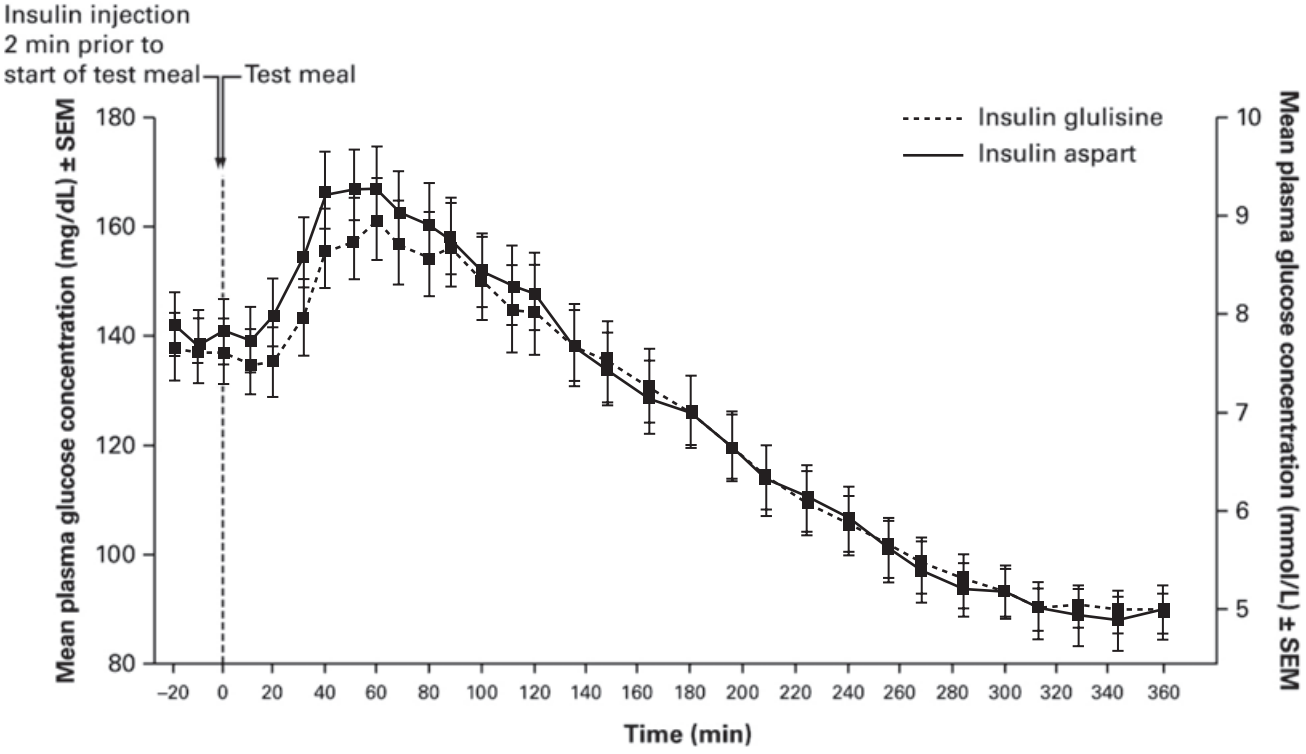

Figure 1. Mean plasma glucose concentrations over time. SEM, standard error of the mean.

of this ratio for glulisine being 127% of the equivalent ratio for aspart (90% CI: 106–152; p = 0.0340; Table 2).

Hypoglycaemia and Safety Parameters

A total of 13 (36.1%) subjects given glulisine and 16 (43.2%) subjects receiving aspart experienced an episode of hypoglycaemia (blood glucose <70 mg/dl with or without symptoms). Among these, 10 and 15 subjects, respectively, experienced an episode of hypoglycaemia 3–6 h after the insulin administration. The remaining episodes occurred 30, 110 and 135 min after glulisine administration and 60 min after aspart administration. Five and eight subjects, respectively, experienced an episode of hypoglycaemia with blood glucose levels below 56 mg/dl. None of the episodes was considered to be severe nor required intervention.

Five treatment-emergent adverse events were reported in four subjects, including injection-site pain (glulisine, one; aspart, one), headache (glulisine, one; aspart, one) and nausea (aspart, one). None of the adverse events was reported as serious.

Discussion

This two-way crossover study is the first to compare the PK/PD profiles of glulisine and aspart in people with T2DM, given a standard meal under identical baseline plasma glucose concentrations. During the first hour following insulin injection, the absolute plasma glucose concentration was significantly lower after administration of glulisine than with aspart (p = 0.0455). Furthermore, the peak glucose concentration was also significantly lower after glulisine administration than after aspart (p = 0.0337). When considering the overall duration of the study, however, the plasma glucose levels and glucose excursions were similar between the two rapid-acting insulin analogues.

Care must be taken when interpreting the PK data, owing to the different assays used for each insulin analogue. As analogue-specific assays were used for determination of aspart and glulisine, the PK data were normalized to a percentage of C_{max} so that the data for the two analogues could be compared. Although there was no difference between groups over the study duration, there was a statistically significant difference in the measured mean insulin concentration over the first 20 min (figure 2b). The C-peptide and NEFA levels throughout the 6-h period were comparable in both groups, indicating that the results were not influenced by changes in endogenous insulin secretion and that both insulins have similar effects on carbohydrate utilization.

Overall, these findings are consistent with previous results obtained in a similar study comparing glulisine and lispro in obese subjects with T2DM [9], which also showed a lower maximum PPG excursion with glulisine. The findings are also consistent with the PD data observed in a study in healthy individuals [13]. These PK and PD differences could be related to the zinc-free formulation of glulisine, which, along with the structural modifications, help to prevent dimerization. Indeed, these changes facilitate the rapid uptake of glulisine from the subcutaneous depot after injection [5,6]. The addition of zinc

Table 2. Pharmacodynamic and pharmacokinetic results.

	Estimated sample mean (n = 30)			Estimate and 90% CI for mean ratios* (glulisine/aspart)	Estimate and 90% CI for mean differences§ (glulisine/aspart)
	Glulisine	Aspart	p value		
Pharmacodynamics results					
$AUC_{0-1\ h}$ (mg·h/dl)	149	158	0.0455	94% (90–99)	—
$AUC_{0-6\ h}$ (mg·h/dl)	738	750	0.5382	98% (95–104)	—
$AUC_{0-1\ h}/AUC_{0-6\ h}$ (%)	20	21	0.0334	95% (92–99)	—
$AUC_{0-2\ h}/AUC_{0-6\ h}$ (%)	41	42	0.0341	96% (94–99)	—
$AUC_{0-4\ h}/AUC_{0-6\ h}$ (%)	74	75	0.0912	99% (97–100)	—
ΔGLU_{max} (mg/dl)	33	40	0.0634	81% (70–100)	−8 (−15 to −10)
GLU_{max} (mg/dl)	170	181	0.0337	94% (90–99)	−11 (−19 to −3)
Time to ΔGLU_{max} (min)	60.0†	59.5†	0.3328	—	−5 (−20 to 5)¶
Time to 10% of total glucose AUC (min)	40.0†	40.0†	0.3566	—	−2 (−6 to 2)¶
Time to 20% of total glucose AUC (min)	67.5†	65.0†	0.9681	—	0 (−4 to 3)¶
Pharmacokinetics results					
$AUC_{0-1\ h}$ (pmol·h/l)	272 (297)‡	138 (167)‡	<0.0001	197% (157–248)	—
$AUC_{0-6\ h}$ (pmol·h/l)	2002 (2077)‡	1289 (1333)‡	<0.0001	155% (141–171)	—
$AUC_{0-1\ h}/AUC_{0-6\ h}$ (%)	14 (2.6)‡	11 (2.4)‡	0.0340	127% (106–152)	—
$AUC_{0-2\ h}/AUC_{0-6\ h}$ (%)	36 (3.6)‡	35 (3.6)‡	0.5566	103% (95–110)	—
$AUC_{0-4\ h}/AUC_{0-6\ h}$ (%)	78 (4.3)‡	77 (4.3)‡	0.3716	101% (99–103)	—
C_{max} (pmol/l)	534 (570)‡	363 (385)‡	<0.0001	147% (133–163)	—
Time to fraction of total insulin AUC (10%) (min)	60.0†	60.5†	0.0372	—	−12 (−26 to −1)¶
Time to fraction of total insulin AUC (20%) (min)	90.0†	91.0†	0.9109	—	0 (−12 to 14)¶
T_{max} (min)	120.0†	93.0†	0.5133	—	17 (−10 to 37)¶

CI, confidence interval; $AUC_{0-X\ h}$, area under the curve for the period 0–X h; ΔGLU_{max}, maximum glucose excursion; GLU_{max}, peak glucose concentration; C_{max}, peak insulin concentration; T_{max}, time to peak insulin concentration.

*For pharmacodynamic parameters, point estimate and 90% CI for the ratio of treatment means according to Fieller's Theorem, based on untransformed data. For pharmacokinetic parameters, point estimate and 90% CI for the ratios of the treatment means, based on ln-transformed data.

†Data are median.

‡Data are sample geometric mean (arithmetic mean).

§Point estimate and 90% CI for the difference of treatment means, from parametric data analysis (analysis of variance), based on untransformed data.

¶Point estimate and 90% CI for the difference of treatment medians from non-parametric analysis (Hodges and Lehmann method).

to the rapid-acting analogues lispro and aspart formulations is necessary to prevent the formation of fibrils [5,16] and to promote the formation of stable hexameric and higher-order aggregates [17,18].

Excess adiposity can adversely affect the PK and PD properties of RHI [19–21]. Indeed, the site of injection may influence the PK and PD of short-acting insulins because body regions with greater skin thickness may show protracted absorption [22]. For example, ter Braak et al. reported that the C_{max} and T_{max} values for insulin (lispro and human insulin) varied between the two types of insulin and between the three injection sites (abdominal, deltoid and femoral sites) [22]. However, in that study, lispro was consistently associated with better PK and PD parameters vs. RHI, irrespective of the site of injection. Based on the results of the present study in obese individuals with T2DM and other studies in lean to obese subjects without diabetes, it transpires that the onset of action of the rapid-acting insulin analogues is not delayed in obese subjects when using a specific injection site [8]. Unfortunately, in both studies, the actual subcutaneous fat thickness was not assessed and BMI *per se* may not be a good marker for subcutaneous fat at the injection site.

Overall, the findings of the present study must be considered in light of the exploratory nature of this study and small sample size. It must also be noted that a strictly defined meal size and content and a fixed insulin dose were used in this study. Therefore, the results should not be generalized to the population as a whole because meal size and content and insulin doses will vary not only between individuals but also according to meals. However, dose proportionality of glulisine has been described in individuals with T1DM [11] and it is possible that a similar effect may be seen in individuals with T2DM; thus, prospectively altering the insulin dose based on meal content may be more appropriate than a predefined titration algorithm for some individuals [23]. In terms of PD, a similar pattern is likely to be seen to that observed in this study, but will clearly depend on the relative carbohydrate and fat content, aside from the effects of insulin resistance in individuals with T2DM.

In conclusion, this study, involving obese subjects with T2DM, showed that, at identical doses, glulisine was associated with a lower plasma glucose level than aspart during the first postprandial hour, in combination with significantly higher glulisine concentrations and when administered by bolus subcutaneous injection. During the remaining period of the test, there were no differences in the glucose profiles and glulisine levels were higher than aspart. Taken together, the lower early and late AUCs for glulisine support the earlier impact of glulisine, compared with aspart, on the PPG profile in response to a standard test meal.

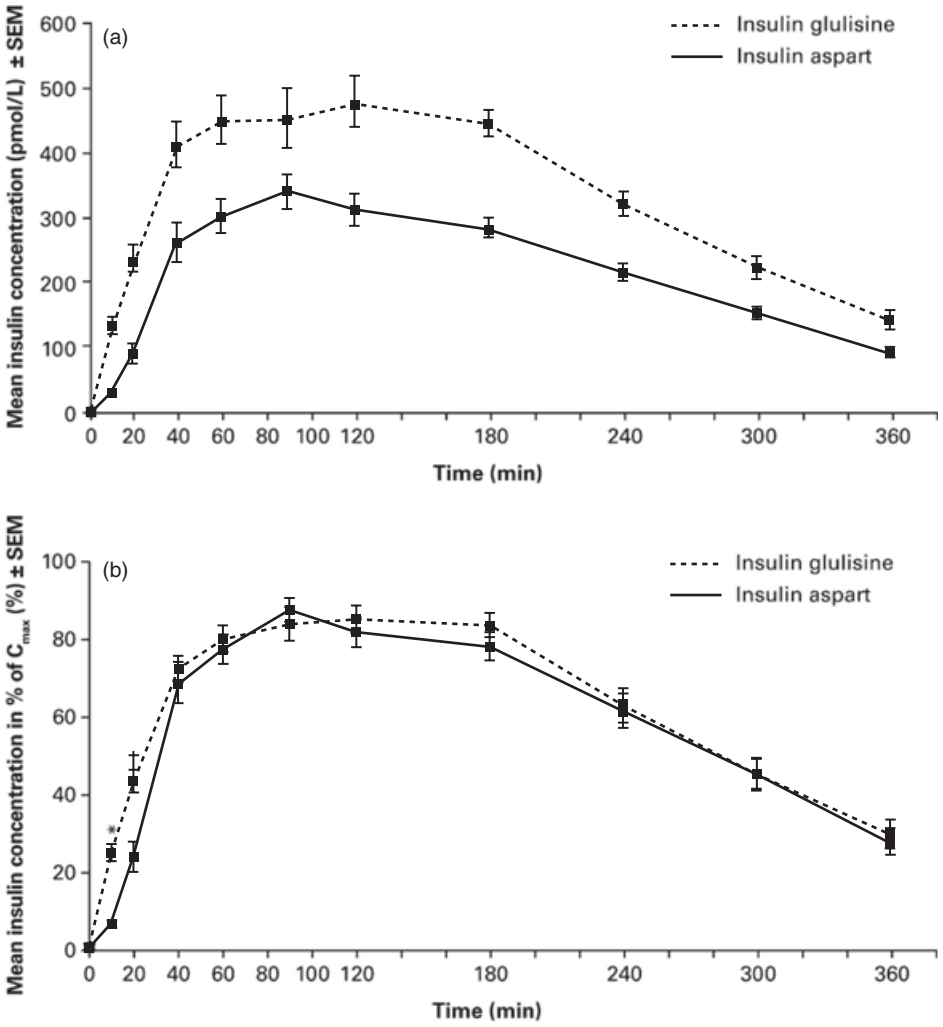

Figure 2. (a) Mean plasma insulin concentrations over time and (b) mean plasma insulin concentrations in percentage of peak insulin concentration over time. *$p < 0.001$ compared with insulin aspart at 10 min and †$p > 0.001$ compared with insulin aspart at 20 min. SEM, standard of the mean; C_{max}, peak insulin concentration.

Acknowledgements

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Conflict of Interest

G. B. B. has received honoraria for consulting and lecturing from sanofi-aventis and Novartis and honoraria for lecturing from Eli Lilly. S. M. has received a grant from sanofi-aventis. C. S.-L. is an employee of sanofi-aventis. B. C. has received fees for consultancy, advisory boards, speaking, travel or accommodation from Takeda, GlaxoSmithKline, Merck Sharpe and Dohme, AstraZeneca, Bristol Myers Squibb, Boehringer Ingelheim, Novo Nordisk, Roche, sanofi-aventis and Novartis. D. O. has lecturing commitments with sanofi-aventis and is an advisory board member for Roche. G. B. B. and C. S.-L. were involved in the study design, critical review of the manuscript and final approval of manuscript prior to submission. S. L., Y. Z. and D. O. were involved in the study design, study conduct, data collection, data analysis, critical review of the manuscript and final approval of manuscript prior to submission. S. M. and F. P. were involved in the study conduct, data collection, critical review of the manuscript and final approval of manuscript prior to submission. B. C. was involved in the study design, data analysis, critical review of the manuscript and final approval of manuscript prior to submission.

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Monomeric Insulins and Their Experimental and Clinical Implications

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Due to the inherent pharmacokinetic properties of available insulins, normoglycemia is rarely, if ever, achieved in insulin-dependent diabetic patients without compromising their quality of life. Subcutaneous insulin absorption is influenced by many factors, among which the associated state of insulin (hexameric) in pharmaceutical formulation may be of importance. This review describes the development of a series of human insulin analogues with reduced tendency to self-association that, because of more rapid absorption, are better suited to meal-related therapy. DNA technology has made it possible to prepare insulins that remain dimeric or even monomeric at high concentration by introducing one or a few amino acid substitutions into human insulin. These analogues were characterized and used for elucidating the mechanisms involved in subcutaneous absorption and were investigated in preliminary clinical studies. Their relative receptor binding and in vitro potency (free-fat cell assay), ranging from 0.05 to 600% relative to human insulin, were strongly correlated (r = 0.97). In vivo, most of the analogues exhibited ~100% activity, explainable by a dominating receptor-mediated clearance. This was confirmed by clamp studies in which correlation between receptor binding and clearance was observed. Thus, an analogue with reduced binding and clearance gives higher circulating concentrations, counterbalancing the reduced potency at the cellular level. Absorption studies in pigs revealed a strong inverse correlation (r = 0.96) between the rate of subcutaneous absorption and the mean association state of the insulin analogues. These studies also demonstrated that monomeric insulins were absorbed three times faster than human insulin. In healthy subjects, rates of disappearance from subcutis were two to three times faster for dimeric and monomeric analogues than for human insulin. Concomitantly, a more rapid rise in plasma insulin concentration and an earlier hypoglycemic response with the analogues were observed. The monomeric insulin had no lag phase and followed a monoexponential course throughout the absorption process. In contrast, two phases in rate of absorption were identified for the dimer and three for the normal hexameric human insulin. The initial lag phase and the subsequent accelerated absorption of soluble insulin can now be explained by the associated state of native insulin in pharmaceutical formulation and its progressive dissociation into smaller units during the absorption process. In the light of these results, the effects of insulin concentration, injected volume, temperature, and massage on the absorption process are now also understood. When given to diabetic patients immediately before a standard meal, the monomeric analogue lowered postprandial glucose excursions by ~50% when compared with human insulin given at the same time. Subsequently, it was shown that three monomeric to dimeric analogues injected separately just before a meal gave glycemic control at least comparable to that of human insulin administered 30 min earlier. Lower plasma glucose concentrations (~50%) were observed with the analogues from 1.5 h postprandially. Thus, monomeric analogues are faster in onset of action, can be given with the meal without losing glycemic control, and have the potential to minimize late hypoglycemia. Therefore, the development of these novel insulins represents a major step in the evolution of insulin preparations to subserve meal-related insulin requirements. *Diabetes Care* 13:923–54, 1990

The introduction of insulin in the 1920s revolutionized the treatment of diabetes (1). Subcutaneous injection therapy has, however, not succeeded in normalizing glycemic control, despite the efforts devoted to improvement in insulin preparations and in-

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jection regimens. During the last decades, the increasing awareness and acceptance of the relationship between metabolic control and the occurrence of devastating microvascular complications have stimulated considerable research into new methods of improving insulin therapy. A major determinant of metabolic control is availability of insulin in the blood, and factors affecting absorption and disposal of insulin have been increasingly studied in recent years.

Normalization of plasma glucose concentrations requires normalization of the plasma insulin profile, with an appropriate elevation in plasma insulin during meals, to prevent unphysiological postprandial glycemia. Therefore, numerous investigations have focused on factors that might influence the rate of absorption of insulin from the subcutaneous injection site. Although the processes conveying the insulin from the injected depot to the blood are not known in detail, many factors relating to insulin formulation, site, method of administration and other conditions have been described.

In the field of pharmaceutical formulation, important improvements have emerged (2). Until recently, such developments have been restricted to improvement in insulin purity; insulin species; and adjustment of the composition of the vehicle with respect to retarding agent, auxiliary substances, and other additives. However, the introduction of recombinant DNA techniques has made it possible to optimize the insulin molecule for substitution therapy.

This article reviews efforts to create, by protein engineering, novel insulins better suited for meal-related therapy than native insulins (3). The physicochemical and biological properties of human insulin analogues, with reduced tendency to self-association, the use of these analogues to elucidate the mechanisms of absorption of unmodified (soluble or regular) insulin, and an early clinical evaluation are presented.

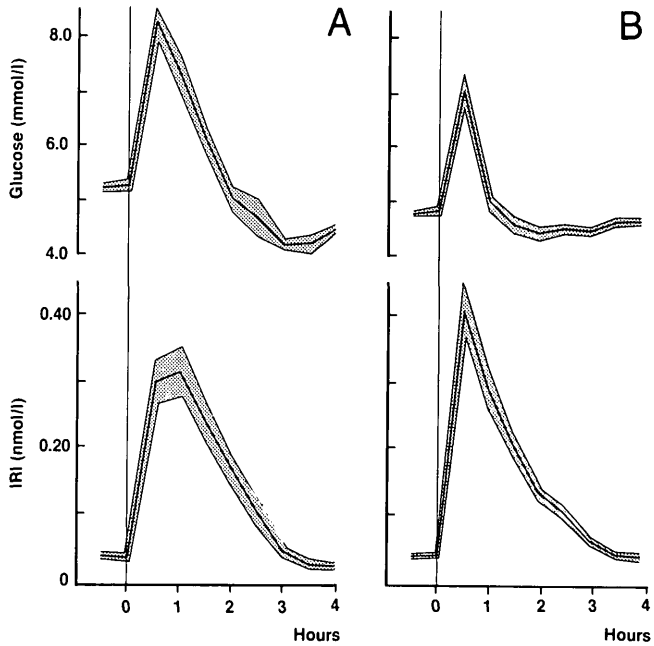

FIG. 1. Mean ± SE plasma glucose and immunoreactive insulin (IRI) concentrations during oral glucose tolerance test (*A*; 75 g glucose, *n* = 24) and standardized meal tolerance test (*B*; 500 kcal, carbohydrate 60% calorie contribution, *n* = 34) in healthy nonobese subjects.

CLINICAL OBSERVATIONS

Physiological meal data in healthy and non-insulin-dependent diabetic (NIDDM) subjects. In healthy people, blood glucose concentrations are maintained within a narrow physiological range by highly efficient homeostatic mechanisms; only insulin lowers blood glucose concentration. The daily plasma glucose and insulin profile in nondiabetic subjects in response to various test meals has been well documented (4–14).

Figure 1 illustrates such observations during the pre- and postprandial state in healthy subjects. The β-cell secretion depicted by plasma immunoreactive insulin levels indicates a low basal level during fasting and a rapid increase in response to nutrient ingestion. Peak insulin levels are achieved within 0.5–1 h from the onset of eating, returning to basal levels within 2–3 h postprandially.

Increasing glucose intolerance is commonly associated with a diminishing insulin secretory response to a glucose challenge (15–21). Similar observations are seen in response to normal meal ingestion, clearly demonstrating an increasing deficit in early insulin secretion with deteriorating glucose tolerance in NIDDM (22,23; Fig. 2). This shows that inadequacies in the temporal and quantitative relationship between nutrient supply and insulin availability can seriously compromise glucose homeostasis. In insulin-dependent diabetes mellitus (IDDM), deficient β-cell secretion is always present, although some patients are still capable of secreting small quantities of insulin (24,25).

Basal insulin secretion and nutrient-stimulated insulin secretion are central ingredients in maintaining normoglycemia in humans, who eat sporadically and depend on carbohydrates as a major source of energy.

Current insulin treatment. Insulin-replacement therapy became a reality after the successful extraction of insulin from animal pancreas glands in 1922 by Banting and Best (1). Since insulin became available in its crudest form, major advances have occurred relating to production, purification, and pharmaceutical formulation (26–35; Table 1).

There is increasingly convincing evidence that poor metabolic control is associated with microvascular complications (36–49). Therefore, the diabetologist is committed to strive for normoglycemia while trying to avoid the dangers of hypoglycemia (44,50). Thus, the routine treatment of diabetic patients with insulin is constantly being reappraised in an attempt to achieve normal physiology and metabolism, as recently reviewed by Pickup

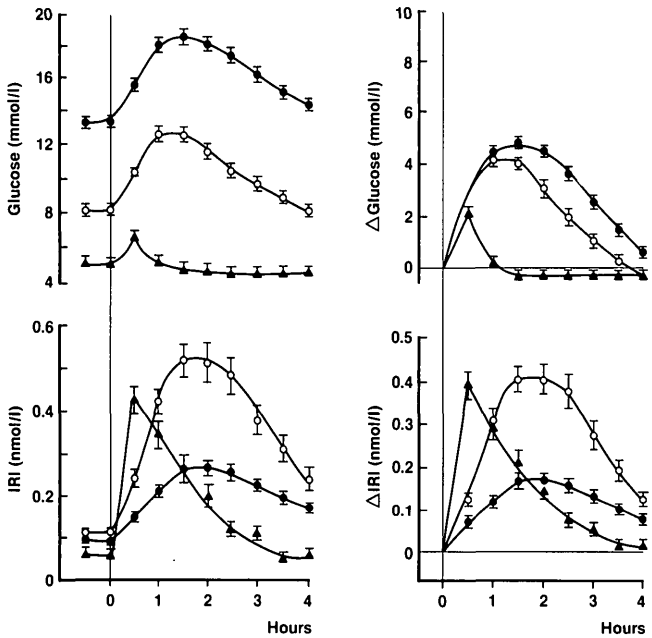

FIG. 2. Mean ± SE plasma glucose (*top left*, absolute values; *top right*, incremental values) and immunoreactive insulin (IRI) concentrations (*bottom left*, absolute values; *bottom right*, incremental values) during meal tolerance test in healthy subjects (▲, *n* = 54) and newly diagnosed previously untreated patients with non-insulin-dependent diabetes mellitus and fasting plasma glucose <10 mM (○, *n* = 71) or ≥10 mM (●, *n* = 150).

(51), Home et al. (52), Skyler (53), Zinman (54), and Berger (55).

The pharmacokinetics after subcutaneous injection of available short-, intermediate-, and long-acting insulin preparations makes it virtually impossible to achieve normoglycemia (52–54,56–60). Attempts to achieve normoglycemia have therefore involved a multiplicity of insulin preparations, regimens, and delivery systems to provide for both basal and meal-related insulin requirements (61–63).

The most common subcutaneous insulin regimen involves twice-daily injection of mixtures of short- and intermediate-acting insulin preparations (62,64–66). Additional efforts to satisfy both basal and meal-related insulin requirements have resulted in the introduction of continuous subcutaneous insulin infusion with a portable pump with adjustable rates (67–71). Comparable glycemic control can, however, be achieved with intensively applied conventional treatment involving long-acting ultralente insulin plus multiple preprandial injections of soluble insulin (72,73).

Insulin regimens and delivery systems, patient education, and self-monitoring techniques are constantly reevaluated to achieve better metabolic control in most patients. In this context, the use of multicomponent insulin regimens has demonstrated the value of providing more physiological insulin therapy in the form of meal-related (bolus) and basal requirements (66,72,74–79).

After subcutaneous injection of soluble insulin into the femoral region, it takes ~2 h for the insulin to be absorbed at maximum rate (80). This slow rise to peak insulin concentration is likely to account for much of the observed postprandial hyperglycemia. Because the insulin concentration falls slowly after the peak, the extended period of elevated insulin concentration results in a tendency toward late hypoglycemia (52). The peak effect may even persist for several hours if circulating insulin-binding antibodies are present (81–84; see IMMUNOLOGICAL ASPECTS). Such plasma insulin patterns bear no resemblance to those in healthy subjects in response to a meal (Figs. 1 and 2).

Although the influence of available short-acting insulins on postprandial glycemia can be improved by subcutaneous administration 30 min to 1 h before eating, the risk of delayed hypoglycemia remains, due to inappropriately high insulin levels persisting in the plasma 3–4 h after injection (56,85–90). Variation in subcutaneous absorption among different insulin formulations, concentrations, dosage levels, sites of administration, and injection techniques and the influence of exercise, massage, and ambient temperature are reviewed by Galloway et al. (56), Berger et al. (57), Binder et al. (58), Owens (59), and Schlichtkrull (80). Measures to alter these factors, to enhance the absorption rate of insulin from subcutaneous tissue, are either impractical, inconvenient, or unsafe to be used by the insulin-requiring diabetic patient on a day-to-day basis.

Subcutaneous absorption process. The absorption of soluble insulin after subcutaneous injection involves a complex series of events influenced by many variables. Although the pharmacokinetics of insulin after subcutaneous administration has been extensively studied, especially over the last decade, understanding of the absorption process, which involves numerous physicochemical and physiological processes, is still far from complete (56–59,91–140; Table 2).

After subcutaneous injection of soluble insulin, an initial lag phase with a low but increasing relative rate of

TABLE 1
Major advances in development of insulin preparations

1922	Banting and Best (1)	Isolation of insulin
1934	Scott (26)	Zinc-insulin crystallization
1936	Hagedorn et al. (27) Scott and Fisher (28)	Protamine insulins
1946	Krayenbühl and Rosenberg (29)	Isophane insulin (NPH)
1952	Hallas-Møller et al. (30)	Lente series
1961	Schlichtkrull et al. (31)	Neutral regular insulin
1972	Schlichtkrull et al. (32)	Monocomponent insulin
1979–1982	Goeddel et al. (33) Chance et al. (34) Markussen et al. (35)	Bio- and semisynthetic human insulin

References are in parentheses.

absorption has been noted and can be observed in most clinical studies (96,101,102,122,141,142). This lag-phase phenomenon has been hypothesized to be due to a local vasoconstrictor effect of insulin or distribution by local diffusion (101,143). This initial delay in absorption is shortened or even disappears with reduced concentration of insulin or decreased volume injected (100,101,144).

Besides the influence of the pharmaceutical formulation of the insulin preparation, many clinical studies have shown that insulin absorption from subcutaneous tissue is to a large extent controlled by local blood flow (101,110,127,145; Table 2). Therefore, factors known to influence blood flow, i.e., site and depth of injection, exercise, smoking, and temperature, also have an effect on the rate of insulin absorption from the subcutis (Table 2). The main influence of blood flow on absorption rate in the low blood flow range is related to the recruitment of capillaries, decreasing the diffusion distance, and to the concentration gradient between interstitial space and blood. However, at higher blood flow rates, factors other than blood flow are limiting for the rate of absorption of soluble insulin (135). These factors include *1*) interstitial transport to the capillaries by diffusion and *2*) the probable restriction for transport over the capillary membrane (total area and permeability). The effect of these factors is governed primarily by the size of the transported molecule.

The hexamer of insulin, the prevailing association unit of insulin in neutral soluble insulin, has a diameter of ~5 nm and a height of ~3.5 nm (146; see INSULIN STRUCTURE). It is assumed that hexameric insulin after injection dissociates in subcutaneous tissue and is transported to the capillaries by diffusion and absorbed in its dimeric or monomeric form (101,147,148). This requires removal of Zn^{2+} and substantial dilution of the insulin depot, which would delay absorption (147; Fig. 3). It is not known if hexameric insulin can actually cross the capillary wall and, if so, whether the passage is more restricted compared with dimeric or monomeric insulin (dimensions of monomer ~2.5 × 2 × 3 nm).

In clinical studies, soluble human insulin has been reported to be absorbed slightly faster, resulting in higher plasma insulin levels than those from soluble pork insulin in healthy subjects and diabetic patients (89,91–96,98,99). However, the clinical significance of this small difference is questionable (59,94,96,149, 150). The mechanism of the increased absorption of human insulin relative to pork soluble insulin remains to be determined but has tentatively been explained by the more hydrophilic character of the human insulin molecule (150).

Many of the factors known to influence insulin absorption also have a more or less pronounced influence on the state of association of insulin or on the ease by which hexameric insulin dissociates into smaller units (Table 2). Concentration of insulin is a main determinant of the association state, and the higher the concentra-

TABLE 2
Factors influencing absorption of regular insulin

Factor	Blood flow relationship	Influence on insulin association state or dissociation rate	Refs.
Insulin formulation			
Species		++	91–99
Concentration		+++	59,100–103
Additives			
Aprotinin	+	BF	57,104–107
Blood serum	?	?	57
Prostaglandin E_1	?	?	108
Phenoxybenzamine	+	BF	109
Injection conditions			
Body posture	+	BF	110
Anatomical region	+	BF	56,57,59,111–113
Depth	+	BF	56,101,102,114–116
Volume (dose)		+	101,117
Jet injection		+	56,118–121
Sprinkler needle		+	122
Other factors			
Exercise	+	BF	12,98,113,123–129
Massage	0	+	57,130,131
Smoking	+	BF	132
Temperature	+	++	57,127,133–135
Epinephrine infusion	0	?	136
Blood glucose concentration	0	?	137
Sense of vibration (neuropathy)	?	?	101

BF, influence via blood flow; ?, unknown or uncertain; 0, no relationship; +, ++, +++, increasing influence.

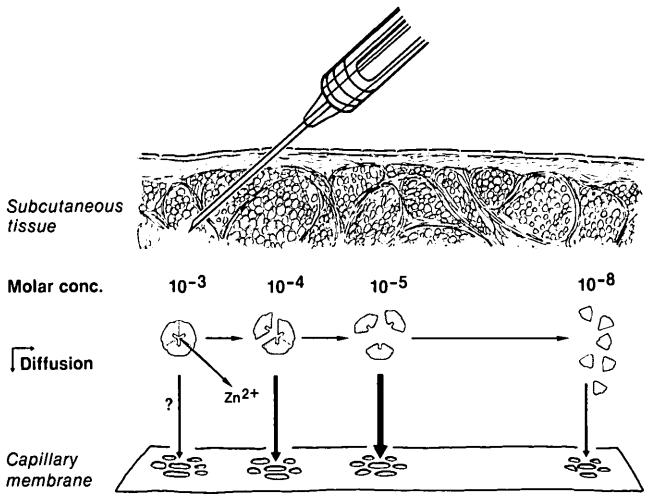

FIG. 3. Schema of putative events in subcutis after subcutaneous injection of soluble (regular) human insulin. Concentration of hexameric Zn insulin, the predominant association state of insulin in soluble insulin in U-40 or U-100 strength (U-100 ~0.6 mM), is lowered by diffusion in interstitial space. During this process, Zn-insulin hexamer complex disintegrates into smaller units. For dissociation into mainly dimeric insulin, 50- to 100-fold dilution is needed, whereas dominant population of monomeric insulin would require further 1000-fold dilution. Passage of more associated forms through capillary membrane is believed to be restricted due to steric hindrance.

tion, the higher the dilution required for dissociation of the insulin hexamer. The species of insulin may also have an influence on the tendency to dissociation of the hexamer on dilution, as shown for pork and human insulin (see PROOF OF CONCEPT AND ELUCIDATION OF ABSORPTION MECHANISM). Factors that influence blood flow and the effect of massage will result in changes in the rate of dispersion and subsequent dilution of the insulin depot and, consequently, alter the rate of dissociation of the oligomeric insulin units. Temperature, in addition to its effect on blood flow, has a direct influence on insulin association, because a shift from ambient to physiological temperatures leads to increased dissociation (J.F. Hansen, unpublished observations). It is probable that protein additives may also interfere with insulin association.

Apparently, absorption can be accelerated by factors with a direct or indirect influence on the association state of insulin or the rate by which associated insulin units disintegrate into smaller elements. Therefore, absorption rate may be increased by reducing the association state and thereby the average volume of the insulin units. Thus, administration of monomeric insulin would be expected to result not only in faster diffusion and less restricted transport but also in the time usually required for dilution and subsequent dissociation of insulin into mainly dimers (100-fold dilution) to reach maximal rate of absorption (lag phase) (Fig. 3).

INSULIN STRUCTURE

Native insulins. In 1928, insulin was found to be a protein (151), but the sequence of its 51 amino acids (primary structure) was first solved by the pioneering work of Ryle et al. (152; Fig. 4). The elucidation of the three-dimensional arrangement of the atoms in insulin rhombohedral crystals was expounded from 1969 to 1988 by the extensive work of Blundell et al. (146),

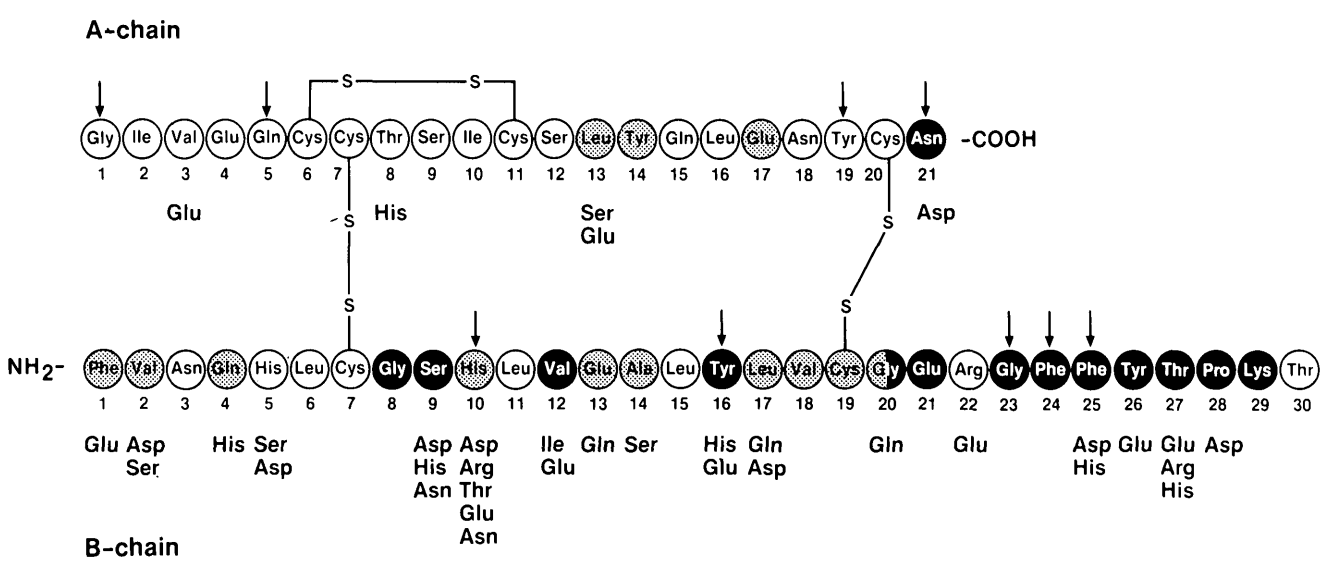

FIG. 4. Primary structure of human insulin with indications of amino acid residues involved in association of 2 insulin molecules into dimer (black residues) and in assembly of 3 dimers and 2 Zn^{2+} into Zn^{2+}-insulin hexamer (gray residues). Putative sites interacting with receptor are indicated by *arrows*. Sites and type of mutation in different analogues are also shown (for composition of individual analogues see Table 4).

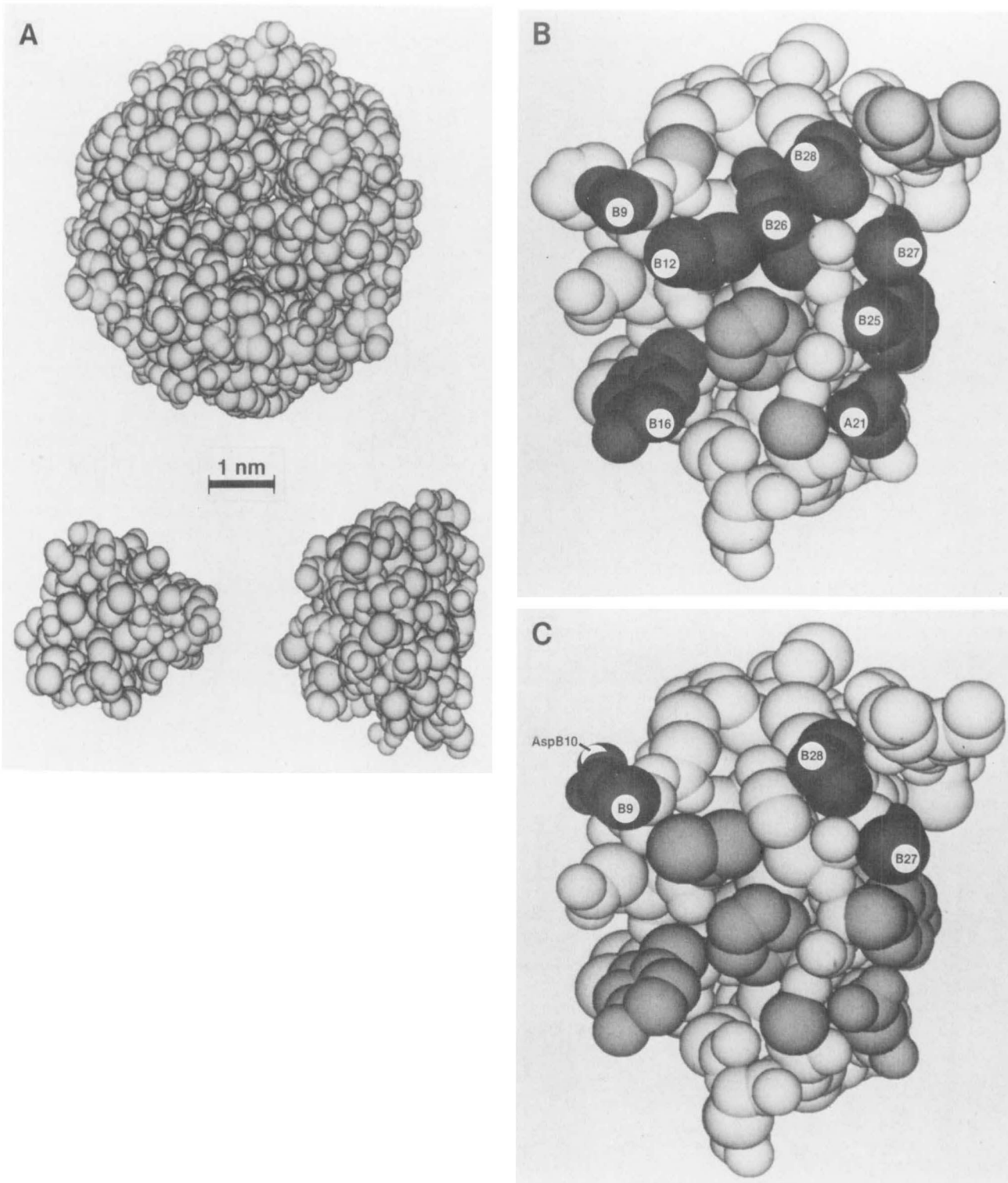

FIG. 5. Space-filling models of human insulin (van der Waal radii). *A*: relative sizes of monomer, dimer, and hexamer of insulin. *B*: view of monomer of insulin showing dimer forming surface. Side-chain atoms (for Gly α-carbon in main chain) of residues with ≤4 Å contacts (ref. 154) in dimer are shown in darkest colors. Side chains substituted in analogues are indicated by darkest color and denoted by number of residue. *C*: monomer of insulin as viewed in *B*. Gray atoms indicate side chains (for Gly α-carbon) of residues believed to be essential for receptor interaction. Sites of substitution in 3 analogues tested clinically are shown with dark-colored side chains and denoted by number of residue (see CLINICAL STUDIES). Actual change of residue is only shown for Asp^{B10} analogue, which allows comparison of its approximate structural changes relative to human insulin (*B*).

Adams et al. (153), and Baker et al. (154), revealing the tertiary and quaternary structures of the insulin molecule in its hexameric, dimeric, and monomeric states (Fig. 5*A*).

The physiological concentration of insulin is normally <1 nM, which ensures that insulin circulates and exerts its biological effects as a monomer (146,155–157). At higher concentrations, insulin dimerizes (association dimer), not to be confused (158) with the covalent dimer formed in small amounts during storage of insulin preparations (2,159). In neutral solution in the presence of Zn^{2+}, three dimers assemble to form a hexamer of insulin monomers (146). Hexamers of insulin, with the shape of a slightly flattened sphere, are the predominant association species of zinc insulin, at least down to a concentration of 0.1 mM, and therefore also the main association state found in neutral soluble insulin (U-100 ~0.6 mM) (160).

Many of the structural properties of insulin have evolved in response to the requirements of biosynthesis, processing, transport, and storage (146). The pattern of assembly facilitates proinsulin conversion and the subsequent crystallization and storage of hexameric insulin in β-cell granules but is evidently not connected to the interaction of insulin as a monomer to its receptor (161).

Exceptional and synthetic insulins. Primary structures have been published for >50 animal species of insulin, but only a few of these insulins have impaired association properties, and only those belonging to the hystricomorph rodent family have been found unable to dimerize. These insulins, however, have an altered tertiary structure and substantially reduced biological potency (162–172; Table 3).

Numerous modifications of the primary structure of insulin have been made by total synthesis or semisynthesis (146,173,174). Few of these derivatives have been reported not to aggregate, but, with the exception of despentapeptide insulin, these insulins have impaired in vivo potency (Table 3). Despentapeptide insulin, however, is not physically stable in solution, especially in the presence of divalent metal ions (175). Sulfated insulin normally consists of a heterogeneous mixture of many different derivatives that are probably partly unfolded (2,171; see PROOF OF CONCEPT AND ELUCIDATION OF ABSORPTION MECHANISMS).

Protein engineering. Advances in genetics and molecular biology have now provided convenient methods for introducing changes into a native protein with the purpose of evaluating how side chains contribute to the physicochemical and biological properties of the protein. Recombinant DNA technology has made it possible to engineer modifications into the amino acid sequence of insulin, enabling production of insulin analogues with one or more changes of amino acid residues with the intention to investigate the role of the individual amino acid in the molecular assembly, biological activity, and therapeutic properties. The pioneering work of Winter and Fersht (176) and Fersht et al. (177) has resulted in research that has revealed that the capacity of a protein to dimerize can be abolished by site-specific mutagenesis (178). These new opportunities have made possible a new approach in optimizing insulin preparations for therapeutic use, i.e., redesign of the active drug for better replacement therapy.

The therapeutic limitations of available insulins were reemphasized by the work group on insulin therapy at the First World Conference on Diabetes Research (Juvenile Diabetes Foundation, 1985), recommending that monomeric insulins and insulin derivatives should be tested. In response, this review relates to the development of monomeric insulins in an attempt to achieve a more physiological, quantitative, and temporal postprandial insulin profile, obviating the need for administration of soluble insulin 0.5–1 h before food, thereby improving compliance and minimizing the risk of delayed hypoglycemia (3,179,180).

Parallel developments in protein engineering are also making it possible to produce insulins with improved properties to simulate basal insulin secretion (181,182).

CREATION OF INSULINS WITH REDUCED SELF-ASSOCIATION

Rationale, concept, and strategy. The concordance between factors with a mutual influence on insulin association and absorption rate (Table 2), together with the fact that smaller molecules or units diffuse more quickly and are less hindered in passing through the capillary membrane, strongly indicates that insulin with reduced tendency to self-association would be more quickly absorbed after injection (see CLINICAL OBSERVATIONS). Therefore, experiments to create insulins with reduced self-association were undertaken with computer-aided molecular modeling and DNA technology (3,179,183).

A rational approach to insulin engineering requires detailed knowledge about the conformation of the molecule and its modes of interaction at the atomic level

TABLE 3
Natural (N) and semisynthetic (S) monomeric insulins

Insulin	Source	Mutations/ changes	Potency (%)		Refs.
			In vitro	In vivo	
Guinea pig	N	18	2	9	163,164
Casiragua	N	21	5		165
Coypu	N	22	3		166
Porcupine	N	8	4		167
Despentapeptide B26–B30	S	6	25	77	168,169
Despentapeptide B26–B30 amide	S	5	105		168
Sulfated	S	4–5		~20	170,171
Tetranitrotyrosine	S	4		50	172

All data relative to human insulin.

within and between subunits. The insulin hexamer found in neutral solution is believed to be the same as that observed in crystals (146,184–186). Therefore, the precise information regarding the arrangement of the individual atoms in the tertiary structure, as determined by X-ray crystallography, has been used to analyze the interactions between insulin monomers in dimers and hexamers and to predict the alterations necessary to produce changes in association pattern (153,154). Model building and computer graphics were essential elements in these considerations.

The aggregation surfaces, which direct the assembly of the insulin molecules into dimers and hexamers, involve mainly B-chain residues that, in several cases, are also included in the putative receptor-binding region of the hormone and are to a greater or lesser extent essential for biological potency of the hormone (187,188; Figs. 4 and 5, *B* and *C*). The amino acid substitutions were selected after reviewing the aggregating surfaces and their interactions in dimeric and hexameric units. Because dimer formation is a necessary requirement for higher aggregation, the main targets have been the residues responsible for dimer formation, including a few of those involved in receptor binding (156; Figs. 4 and 5*B*; Table 4). An important requirement for alterations in amino acid residues has been to retain the integrity and overall tertiary structure of the monomer to avoid a large reduction in bioactivity, which is the case with the naturally occurring monomeric insulins (162; Table 3).

The different strategies used for counteracting asso-

TABLE 4
Association behavior and biological characteristics of analogues

Human insulin analogue	Association state when zinc free (osmometry 1 mM; 21°C)*	Biological potency (%)			Negative cooperativity low/high concentration§	Code used in Fig. 7
		FFC†	MBG†	RBA‡		
Asp^{B9}	1.1	26	79		+/−	5
Asn^{B9}	~4	47				
His^{B9}	4.5	69			+/−	
Asp^{B10}	2.2	207	98	315	+/−	T
Arg^{B10}	4.2	50	90			E
Thr^{B10}	3.5	72				
Ile^{B12}	3.3	29	86		+/−	3
Glu^{B12} (plus des-B30)	1.0	0.04		0.15		9
His^{B16}	1.0	43		35		
Gln^{B17}	2.3	13			+/+	R
Gln^{B20}	3.7	88	73			
Asp^{B25}	2.2	0.10		0.05	(−)/−	
His^{B25}	3.9	45		28	+/−	
Glu^{B26}	2.0	125	104	158		A
Glu^{B27}	4.0	108	110	87	+/−	1
Asp^{B28}	1.3	101	104	88	+/−	F
Glu^{A13},Glu^{B10}	1.9	14		27		
Ser^{A13},Glu^{B27}	2.9	11			+/+	
Asp^{A21},Glu^{B27}	1.5	87	61		+/−	4
Glu^{B1},Glu^{B27}	2.6	97				
Ser^{B2},Asp^{B10}	~2	253		385		
Asp^{B5},Asn^{B10}		0.13				
Asp^{B9},Arg^{B27}	1.6	40	77			
Asp^{B9},Glu^{B27}	1.1	31	93	20	+/−	2
Asp^{B10},Asp^{B28}	1.6	201				X
Glu^{B12},Gln^{B13}	1.2	0.05		<0.05		
Ser^{B14},Asp^{B17}	~2	6	30			
Glu^{B16},Glu^{B27}	1.1	13	55		+/−	8
Asp^{B2},Ser^{B5},Glu^{B27}	4.0	30	55	23		0
Glu^{A3},Glu^{B10},Glu^{B22}	1.2	0.06				
His^{A8},Asp^{B9},Glu^{B27}	1.1	116	114	94		7
His^{A8},Asp^{B10},His^{B25}	1.7	203			+/−	
Asp^{A21},Asp^{B9},Glu^{B27}	1.1	25	75			6
His^{A8},His^{B4},Glu^{B10},His^{B27}	~2	573	86	503	+/−	
Human, Zn^{2+} free	4.4					Z
Human, 2 Zn^{2+}/hexamer	6	100	100	100		H

*Data from refs. 3, 183; J.F. Hansen, unpublished observations.
†FFC, free–fat cell assay; MBG, mouse blood glucose assay. Data from refs. 3, 183; A.R. Sørensen, unpublished observations.
‡RBA, receptor-binding affinity (human hepatoma cell line). Data from refs. 3, 183, 214; K. Drejer, unpublished observations.
§ +, Presence; −, absence of negative cooperativity. Data from ref. 217.

ciation are shown in Table 5. The primary strategy followed was to introduce charge repulsion into the interfaces. Addition of positive charge will, because the isoelectric point of insulin is ~5.4, tend to decrease solubility of the resulting insulin at physiological pH. Therefore, negative charges (amino acids with carboxylic acid in the side chain) have been used in most cases. Side-chain carboxyl groups (Asp or Glu residues) in some cases already exist adjacent to the interface and have been chosen as opponents to an inserted negative charge in the opposite unit. Examples of analogues produced according to the different strategies are shown in Table 5.

Association state and tendency to dissociation. The association pattern of the analogues was assessed by various physicochemical methods including osmometry and size-exclusion chromatography (SEC) (3,189).

The osmotic pressure over a semipermeable membrane, impermeable to insulin and separating a solution of insulin from the pure solvent, is a function of the number of solute particles per unit volume. Therefore, measurement of the osmotic pressure of an insulin analogue relative to that of hexameric human insulin gives an estimate of the mean association state of the insulin analogue at a particular concentration. The results of such measurements at 1-mM concentration, which is well above the normal pharmaceutical strength (U-100 ~0.6 mM), are given in Table 4. It appears that 33% of the analogues are essentially monomeric and another 33% primarily dimeric. The remaining analogues are more associated, although less than the parent molecule, which has a mean association state of 4.4 in its Zn^{2+}-free state. Note that, if the temperature is increased to 37°C, then the dimeric Asp^{B10} analogue also becomes more widely dissociated (J.F. Hansen, unpublished observations).

The mean association state, however, does not give information about the strength by which the individual units are held together. The tendency of the assembled insulin to dissociate into smaller units during lowering of the insulin concentration can be evaluated in SEC experiments. The time of elution and the elution pattern give a qualitative measurement of this property and can be used for differentiating insulins with the same mean association state as deduced from osmometry. The elution profiles of different insulins are shown in Fig. 6. Whereas monomeric analogues elute late in the chromatogram as a symmetric peak, dimeric analogues and human insulin elute earlier with asymmetric distribution dependent on the Zn^{2+} concentration (see PROOF OF CONCEPT AND ELUCIDATION OF ABSORPTION MECHANISMS).

Structure and stability. All current mutations are on the surface of the monomer and are not likely to grossly

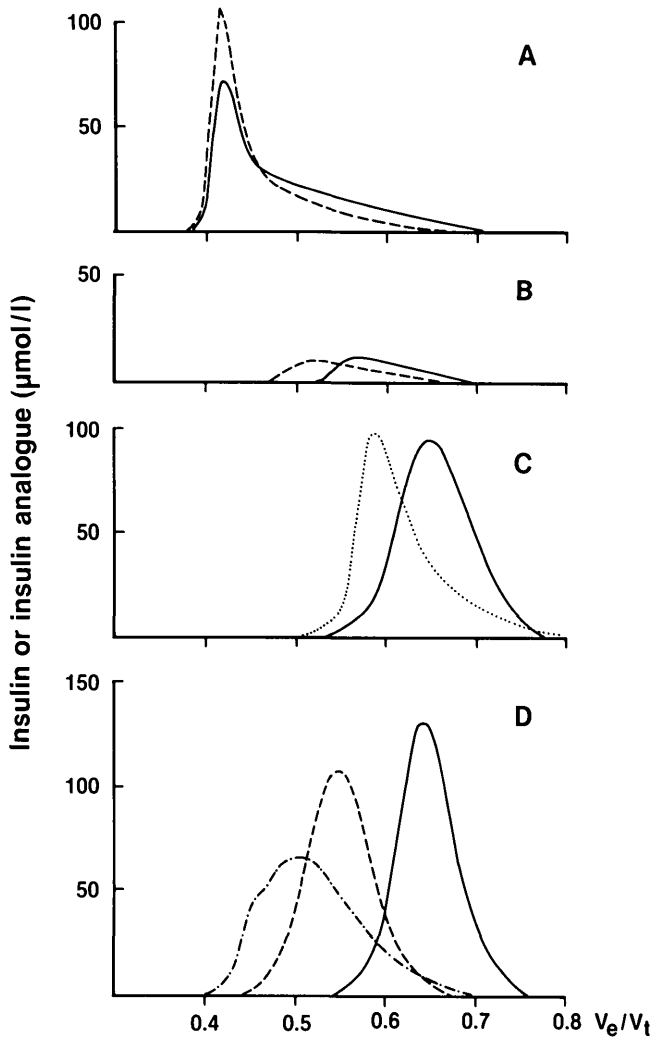

FIG. 6. Size-exclusion chromatography on Bio-Gel P-60, 100–200 mesh (25 × 440 mm). Eluent, 0.04 M Tris, 0.01 M NaCl, pH 8.0; elution rate, 3 cm/h. Photometer readings at 276 nm were transformed to molar concentration with molar absorption coefficient of $6.2 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$. V_e, elution volume; V_t, total column volume. *Curves in each panel (A–D)* represent consecutive runs on same column. *A*: applied 1.3 μmol pork (*dashed line*) and human (*solid line*) 2 Zn^{2+} insulin (10 mg in 1 ml ~U 220 ~1.3 mM). *B*: applied 0.3 μmol pork (*dashed line*) and human (*solid line*) 2 Zn^{+} insulin (2 mg in 1 ml ~U 50). *C*: applied 1.7 μmol Asp^{B10} (*dotted line*) and Asp^{B28} (*solid line*) analogue each in 1 ml. *D*: applied 2.0 μmol sulfated beef insulin (*dashed line*); 2.0 μmol Asp^{B9},Glu^{B27} analogue (*solid line*); and 1.7 μmol Zn^{2+}-free human insulin (*dashed and dotted line*) all in 1 ml. Dilution taking place during passage of column (peak concentration) will appear to be 10- to 20-fold.

TABLE 5
Counteraction of insulin association

Strategy	Examples
Charge repulsion	
With already existing charge	Asp^{B9} (Ser), Glu^{B12} (Val), Asp^{B25} (Phe), Asp^{B28} (Pro)
Introducing charge counterparts	Glu^{B27} (Thr) + Asp^{A21} (Asn)
Steric hindrance	Ile^{B12} (Val)
Hydrophilicity into hydrophobic interfaces	Glu^{B16} (Tyr), Gln^{B17} (Leu), Glu^{B26} (Tyr)
Removal of metal-binding sites	Asp^{B10} (His), Thr^{B10} (His)

Amino acid residue in parentheses is residue in human insulin.

influence the three-dimensional structure of the mutants relative to that of native insulin. A few of the analogues have been crystallized, and X-ray crystallographic analysis revealed small structural changes that generally only occur in the neighborhood of the altered residue when compared with human insulin (190; G. Dodson, unpublished observations). Although the Asp^{B9},Glu^{B27} analogue is essentially monomeric at 1-mM concentration, it does crystallize as a hexamer in the presence of 1% phenol in addition to Zn^{2+} and Ca^{2+}. Crystallographic studies have shown that the structure is similar to the native monoclinic phenol structure described by Derewenda et al. (191), except for several Ca^{2+}-binding sites situated in the core of the mutant hexamer (J.P. Turkenburg, unpublished observations). The presence of Ca^{2+} in the structure explains the formation of the hexamer with 12 negatively charged carboxylic acids closely packed together. Preliminary crystallographic studies on crystals of the Asp^{B10} analogue have indicated that the molecules are arranged as dimers with a structure similar to that seen in the cubic structure of native insulin (192; J.P. Turkenburg, unpublished observations).

Studies by nuclear magnetic resonance (NMR) spectroscopy of the monomeric Asp^{B9} analogue in neutral solution have shown that this mutant and monomeric human insulin (low concentration) exhibit nearly identical proton NMR spectra. This strongly indicates that the Asp^{B9} mutant and nonaggregated human insulin have similar three-dimensional structures in solution, whereas the free-monomer conformation of native human insulin is not the same as that in the associated state (193,194). This result is in agreement with earlier conclusions by Bi et al. (185) that monomeric despentapeptide insulin has a definite three-dimensional structure in solution, essentially identical to that of insulin in its hexameric crystalline form (153,154). Whether a similar unaltered tertiary structure also applies to other analogues with reduced association tendency remains to be seen.

The physical stability (resistance to precipitation as insulin fibrils) of insulin in solution has been demonstrated to increase by stabilization of the hexameric state of insulin (195,196). This effect is explained by insulin fibrils being formed via the monomeric state (197). The chemical stability (resistance to degradation by chemical reactions) of insulin has been shown to improve when the flexibility of the molecule is reduced on association and crystal formation (J.B., unpublished observations).

Consequently, the stability of a nonassociating insulin would be expected to be less than that of an associated insulin. Preliminary investigations have shown that this is the case for many of the analogues; however, a pharmaceutical formulation with satisfactory physical and chemical stability has been developed for the Asp^{B10} analogue (S. Havelund, unpublished observations). Selected analogues with comparable and, in some respects, even largely improved stability relative to human insulin are under development (L. Langkjaer, unpublished observations).

BIOLOGICAL AND PHARMACOLOGICAL PROPERTIES

Receptor binding. It is generally accepted that binding of an insulin molecule to its receptor is the initial event for eliciting biological responses. It also appears that receptors from different cell types within the same subject have similar binding affinities but differ with respect to the binding capacity or number of surface-active receptor-binding sites. Because of this variation, binding studies are often conducted as comparative assays, in which the binding properties of a test insulin are compared with those of a reference.

The relative receptor-binding affinities were obtained with the human hepatoma cell line (HepG2) (198; Table 4). The assays were performed at 4°C to minimize internalization and degradation of insulin. The displacement of ^{125}I-labeled human insulin by increasing concentrations of the analogue and cold human insulin was analyzed according to the dose-response relationship to yield an estimate of the relative equilibrium-binding affinity.

Substitution of a few amino acids can reduce the binding affinity by a factor of ~2000 or increase it sixfold (Table 4). The Asp^{B10} analogue has also been studied by Schwartz et al. (199), who found a potency of 534 ± 146% relative to bovine insulin in receptor-binding assays with rat liver plasma membranes. Previously, the D-Phe^{B24} analogue of human insulin had been found to have increased binding (180%) to cultured human lymphocyte receptors relative to human insulin (200). Of the native insulins, only those from turkey and chicken have been found to have increased binding affinity to mammalian receptors (201). These insulins differ from human insulin in seven positions, but the substitution (to His) in position 8 in the A chain is the predominant explanation of the increased affinity (174). Note that pork and beef insulin, which differ in exactly the same position (Thr and Ala, respectively), have been found to have the same receptor-binding affinity (202).

The question of how several substitutions modify receptor binding has been studied, and it was found that the effect of two or more substitutions could be approximately described by multiplication of the relative potencies associated with the single-site substitutions (P. Hougaard, unpublished observations). For example, realizing that the His^{A8} substitution enhances the receptor-binding affinity as mentioned above, the reduced affinity (20%) of the disubstituted Asp^{B9},Glu^{B27} analogue is compensated in the trisubstituted His^{A8},Asp^{B9},Glu^{B27} analogue with 94% binding affinity (Table 4). Likewise, by combining the two affinity-enhancing substitutions, His^{A8} and a negative charge (Asp or Glu) in position B10, in the same analogue (His^{A8},His^{B4},Glu^{B10},His^{B27}), a high-affinity analogue is obtained (Table 4). This general multiplicative rule for combination of effects of substi-

tutions indicates independence between the individual sites in binding to the receptor.

Most of the insulin analogues or derivatives that have been studied have exhibited reduced receptor binding in various assay systems (203–212). The availability of a long series of insulin analogues with a range of binding affinities may have potentially important applications as tools to analyze the structural determinants for binding of insulin molecules to receptors and modifying post–receptor-binding events. Because it would be expected that modified-binding affinity would be associated with modified biological effects, insulin analogues with increased receptor binding may also exhibit increased biopotency.

Because insulin also has some ability to bind to the insulin growth factor (IGF) receptors, it would be of interest to determine whether the monomeric analogues have similar low binding affinities (212,213). Drejer et al. (198) tested a subset of the analogues relative to IGF-I and found a similar ranking as shown for binding to the insulin receptor (K. Drejer, V. Kruse, U.D. Larsen, S. Gammereltoft, unpublished observations; Table 4). However, the binding affinities of the insulin analogues were at least a factor of 1000 lower than that of IGF-I, suggesting that the analogues are not expected to change the balance between metabolic and growth-promoting actions mediated via insulin and IGF-I receptors. Binding of the analogues Asp^{B9}, Asp^{B10} and Asp^{B28} to the IGF amniotic fluid–binding protein has been found to be undetectable. Even extraordinarily high doses of the insulin analogues could not displace labeled IGF-I from the binding protein (E.M. Spencer, unpublished observations).

An interesting feature of the binding of insulin to cell surface receptors is the phenomenon of negative cooperativity, meaning that insulin binding induces a loss of affinity of the other receptor sites for insulin due to an accelerated dissociation rate of the insulin-receptor complex (215,216). This is assumed to be related to site-site interactions between cell surface–located insulin-receptor complexes and secondary conformational changes after initial binding. Because the physiological significance of this phenomenon is not clear, it is important to determine whether the monomeric insulin analogues exhibit negative cooperativity to the same extent as human insulin. Animal insulins, including the monomeric guinea pig insulin, show intact negative cooperativity, whereas despentapeptide insulin has an impaired capacity to elicit negative cooperativity (216; Table 3). Of the 15 analogues analyzed, only the Asp^{B25} analogue, with low binding affinity for the receptor, revealed a probable deviation from full ability to induce negative cooperativity (217; Table 4). The negative cooperative effect normally decreases when the insulin concentration in the medium exceeds 10^{-7} M (215). Because the two nonaggregating insulins, guinea pig and tetranitrotyrosyl insulin, show no tendency to such a decrease, this disappearance of cooperativity at high concentrations has been explained by insulin-dimer formation (216; Table 3). The results obtained with current monomeric analogues clearly indicate that the fall in negative cooperativity at increased insulin concentration is unrelated to the capacity of insulin to form dimers (217; Table 4; M. Kobayashi, unpublished observations). The only two analogues that did not show the disappearance of negative cooperativity at high concentration were substituted (A13 and B17 residues) in the surfaces interacting when dimers form a hexamer. This result indicates that interaction of the unlabeled insulin (with prebound ^{125}I-labeled insulin or with another receptor domain) stabilizes, at high concentration, a tightly bound state of the ^{125}I-labeled insulin-receptor complex and abolishes the negative cooperativity (217).

In vitro biological activity. In addition to receptor-binding activities, relative potencies of the analogues determined in the free–fat cell bioassay are also shown in Table 4. The assay is identical to the method developed by Moody et al. (219), which is based on the incorporation of [3-^{3}H]glucose into lipids during a 2-h incubation of free fat cells from mice at 37°C. It appears from the results that the free–fat cell and the receptor-binding activities are closely correlated (Table 4). Calculation of the correlation coefficient yields a value of 0.97 ($P < 0.001$). Similar close relationships between receptor binding and biological effects in the same cell systems have been reported earlier (206,220).

This relationship is fundamental for the above-mentioned concept that the metabolic effects of insulin require binding of the molecule to its receptor. Maximal metabolic effects are attained at relatively low binding levels corresponding to a receptor occupancy of ~5–10%. Several studies that used partially overlapping methods for determining receptor binding to different cell types and biological activities (with various cell systems and metabolic responses) have given similar results for a range of insulin analogues and derivatives (3,199,200,203–206,208–212,220). Thus, it seems reasonable, as a useful approximation, to characterize insulin analogues or derivatives by in vitro potency, regardless of which method was used. Nevertheless, new analogues should be tested for differential effects on various cell types or metabolic processes. For example, among the analogues analyzed there are two (Glu^{A13},Glu^{B10} and His^{B25}) that differ by a factor of almost two in free–fat cell activity relative to receptor binding (Table 4). These analogues need further testing in other systems.

Another aspect of differential activity of insulin analogues with regard to clinical use for treatment of diabetic patients is whether two different insulins, e.g., human insulin and one of the monomeric analogues, have additive effects when both are present. Mixtures of beef insulin, four insulin derivatives, and pork proinsulin have been investigated in the rat free–fat cell assay and found to deviate significantly from additivity (221). Secretion of normal human insulin (40%) and a mutant insulin (60%), in which Phe^{B24} or Phe^{B25} was substituted by Leu, has been reported in a diabetic patient (222).

The mutant insulin had only 10% receptor-binding affinity (human IM-9 lymphocytes). However, whereas the receptor-binding affinity of the mixture of mutant and human insulin was ~45%, the in vitro biological potency of the mixture was significantly reduced to 12% with regard to glucose transport or oxidation in rat adipocytes. This substantial antagonistic effect with respect to biopotency could explain the apparent insulin resistance of this patient, who was characterized as hyperinsulinemic and nonketotic.

Some of the monomeric analogues have also been tested for additive effects in mixtures with human insulin (223). With the mouse free–fat cell bioassay system, it was found that analogue Asp^{B9},Glu^{B27} showed a statistically significant but relatively small antagonistic effect (~10% loss of activity), whereas three other analogues (Asp^{B10}; Asp^{B28}; and $His^{A8},His^{B4},Glu^{B10},His^{B27}$) showed no significant deviations from additivity. It was concluded that the magnitude of the deviations from additivity seen with these analogues would not be of clinical significance.

Hypoglycemic effects (in vivo potency). In vivo animal bioassays have been used to assess the potency of insulin since insulin was first isolated. A series of successive international standards of insulin have been established to define the unit, and insulin manufacturers have been required to conduct in vivo assays of new insulin batches according to pharmacopoeial methods. Small species differences in relative potency of insulin in the rabbit assay and the use of single-species insulin preparations have led to the establishment of the current human, pork, and beef international standards (224,225). In past years, there has also been a trend toward replacing the laborious in vivo assays with quantitative high-performance liquid chromatography methods that can check the potency (strength) of the insulin preparation and its identity and purity (226).

Most analogues, including those with in vitro potency >100%, have in vivo potencies near 100% when assayed relative to the human international standard, according to the mouse blood glucose bioassay (227; Table 4). Some of the analogues with reduced in vitro potencies at least down to ~30% have retained nearly 100% in vivo potency. Similar results have been observed previously and explained by Jones et al. (208), who studied four insulin derivatives and pork proinsulin by successively increasing the intravenous infusion rate in dogs. They were able to express the in vivo potency based on the hypoglycemic response relative to either the dose or the steady-state molar concentration. The estimated in vivo potency relative to the circulating concentration was in close agreement with the in vitro potency determined in the free–fat cell and receptor-binding assays, which gave results in the range from ~2 to 30% relative to beef insulin. With in vivo potency expressed in the usual way, relative to the infused doses, much higher potencies ranging from 29 to 97% were obtained. Because the reduced in vivo potency based on the circulating concentration was due to the markedly increased steady-state molar concentration, the most likely explanation was that the analogues with low receptor binding and in vitro potency had a lower metabolic clearance rate. The resulting higher in vivo concentrations would then counterbalance the reduced in vitro or intrinsic potency. This explanation would also apply to the analogues, provided they also exhibit proportionality between plasma clearance and in vitro potency (Table 4). However, Jones et al. (208) did not show whether analogues with >100% in vitro potency are cleared more rapidly from the circulation than human insulin.

Clamp studies and clearance determinations. The euglycemic-hyperinsulinemic clamp technique can provide information about the clearance of insulin infused intravenously and the amounts of glucose required to balance the effect of the insulin (228). Ribel et al. (229,230) conducted clamp studies in pigs with human insulin, with the Asp^{B9},Glu^{B27} and Asp^{B10} analogues infused at a constant rate of 6 pmol · min^{-1} · kg^{-1} for 2 h. The steady-state glucose infusion rates required to maintain euglycemia during the last 30 min of the 2-h infusion did not differ significantly and neither did the total amounts of glucose required during the 2-h infusion and 2-h postinfusion follow-up. These results confirm the similarity of the in vivo potencies of the two analogues and human insulin. However, the steady-state concentration of the insulins was different. The Asp^{B9},Glu^{B27}–substituted analogue achieved a two to three times higher plasma concentration and the Asp^{B10} analogue a significantly reduced steady-state level compared with human insulin. When the metabolic clearance rates of the insulins were calculated, the mean values were ~20, 7, and 26 ml · min^{-1} · kg^{-1} for human insulin, Asp^{B9},Glu^{B27}–, and Asp^{B10}-substituted analogue, respectively. Thus, these results are in complete agreement with the expected relationship between in vitro potency and metabolic clearance.

Another clamp study has been conducted in minipigs with the same dose of the same two analogues and human insulin but with a 4-h infusion period and simultaneous infusion of [3-^{3}H]glucose to allow for calculation of rate of hepatic glucose output (R_a) and rate of glucose uptake (R_d) (231). Whereas the metabolic clearance rates determined in these pigs were two to three times higher than the values quoted above, the ranking between the values for human insulin and the two analogues was similar. Tracer data showed that both analogues and human insulin were equally efficient with respect to suppressing R_a and enhancing R_d. A clamp study in rats also confirmed the differences in clearance between human insulin and the same two analogues (Asp^{B9},Glu^{B27} and Asp^{B10}) (232). No differences were found with respect to R_a and R_d.

These clamp studies indicate that the similar in vivo potency relative to human insulin of the Asp^{B9},Glu^{B27} analogue (with low in vitro potency) and the Asp^{B10} analogue (with high in vitro potency) can be explained on the basis of the differences in metabolic clearance.

Thus, the analogue with low in vitro or intrinsic potency exerts full in vivo action, because it reaches higher concentration levels due to its reduced plasma clearance, which again presumably is a consequence of its reduced receptor-mediated elimination.

Metabolism and postreceptor effects. The distribution kinetics of certain insulin analogues has been studied in rats by whole-body scintigraphy, as described by Sodoyez et al. (233). A14-Tyr-^{123}I-labeled insulin analogues and human insulin were administered by intravenous bolus injection, and the counts over the liver, kidneys, heart, and background tissues were followed for 30 min (234). It was observed that low-affinity analogues, such as the Asp^{B9},Glu^{B27} analogue, were predominantly taken up by the kidneys. The high-affinity analogues, such as Asp^{B10} and His^{A8},His^{B4},Glu^{B10},His^{B27}, showed higher uptake by the peripheral tissues and less by the kidneys and liver, where the count tended to be lower than with human insulin and the Asp^{B28} analogue.

After binding of insulin to its receptor, the hormone-receptor complex is transported across the plasma membrane (internalized), and insulin is subsequently degraded by insulin proteases. These processes were studied in HepG2 at 4°C with A14-Tyr-^{125}I-labeled insulin analogues and human insulin, and it was found that the two high-affinity analogues mentioned above were internalized to a greater extent and showed less degradation after internalization than low-affinity analogues (V. Kruse, unpublished observations).

A major initial cleavage site in the degradation of insulin has been suggested to be the B16–B17 peptide bond (235). Degradation by insulin proteinase of the two B16-substituted analogues has been studied and shown to be decreased with the Glu^{B16},Glu^{B27} analogue, whereas the analogue with a B16 Tyr-to-His replacement had unchanged susceptibility to cleavage by the enzyme compared with native insulin (M. Kobayashi, unpublished observations; Table 4).

In addition to the effects on glucose uptake and suppression of hepatic glucose production, the above-mentioned clamp studies also provided data on the entrapment of [^{3}H]-2-deoxyglucose by different tissues (232). No significant differences were observed between human insulin and the two analogues in various muscle tissues of different fiber composition or in brown and white adipose tissue.

Another approach to study differential effects of insulin analogues on intermediary metabolism was used by Falholt et al. (236). Normal pigs were given twice-daily injections of 120 nmol s.c. (20 IU) human insulin or the analogues at mealtimes for 4 wk. Tissue samples of muscle, liver, and aorta were then analyzed for enzyme activities and metabolites. All three analogues studied (Asp^{B9},Glu^{B27}; Asp^{B10}; and Asp^{B28}) showed a significantly reduced triglyceride content in all three types of tissues compared with injection of either soluble human insulin or medium (saline). In keeping with this result, glucose-6-phosphate dehydrogenase activity was also significantly diminished in the same groups. In contrast, the glycogen content of muscle and liver was increased with the analogues relative to the increase found with the human insulin preparation. Parallel changes in glycogen synthase activity were also observed, but there were no consistent effects on the other enzymes studied, i.e., phosphofructokinase, hexokinase, and pyruvate kinase. It was concluded that the differential effects were probably due to the fast absorption of the analogue preparations, including the faster return to basal insulin levels or the faster and more pronounced hypoglycemia induced by the analogues. Whether a more rapid absorption of insulin can lead to preferential deposition of glycogen relative to lipids in the treatment of diabetic patients remains to be studied.

Summary. It is clear from the results obtained with various insulin analogues and derivatives that their in vivo biological potencies relative to human insulin cannot be accurately predicted from various in vitro assay systems. These in vitro potencies do, however, agree (usually within a factor of 2) regardless of the in vitro assay system used. Because binding to the insulin receptor is the initial event, it seems reasonable to assume that the similarity of the in vitro potencies reflect similarities between insulin-receptor binding sites from different species and cell types.

Most of the insulin analogues or derivatives that have retained a substantial part, i.e., at least 20% of the in vitro potency, possess nearly 100% in vivo potency. This can be explained by a dominating receptor-mediated clearance of these analogues, which leads to higher circulating concentration of the low-affinity analogue, which in turn counterbalances its reduced in vitro activity and results in nearly 100% in vivo potency. Similarly, a high-affinity analogue will be cleared more rapidly and give a lower in vivo concentration, resulting in approximately the same in vivo bioactivity as human insulin.

These results and the underlying concepts do not leave much hope for finding insulin analogues with differential effects on different organs or metabolic pathways (208). On the other hand, such possibilities should not be ruled out in advance because they could have important implications.

Although insulin receptors may not be able to discriminate qualitatively between different insulin analogues, it may still be possible to modify postreceptor events by changing the time course of insulin-receptor stimulation, as suggested by some of the experimental results (236). Such differential dynamic effects would be difficult to detect in steady-state in vitro or in vivo experiments.

IMMUNOLOGICAL ASPECTS

Various unwanted effects associated with insulin therapy are attributable to insulin-antibody production. These immunological side effects include lipoatrophy, local and systemic allergy, and immunological insulin resist-

ance (237–239). The introduction of highly purified monocomponent insulins in the 1970s and the availability of human insulin in the 1980s have led to a decreased prevalence and lower titers of insulin antibodies and, consequently, less severe immunological complications. However, circulating insulin antibodies are still seen in patients treated solely with homologous human insulin (84,240–244). The reasons formulations of human insulin administered subcutaneously induce antibody production in contrast to the endogenous hormone remain poorly understood. The residual immunogenicity of human insulin preparations has been assumed to be due to the chemical alterations of the molecule that develop during storage or after injection, especially when higher molecular transformation products are formed (245–249).

Immunogenicity (antibody formation). In addition to purity, species, and stability, factors influencing the immunogenicity of an insulin preparation include pH, physical state of the insulin, retarding agent, and the degree of protraction (length of stay in subcutis) (237,245,250–255). The influence of the latter factor is supported by the observation that subcutaneous continuous pump infusion gives rise to higher antibody levels than conventional injection of the same soluble insulin (256,257).

Association of insulin with its antibody is a reversible process dependent on the capacity and affinity of the antibody. Therefore, circulating antibodies sequester injected insulin within the vascular compartment, and this complex acts as an unphysiological buffer reservoir of insulin (258). This process has the effect of reducing the availability of administered insulin and damping oscillations in free-insulin levels, also resulting in an apparent increase in half-life of free insulin (81,238,259–272). Thus, the higher the titer of insulin antibodies, the slower the increase in plasma free-insulin concentration and, consequently, the greater the postprandial plasma glucose after a meal bolus injection of insulin.

The antibody-bound insulin reservoir also leads to a prolongation of action of injected soluble insulin, a delayed return to baseline free-insulin levels, and increased risk of delayed hypoglycemia (81,265,268,273). The higher the titer, the slower the plasma glucose recovery after hypoglycemia (265). The clinical relevance of low-affinity antibodies is still being debated, but it is evident that the presence of high-affinity antibodies with sufficient capacity to blunt and delay the free-insulin peaks is a disadvantage in the attempt to achieve normal physiological insulin profiles in relation to meals (84,262,274–277). Consequently, when near normoglycemia is the goal, the presence of insulin antibodies is undesirable, and their plasma concentration should be kept below a clinically significant level. This can only be achieved if insulin preparations of low immunogenicity are used for treatment.

In the past, a rabbit model was developed for evaluating the influence of varying levels of impurities in insulin (32,278). With this model, conventional insulin crystallized five times, and highly purified beef and pork insulins did not result in antibody formation when administered in neutral solution (32,279). Only when administered at high dose levels and incorporated into an oil emulsion (Freund's incomplete adjuvant), primarily having the effect of prolonging the stay in subcutis and thereby the stimulus to the immune system, was significant antibody formation seen (32,278). A similar difference in response between insulin in solution and as a protracted preparation was observed in subjects injected with conventional beef insulin. The protracted preparation resulted in high-antibody titers, whereas the short-acting beef preparation gave a low response comparable to that of pork short-acting insulin (250,252, 275). Because of the lack of a response without adjuvant, it is questionable whether the rabbit model is suitable for predicting the clinical immune response to subcutaneously administered short-acting novel insulins. It may, however, be useful to identify and reject highly immunogenic insulin analogues for screening purposes.

Realizing the shortcomings of the rabbit model, an alternative and clinically more relevant in vitro method that uses human lymphocytes has recently been developed for the assessment of immunogenicity of novel insulins in humans (280). Lymphocytes from healthy subjects are primed with insulin before a secondary challenge with the same insulin in conjunction with autologous antigen-presenting cells. The response of this assay to heterologous insulins is in keeping with the ranking order observed for antibody production in the clinic (280). The immunogenicity of several of the monomeric analogues has been tested in this way with lymphocytes from five donors previously shown to respond positively to animal insulins. Only analogues with a substitution within the A-chain loop (A8–A10) elicited a substantially increased response similar to that of beef insulin. Analogues with substitutions at the COOH-terminal end of the B chain gave intermediate responses, whereas substitutions in B9 or B10 resulted in the lowest responses comparable to that of pork or even human insulin (B.A. Parkar, W.G. Reeves, unpublished observations).

The presence of insulin in monomer form instead of aggregated form is likely to result in less antibody production, and the shortened stay in subcutis will probably have the same effect (240,281,282). However, only long-term clinical trials will be able to confirm this expectation and establish whether monomeric insulin analogues are sufficiently low in their immunogenic potential for general clinical use.

Antigenicity (binding to antibodies). Insulin resistance due to the presence of high titers of insulin antibodies is a rare complication of insulin treatment. The management of immunological resistance involves changing therapy to insulins with reduced affinity to the circulating antibodies (239,283). Sulfated insulin combines less avidly with antibodies induced by beef and pork insulin and has been used successfully for treatment for resistant patients (170,284–287; Table 3). Several of the ana-

logues described herein share with sulfated insulin the presence of negatively charged residues in positions B9 and B27 and have the potential for reduced binding to antibodies against native insulins (Table 4). Indeed, the Asp^{B9},Glu^{B27} analogue has been shown to have reduced affinity to such preformed antibodies (I. Jensen, unpublished observations); therefore, this or a similar analogue might be a possible future substitute for inhomogeneous sulfated insulin in the treatment of immunological insulin resistance.

PROOF OF CONCEPT AND ELUCIDATION OF ABSORPTION MECHANISMS

Provided active transport of insulin in subcutaneous tissue can be excluded, the absorption of soluble insulin is determined solely by factors influencing diffusive transport. These include the insulin-concentration gradient, total area and permeability of the absorbing capillary membrane, diffusion capacity of the injected insulin, and the distance for diffusion. Of these factors, diffusion capacity is governed by the form and volume of the diffusing substance (diffusion coefficient with free diffusion) and by the steric hindrance of free diffusion. Therefore, the association state of insulin together with the tendency of the oligomeric units to dissociate when diluted during transport are expected to be of paramount importance for the diffusion capacity (see CREATION OF INSULINS WITH REDUCED SELF-ASSOCIATION).

To assess the importance of diffusion capacity relative to the other factors mentioned and to evaluate the influence of insulin association state on subcutaneous absorption of soluble insulin, a series of in vitro and in vivo experiments have been undertaken (189,288,289).

Chromatographic investigations. The different native insulins used in therapy are all hexameric when examined by osmometry but may differ in their ability to remain in the hexameric state when the concentration of insulin is lowered. The dilution of the injected depot occurring in the subcutis during absorption can be imitated in SEC experiments in which differences with respect to insulin-dissociation behavior are revealed as differences in time of elution and elution profile of the insulin. Examples of such experiments are shown in Fig. 6 (183,189). Whereas an essentially monomeric analogue elutes as a symmetrical peak late in the chromatogram, human insulin elutes earlier as associated molecules dependent on the Zn^{2+} content. A difference can be observed between the elution profiles of 2 Zn^{2+} pork and human insulin (Fig. 6, *A* and *B*). Human insulin dissociates more than pork insulin during the passage of the column, and this difference is even more pronounced when the insulins (1 ml) are applied at a lower concentration (U-50 ~0.3 mM).

Monomeric sulfated insulin elutes relatively early at the approximate site of a dimer as a symmetrical peak, indicating that no further dissociation is taking place (Fig. 6*D*). A possible explanation for this occurrence is that the bulky sulfate groups increase the volume of the molecule either directly or by causing an unfolding of the monomer as also indicated by the low in vivo potency (Table 3).

Animal studies. To assess whether the association state (or the tendency to dissociation of the associated insulin molecules) represents a rate-limiting step in the entire process of absorption of insulin from the subcutaneous injection site, a series of analogues with varying association properties and different animal insulins (with and without metal-induced stabilization of the hexameric structure) was investigated in pig absorption experiments (3,189). The mean association state of the insulin analogues was deduced from osmometry, and the stability of the different native hexameric insulins in relation to dilution was ranked according to their behavior in SEC experiments (see CREATION OF INSULINS WITH REDUCED SELF-ASSOCIATION).

The disappearance of the different insulins (containing A14-mono-^{125}I-insulin tracer) after subcutaneous injection was determined by local external γ-counting (101,148). The experiments were conducted with two standards, the monomeric Asp^{B9},Glu^{B27} analogue and normal hexameric 2 Zn^{2+} human insulin, in a crossover design with at least 6 pigs/study. The T_{50} values of the disappearance of radioactivity from the site of injection were calculated relative to 2 Zn^{2+} human insulin with data from both standards to adjust for systematic variations between experiments.

The results of these studies are summarized in Fig. 7. The relative T_{50} values of the analogues and human insulin with and without Zn^{2+} are strongly correlated to the degree of insulin self-association ($r = 0.96$, $P < 0.001$), i.e., an inverse relationship between subcutaneous absorption rate and the average size of the insulin units. In the upper-right section in Fig. 7, a similar close rank correlation between T_{50} and the dissociation tendency of native Zn^{2+} and Co^{2+} insulins during SEC experiments is seen. It can also be observed that the non-dissociating cobalt (CoIII) insulin hexamer (290) can actually be absorbed from the subcutis but at a rate three to four times slower than that of monomeric insulins.

Similar investigations on the monomeric insulin derivatives despentapeptide B26–B30 insulin and sulfated insulin have revealed relative T_{50} values of 81 and 82%, respectively, which do not fit into the relationship shown in Fig. 7 (288; Table 3). These deviations are probably explained by sulfated insulin being more voluminous than a normally folded monomer and by despentapeptide forming precipitates at the injection site (see above and INSULIN STRUCTURE). The latter explanation is supported by the fact that despentapeptide in the initial phase of subcutaneous absorption disappears relatively fast from the injection site, whereas the rate slows down thereafter (U. Ribel, unpublished observations).

The pig studies have confirmed the slightly faster absorption of human relative to pork soluble insulin repeatedly seen in clinical studies (91–96,99; Fig. 7).

Discussion and conclusions. Mathematical modeling

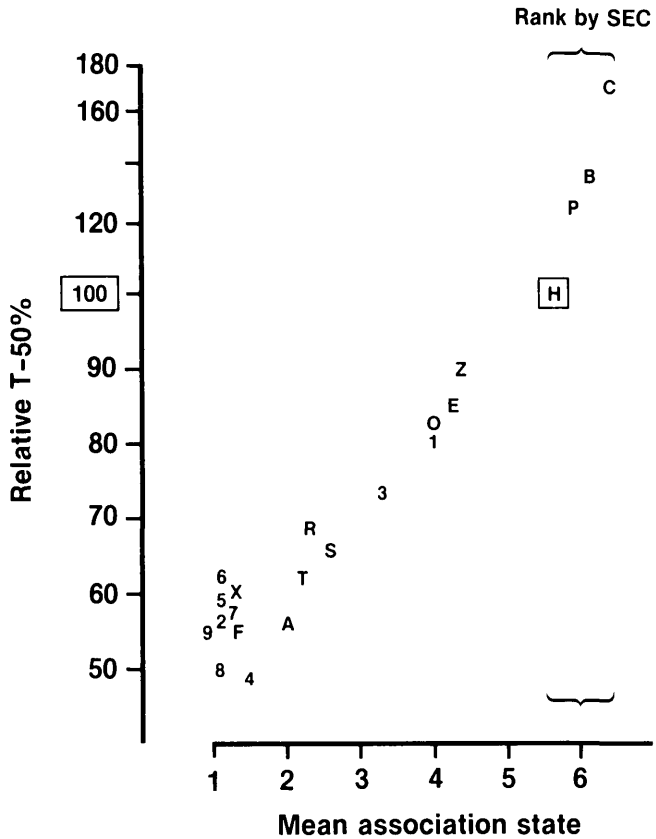

FIG. 7. Correlation between T_{50} disappearance from injection site in pig studies (for further details see text) and mean association state at 1 mM of various insulins. Association state was deduced from osmometry (see CREATION OF INSULINS WITH REDUCED SELF-ASSOCIATION), and in case of hexameric insulins, tendency to dissociation on dilution was assessed by size-exclusion chromatography (see PROOF OF CONCEPT AND ELUCIDATION OF ABSORPTION MECHANISMS). Each figure and letter in diagram represents mean results for 1 insulin or insulin analogue. B, beef; P, pork 2 Zn^{2+} insulin; C, cobalt (CoIII) human insulin; for other codes see Table 4. Note that $T_{50\%}$ >100 is log scale.

techniques that use various approaches to quantitatively study subcutaneous insulin absorption have been applied by several investigators (80,138,144,147,148, 291–297).

The use of theoretical models for studying the absorption of insulin is dependent on many assumptions and approximations. Based on the hypothesis that injected soluble insulin is present in the subcutaneous tissue primarily as two oligomeric forms (hexamer and dimer) and that only dimers can penetrate the capillary membrane, Mosekilde et al. (144) constructed a model that was able to explain the existence of an initial slow absorption phase (lag phase) and the variation of the absorption rate with insulin concentration and injected volume. Their model describes how diffusion and absorption gradually reduce insulin concentration in the subcutaneous depot, whereby the equilibrium between hexamers and dimers in accordance with the law of mass action is shifted toward the dimer. These assumptions are in keeping with an earlier theory by Binder (147), who assumed that insulin is absorbed in no more than a dimeric state, and supported by later studies by Ribel et al. (148). In contrast, Hildebrandt et al. (135), based on calculations of capillary diffusion capacity of the injected insulin, suggested that insulin is transported to the bloodstream in a polymeric form.

The above studies demonstrate a major influence of the insulin-association state on the rate of absorption of subcutaneously injected soluble insulin. This factor seems to be the main determinant in the influence of insulin species, insulin concentration, and injected volume, the latter also relating to the effect of using jet injection or sprinkler needle (relatively faster dilution, the smaller or more dispersed the volume injected). Also, the effect of increased temperature and massage (faster dispersion and dilution of the insulin depot) can be explained by increased or faster dissociation of hexameric insulin.

The other important factor, blood flow (recruitment of available capillaries), functions by influencing the diffusion distance and the insulin concentration gradient. Because the rate of transport by linear diffusion is proportional to the concentration gradient, an increase in blood flow will also contribute to the velocity by which associated insulin is diluted and dissociated after injection. This interaction between dominating factors that influence the rate of subcutaneous absorption of insulin emphasizes the complexity of the entire absorption process.

The initial delay (3–4 h) in reaching maximal absorption rate (lag phase), i.e., the time until the absorption follows first-order kinetics, can now be explained by the time necessary for sufficient dilution and the resulting dissociation of hexameric insulin into smaller units (101). Because of the low concentration required for further dissociation into monomers, the fraction of soluble native insulin absorbed in monomeric form is small (Fig. 3). The monomeric insulin analogues follow a monoexponential disappearance course from the time of injection (see CLINICAL STUDIES).

The observation that hexamers can actually be absorbed from the subcutis indicates that soluble insulin is also partly absorbed while in its hexameric state (289; Fig. 7). Because the rate of free diffusion is inversely related to the radius of the diffusing unit, the hexamer with approximately twice the diameter of the monomer would, provided unrestricted diffusion, be expected to be absorbed at half the rate of that of the monomer. However, the difference in the rate of absorption is three- to fourfold (Fig. 7). Consequently, the hexamer must, in addition to its slower free diffusion, be sterically more hindered than the insulin monomer during the diffusional transport in the subcutis and/or during its passage through the capillary membrane.

The slightly faster subcutaneous absorption of human soluble insulin relative to the animal insulins can now be explained by a less stable hexameric structure of human insulin as reflected by the greater tendency to dis-

sociation with decreasing concentration of insulin. This property is basically due to the more hydrophilic amino acid in position B30 (Thr instead of Ala), resulting in a changed solvent structure in the B28–B30 region and alterations in the intermolecular contacts (298). These changes apparently have a weakening effect on hexamer stability. Therefore, the faster absorption of human insulin is probably not caused by the increased hydrophilicity but rather is due to the effect the amino acid change in position B30 has on the strength by which the dimers are held together within the hexamer.

From these animal and chromatographic studies, it can be concluded:

1. The size of the insulin unit (association state) and the ease by which assembled molecules dissociate play an important role in determining the rate of absorption after subcutaneous injection of soluble insulin.
2. The rate of absorption is inversely correlated to the degree of insulin self-association and the ease by which associated insulin dissociates.
3. Two semisynthetic monomeric insulin derivatives (sulfated insulin and despentapeptide B26–B30 insulin) deviate from this relationship, possibly because of unfolding to a more voluminous molecule and precipitation at the injection site, respectively.
4. Monomeric insulins are absorbed from the subcutis three to four times faster than a nondissociating hexamer, indicating that steric restriction of transport in the tissue and/or through the capillary membrane is also a limiting factor in the absorption process.
5. The slightly faster absorption of human insulin compared with pork soluble insulin after subcutaneous injection is due to a small difference in the tendency to dissociation of their respective hexamers.
6. The lag phase in absorption of soluble insulin; the effect of insulin concentration, injected volume, and temperature; and the influence of massage on the absorption process are now easily understood in the light of these results.
7. Monomeric insulins are promising candidates for quicker delivery of insulin by the subcutaneous route and, consequently, for minimizing postprandial glucose increase and reducing between-meal hypoglycemia in IDDM subjects.

CLINICAL STUDIES

Clinical evaluation of the human insulin analogues intended for meal-related insulin requirements began in 1987 after the demonstration in pigs that the subcutaneously administered disubstituted monomeric analogue Asp^{B9},Glu^{B27} was absorbed faster with an earlier effect on plasma glucose than the reference soluble human insulin (3). A series of single-dose studies was conducted in healthy subjects with subcutaneous administration of ^{125}I-labeled human insulin (Actrapid) and three candidate insulin analogues Asp^{B9},Glu^{B27}; Asp^{B10}; and Asp^{B28}, followed by studies in insulin-treated diabetic subjects to evaluate the influence of the insulins on postprandial glycemic excursions after ingestion of a standardized test meal (299–303; S.K., F.M. Creagh, J.R. Peters, J.B., A.V., D.R.O., unpublished observations). All studies were approved by the local ethical committee and performed in accordance with the Declaration of Helsinki.

Additional clinical studies have been undertaken elsewhere involving both healthy subjects and IDDM patients (304,305; S. Jørgensen, G. Petranyi, unpublished observations).

Studies in healthy subjects. The data presented from our own studies are from seven healthy male volunteers not receiving concomitant medication who received, in the course of a series of studies over 6 mo, the following four test preparations in the same formulation: soluble human insulin and human insulin analogues Asp^{B9}, Glu^{B27}; Asp^{B10}; and Asp^{B28} (S.K., J.B., A. Burch, A.V., D.R.O., unpublished observations).

Each study was performed with subjects fasted overnight, resting in a supine position during the study in a constant-temperature environment (22°C); smoking was not permitted. The bolus subcutaneous injections were sited in the anterior abdominal wall midway between the umbilicus and the anterosuperior iliac spine. The residual radioactivity at the injection sites was determined by external counting, with the detector consisting of a thallium-activated sodium iodide crystal and photomultiplier tube connected to a single-channel analyzer. The scintillation detector was attached to a cylindrical lead collimator and fixed 50 mm from the skin surface. Residual radioactivity was measured continuously for the first 2 h after injection of the ^{125}I-insulin preparations and thereafter during 5-min intervals corresponding to blood sampling times. Counts were corrected for background and the results expressed as percentage of initial values. Frequent mixed venous blood samples were obtained throughout the 6-h study for the determination of plasma glucose, insulin, and insulin-analogue concentrations. Immunoreactive insulin–analogue analyses were performed with selected antibodies and the respective insulin analogues for the standard curves.

All preparations were well tolerated by all subjects, with no clinical evidence of local or systemic adverse events. Figure 8 illustrates the residual radioactivity at the injection site and the plasma insulin, insulin analogue, and glucose levels after subcutaneous administration of the four test preparations at a dose of 0.6 nmol/kg (0.1 U/kg).

The disappearance of the three insulin analogues from the subcutis, as depicted by the residual radioactivity at the site of injection, was faster than that of the reference soluble human insulin. The calculated times to T_{50} residual radioactivity for human insulin and insulin ana-

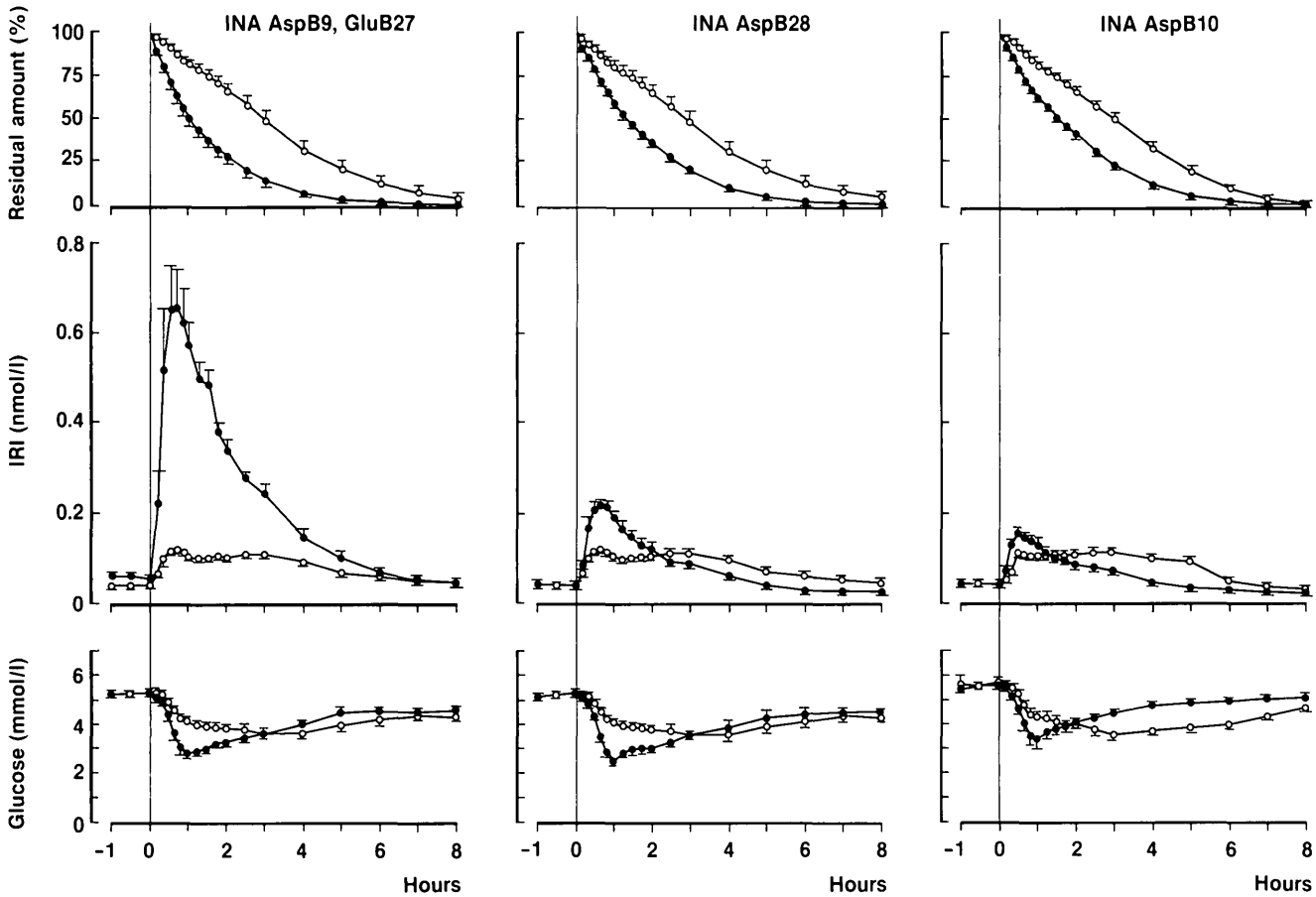

FIG. 8. Mean ± SE residual radioactivity at injection site and plasma immunoreactive insulin (IRI), insulin analogue (INA), and glucose concentrations after subcutaneous injection of 0.6 nmol/kg of soluble human insulin (○) or insulin analogue (●) into healthy subjects (n = 7). For further details see text.

logues Asp^{B9},Glu^{B27}; Asp^{B10}; and Asp^{B28} were ~180, 60, 90, and 80 min, respectively. Consequently, a more rapid rise in plasma concentrations of the three analogues is observed compared with the reference human insulin. The differences between the plasma insulin profiles of the analogues also reflect the influence of their respective metabolic clearance rates (see BIOLOGICAL AND PHARMACOLOGICAL PROPERTIES). Each of the three insulin analogues resulted in an earlier and more pronounced hypoglycemic response, reaching a nadir at 1 h after administration, followed by a quicker return toward preinjection levels. This contrasts with the much slower fall in plasma glucose after soluble human insulin, with a less well defined nadir between 3 and 4 h and a slower return toward fasting levels.

The percentage of the test preparations of insulin absorbed and the corresponding hypoglycemic responses for the first 3 h and last 5 h of the study are shown in Fig. 9. Approximately 80% of the analogues are absorbed during the first period, in contrast to only 50% of human insulin. From 3 to 8 h, the percentage of human insulin absorbed is two to three times higher than that of the analogues. The cumulative hypoglycemic effects (*lower panel*) are also higher for the analogues during the first 3 h, and a more prolonged effect (3–8 h) can be observed with human insulin.

From the above disappearance studies, a strong relationship between the association state of insulin and the rate of disappearance from the subcutaneous injection site is observed, confirming in humans the results found in pigs (189,288,289,300; S.K., J.B., A. Burch, K.H. Jorgensen, A.V., D.R.O., unpublished observations; Fig. 7).

The observations of Heineman et al. (306), who used the euglycemic clamp technique to compare subcutaneously administered human insulin analogues Asp^{B9}, Glu^{B27} and Asp^{B10} in healthy volunteers given 72 nmol (~12 U) of each indicate that the glucose infusion rates were higher with the analogues from as early as 20–30 min postinjection. An earlier and faster decrease in the glucose infusion rate was also observed with the analogues between 5 and 6 h after administration. These findings confirm the earlier onset and shorter duration of action of the insulin analogues after subcutaneous injection in healthy subjects.

Intravenous infusion over 90 min at two dose levels (1 and 2 pmol · kg^{-1} · min^{-1}) of human insulin and the analogues Asp^{B9},Glu^{B27} and Asp^{B10} in 10 healthy subjects

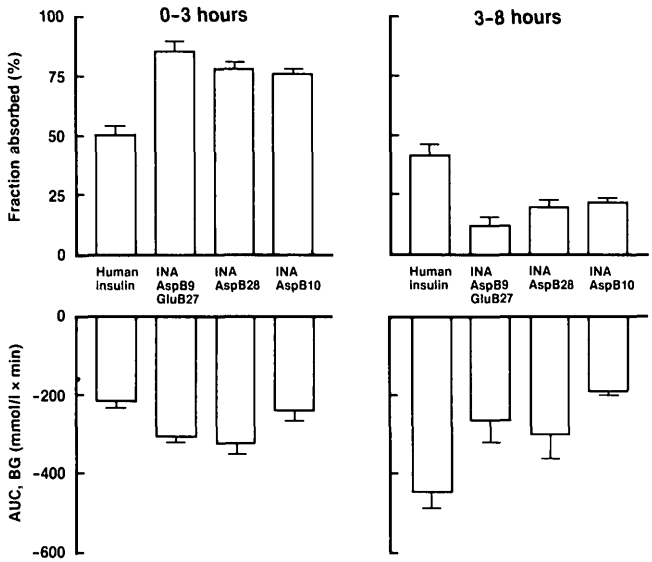

FIG. 9. Comparison of absorbed percentages (means ± SE) of human insulin and insulin analogues (INAs) Asp^{B9},Glu^{B27}; Asp^{B28}; and Asp^{B10} during first 3 h and last 5 h of study in healthy subjects (*upper panels*; see Fig. 8). *Lower panels*, areas under the curve (AUC; in negative values to indicate hypoglycemic effect) between blood glucose (BG) curves after injection and initial glucose levels (means ± SE).

gave a similar fall in blood glucose levels (304,305). The steady-state plasma levels at the 2-pmol · kg^{-1} · min^{-1} dose level for human insulin and insulin analogues Asp^{B9},Glu^{B27} and Asp^{B10} were ~110, 160, and 80 pM, respectively, reflecting their different metabolic clearance rates (corrected for C-peptide) of 20, 13, and 26 ml · kg^{-1} · min^{-1}, respectively.

It has been argued that substantial degradation of insulin takes place at the subcutaneous injection site (57). If local enzymatic degradation was a major problem, it would be expected that the monomeric insulin would be even more susceptible to such degradation than the hexamers in current soluble insulin. A comparison of the measured appearance in the blood (corrected for endogenous insulin secretion) and appearance calculated from the disappearance of radioactive-labeled insulin analogue from the injection site (based on the data for insulin analogue Asp^{B9},Glu^{B27} from Fig. 8) is shown in Fig. 10. Assuming that elimination from plasma is first order, the predicted appearance curve agrees with the actual measured values, and a corresponding agreement has also been found for human insulin (P. Hougaard, unpublished observations). Therefore, the use of ^{125}I-insulin represents a reliable comparative method to study absorption kinetics of the rapid-acting analogues.

The initial lag phase in the absorption of soluble human insulin is evident also in these studies when the disappearance data are depicted on a log scale (Fig. 11; see CLINICAL OBSERVATIONS and PROOF OF CONCEPT AND ELUCIDATION OF ABSORPTION MECHANISMS). In contrast, the monomeric insulin analogue Asp^{B9},Glu^{B27} does not show such a delay in reaching maximal rate of disappearance. It follows a monoexponential course during the entire absorption process, and the slope of the disappearance curve, i.e., rate of disappearance, is much steeper than that of the initial part of the disappearance curve for human insulin. The essentially dimeric insulin analogue Asp^{B10} initially (0–2 h) disappears at a rate similar to that of human insulin in its intermediate phase of absorption (2–4 h) (Fig. 11; Table 6). Later (3–8 h), insulin analogue Asp^{B10} disappears at a rate close to that of the monomeric analogue (insulin analogue Asp^{B9},Glu^{B27}). The last 20% of human insulin (5–8 h) also disappears with a rate near to that of monomeric insulin. Assuming that the initial disappearance rate of human insulin represents the rate of absorption of hexameric insulin, it can be deduced that a monomer of insulin is absorbed 3.3 times faster than hexameric insulin (Table 6). This value is in agreement with the ratio of ~3.2 between the T_{50} values for the nondissociating Co^{2+} hexamer and the monomeric insulins in pig studies (Fig. 7).

It is now evident that the long T_{50} for the absorption process, based on disappearance studies, and the deviation from a monoexponential disappearance curve are not, as alleged by Berger et al. (57), due to the indirect method of measuring insulin absorption from the subcutaneous tissue. These phenomena can be explained by the associated nature of the native insulin molecule in pharmaceutical formulations and the dissociation into smaller units occurring during the absorption process.

For soluble human insulin, the duration of the lag phase (i.e., time to dissociation of hexameric insulin) varies with volume and concentration and would also be expected to vary with the region and depth of injection and with the blood flow and all its related factors (Table 2; CLINICAL OBSERVATIONS and PROOF OF CONCEPT

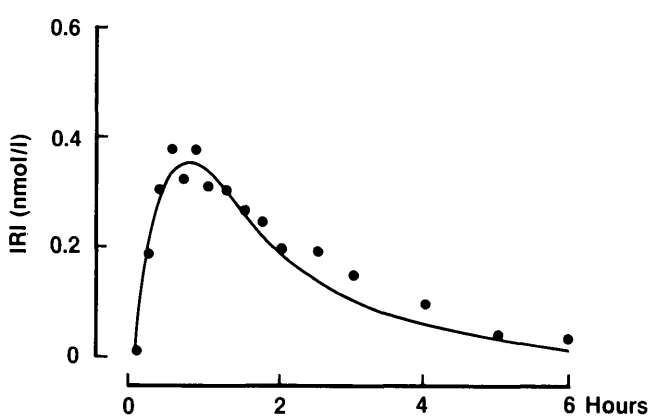

FIG. 10. Comparison of measured appearance (●) of immunoreactive insulin (IRI) in blood (corrected for endogenous insulin secretion based on C-peptide analyses) and appearance calculated from measurement of residual radioactivity at injection site (*solid line*) after subcutaneous injection of monomeric insulin analogue Asp^{B9},Glu^{B27} 0.3 nmol/kg into healthy subjects (n = 7).

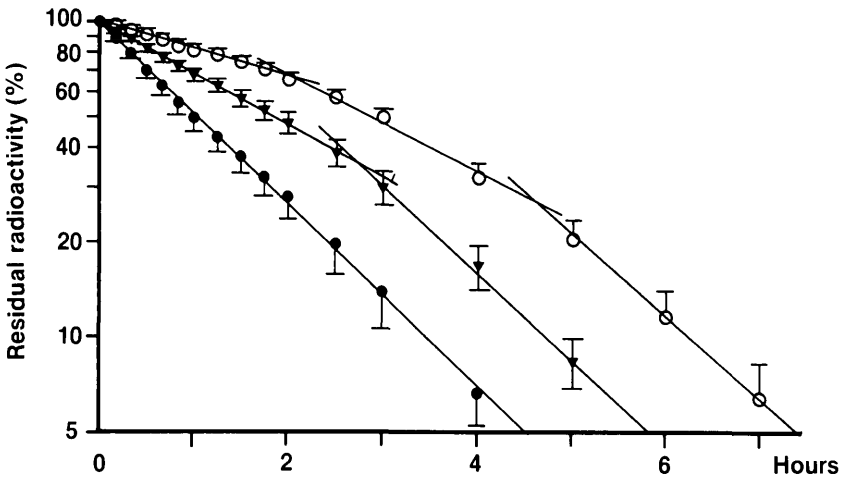

FIG. 11. Replot of disappearance curves of human insulin (○), insulin analogues Asp^{B9},Glu^{B27} (●), and Asp^{B10} (▼) in logarithmic scale (means ± SE; see Fig. 8). Straight line segments were calculated by linear regression analysis with data from relevant time intervals (see Table 6 for listing of relative rates of absorption corresponding to slopes of lines).

AND ELUCIDATION OF ABSORPTION MECHANISMS). These relationships, contributing to the day-to-day variation in insulin absorption, are likely to be of less importance when the lag phase is reduced or eliminated. In agreement with this theory, the study by Vora et al. (299) indicates a lower dose-related variation in the absorption rate of the monomeric (Asp^{B9},Glu^{B27}) analogue compared with soluble human insulin.

Studies in diabetic subjects. The availability of insulin preparations with faster absorption rates from the subcutis and a shorter duration of action than currently available insulins offers many potential advantages. The delivery of early prandial insulin and avoidance of prolonged hyperinsulinemia with the monomeric insulin analogues may limit excessive postprandial glycemic excursions and the predisposition to late hypoglycemia incumbent with currently available short-acting insulins. Another benefit would be the possibility of administration just before the meal.

In an attempt to examine the first of these hypotheses, a pilot study was conducted with the disubstituted insulin analogue Asp^{B9},Glu^{B27} in a few insulin-treated diabetic patients (302,303). Neutral soluble human insulin (Actrapid) and the insulin analogue were each given by subcutaneous injection into the anterior abdominal wall in six male insulin-treated patients at a dose level of 60 nmol (10 U) immediately before a standard test breakfast. All patients were previously treated with a basal (Ultratard once daily before bed) bolus (Actrapid 3 times daily before meals) insulin regimen. Basal insulin was discontinued 72 h before the study, with glycemic control maintained by frequent soluble insulin injections as required. An intravenous infusion of insulin was commenced (0.12 mU · k^{-1} · min^{-1}) on the last evening and continued up to 30 min before the test meal. The pre- and postprandial plasma glucose, free-insulin, and insulin-analogue concentrations after bolus subcutaneous administration are shown in Fig. 12. Plasma insulin and analogue profiles agree with those previously seen for the same dose of the two preparations in healthy subjects (299). Incremental postmeal glucose levels were lower with insulin analogue Asp^{B9},Glu^{B27} from 1 h onward, and the cumulative area under the incremental glucose curve for the 4-h study was 45% lower with the analogue ($P < 0.01$).

TABLE 6
Rates of subcutaneous absorption (%/h)

Rate	Human insulin	Insulin analogues	
		Asp^{B10}	Asp^{B9},Glu^{B27}
Initial (0–2 h)	20.1	36.7	66.2 (0–8 h)
Intermediate (2–4 h)	35.9	63.4 (3–8 h)	
Final (5–8 h)	60.5		

Rates were calculated as slope of regression lines for logarithms of residual amount at injection site versus time with all measurements in indicated time interval (Fig. 11).

A second study was performed with the reference human insulin administered 30 min before the test breakfast, and the three insulin analogues Asp^{B9},Glu^{B27}; Asp^{B10}; and Asp^{B28} individually injected immediately before food on separate study days in six IDDM subjects usually treated with a basal bolus regimen (301; unpublished observations). The same protocol as above was used, with the exception that the insulin infusion commenced the previous evening was continued throughout the study. Plasma glucose profiles are shown in Fig. 13. All three insulin analogues injected immediately before the meal achieved glycemic control comparable to that of soluble human insulin administered 30 min earlier. The reduction in the incremental area under the postprandial glucose curve compared with that of human insulin for the 4-h study for insulin analogues Asp^{B9},Glu^{B27}; Asp^{B10}; and Asp^{B28} was ~30, 20, and 45%. The differences in glucose concentrations between analogues and human insulin were most pronounced when considering the 1.5- to 4-h postprandial period (Fig. 14). Although mean increments with the analogues were only approximately half that of human insulin, the difference did not reach statistical significance due to the few patients tested.

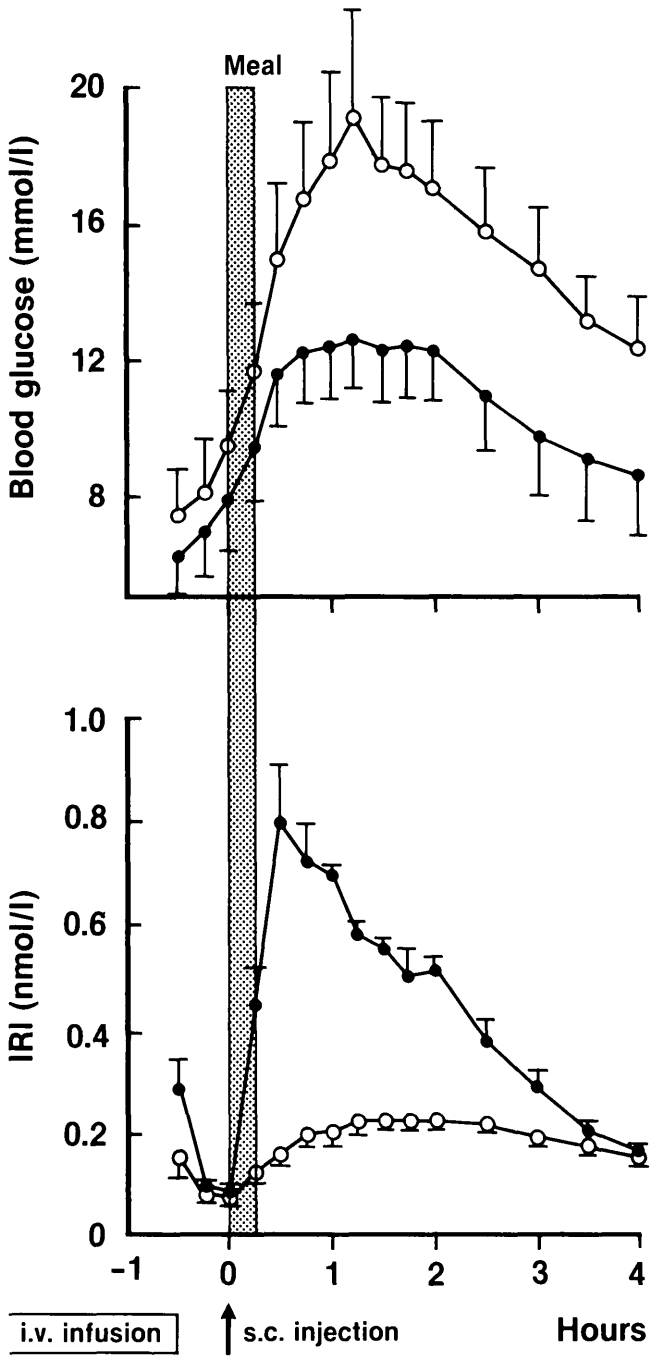

FIG. 12. Mean ± SE plasma glucose and plasma free-insulin (IRI) or insulin analogue levels after test meal (500 kcal) and subcutaneous injection of soluble human insulin (○) or insulin analogue Asp^{B9},Glu^{B27} (●) in equimolar doses (10 U) into insulin-treated patients (n = 6) at 0 min.

Similar postmeal glucose profiles with human insulin given 30 min before mealtimes and the insulin analogue Asp^{B10} injected immediately before the meal were observed by Jørgensen et al. (unpublished observations).

Additional kinetic and metabolic studies in IDDM subjects with the glucose-clamp technique and intravenously administered human insulin analogues Asp^{B9}, Glu^{B27} and Asp^{B10} (G. Petranyi, unpublished observations) confirm the bioequivalence of these preparations as observed earlier in animals (229,230) and humans (304,305).

These preliminary findings demonstrate that, in both healthy subjects and diabetic patients after subcutaneous injection, the three analogues achieve a similar hypoglycemic effect, being faster in onset and shorter lived than soluble human insulin.

Clinical implications. There is compelling evidence that there is a relationship between diabetic complications and the degree of metabolic derangement that exists in diabetes mellitus (36–38,307–309). Several studies have demonstrated that improvement in glycemic control can result in morphological and functional improvements, emphasizing the need to strive for the best possible metabolic control in the insulin-requiring diabetic patient (39–42,78). Due to their inherent pharmacokinetic properties, currently available insulin preparations given subcutaneously can at best only achieve near normoglycemia in a few patients. Currently, meal-related soluble insulin needs to be injected 0.5–1 h premeal, attempting to supply some insulin during the early prandial phase to minimize the postmeal glycemic excursion. Consideration must also be given to regional differences in the rate of insulin absorption, which is known to be slower from the thigh than from the abdomen. Despite using the abdomen, prolonged hyperinsulinemia predisposes the patient to delayed postprandial hypoglycemia.

The preliminary clinical experience summarized above confirms that monomeric insulin analogues administered by subcutaneous injection immediately before the meal are at least as good as soluble human insulin administered 30 min earlier. Therefore, they are promising candidates for limiting both excessive postprandial glucose concentrations and delayed hypoglycemia. A greater benefit in glycemic control may also be expected from injecting the analogues 10–15 min before a meal. Less reliance on patient compliance in relation to injection times must also be an advantage. Hyperinsulinemia can subject patients to both acute (hypoglycemia) and, possibly, long-term atherosclerotic risks (310–317). Thus, when insulin is required, the monomeric analogues are more suitable also for use in NIDDM subjects compared with soluble human insulin. Observations from clinical studies of NIDDM and IDDM subjects could be interpreted to suggest that they might benefit from insulin effects that appeared and disappeared more rapidly in connection with meals (318,319). This could represent important clinical applications for monomeric insulin analogues.

Therefore, the development of these novel insulins represents a major step in the evolution of insulin preparations to subserve meal-related insulin requirements. However, a final candidate for large-scale clinical studies must satisfy, in addition to efficacy, both safety and stability requirements.

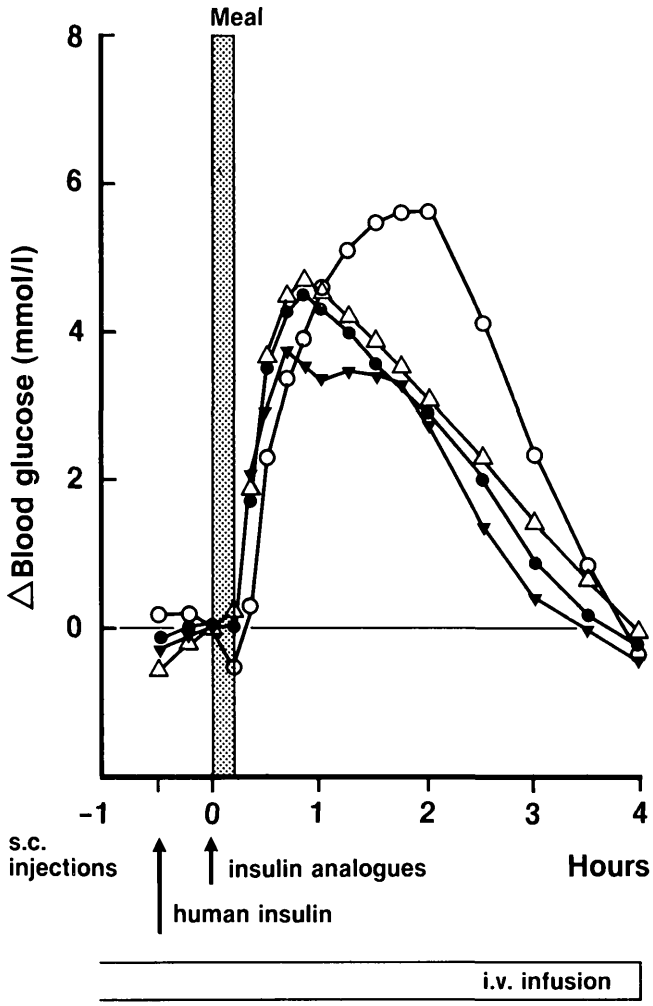

FIG. 13. Mean incremental plasma glucose concentrations during meal tolerance test (500 kcal) in insulin-dependent diabetic patients (n = 6) receiving constant insulin infusion (0.12 mU · min^{-1} · kg^{-1} human soluble insulin, Actrapid) and given insulin analogues Asp^{B9},Glu^{B27} (●); Asp^{B28} (▼; n = 5); and Asp^{B10} (△) immediately before test meal and human soluble insulin (○) 30 min earlier, all by bolus subcutaneous injection into anterior abdominal wall.

SUMMARY AND FURTHER PROSPECTS AND DIRECTIONS

Important advances in the understanding of physiological effects, chemistry, kinetics, and action of insulin have emerged since the hormone was first isolated in the early 1920s. However, major deficiencies of today's therapeutic regimens are still evident. The physiological replacement of insulin remains an elusive goal, although the usefulness and perspectives of the most recent milestone in the development of insulin for clinical use, the genetically engineered insulin analogues, are yet to be established (54).

Glucose intolerance and frank diabetes mellitus are recognized risk factors for cardiovascular disease, especially coronary heart disease, which claims more than half of the deaths in diabetic patients in westernized cultures (320–322). Common among the risk factors for coronary artery disease in individuals with or without glucose intolerance are insulin resistance and hyperinsulinemia and their metabolic and morphological consequences (317,323). In the pursuit of normoglycemia without coexisting hyperinsulinemia, the requirements of insulin therapy are exacting and the goal is rarely, if ever, reached with currently available insulin preparations and/or methods of delivery (72). The avoidance of microvascular complications of diabetes also requires the achievement of good metabolic control (36).

The early results with the new insulin analogues for meal-related insulin requirements in IDDM patients are encouraging, but more extensive studies are needed to better evaluate their full clinical potential. The rapid resorption from the subcutis offers considerable advantages over current soluble insulins, provided chronic treatment does not induce the formation of antibodies capable of obtunding the early availability or serving as an unphysiological reservoir of insulin in the circulation. One benefit of such insulin analogues for the diabetic patient is the possibility of administration much nearer to mealtimes, contrasting with the current recommendation of injecting soluble insulin 0.5–1 h preprandially. The potential for minimizing the risk of delayed hypoglycemia with such insulin analogues will also require careful evaluation. Moreover, the full impact of these novel short-acting insulins for IDDM subjects, who

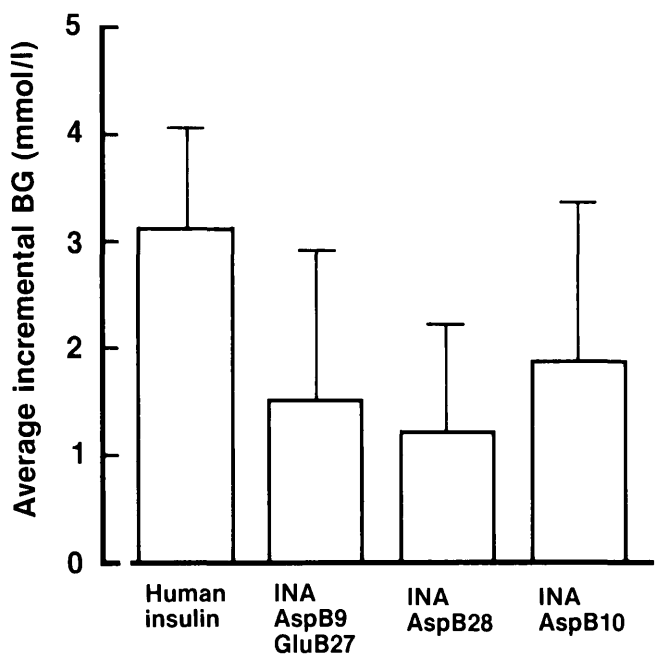

FIG. 14. Average incremental blood glucose (BG) from 1.5 to 4 h after meal tests in diabetic patients given subcutaneous injections of human insulin (12 U) 30 min before meal or equimolar doses of 3 different insulin analogues (INAs) immediately before meal (means ± SE; see Fig. 13).

also need basal insulin supplementation with currently available intermediate- or long-acting insulin preparations, will require long-term clinical evaluation. The future availability of new insulins for the delivery of a more physiological basal insulin supply would be complementary to the aim of achieving normoglycemia without hyperinsulinemia (181,182).

In the case of NIDDM, the new insulin analogues may be used to supply prandial insulin requirements, especially in the nonobese insulinopenic patient. The opportunity to overcome the deficit in the early insulin-secretory response to food in NIDDM subjects with the quickly absorbed insulin analogues is promising and requires careful evaluation.

Current absorption studies in animals and the clinical investigations in humans have contributed significantly to the understanding of subcutaneous insulin absorption. Several of the hitherto puzzling phenomena in relation to the absorption process can now be explained by the associated nature of native insulin in pharmaceutical formulation and the tendency of such assembled insulin molecules to dissociate on dilution during the absorption process. Availability of the monomeric insulins for clinical use will make it possible to clarify and quantify more precisely the role of the many factors influencing subcutaneous absorption of insulin and thereby contribute further to the elucidation of the exact mechanisms of subcutaneous absorption of insulin.

In addition to the prospects of optimizing parenteral insulin therapy, the new insulins with reduced tendency to association might also have important potential for improving delivery of insulin by alternative routes of administration, e.g., nasal and transdermal delivery. However, preliminary investigations have shown that the absorption of monomeric insulins through the nasal mucosa is absent or insufficient when administered without the use of absorption-promoting adjuvant to rats (J.L. Bolaffi, G. Grodsky, unpublished observations) or rabbits (A.R. Sørensen, unpublished observations). In contrast, transdermal transport by iontophoresis through mouse skin seems to be enhanced with monomeric insulins compared with human insulin (P.G. Green, R. Guy, unpublished observations).

Experimentally, the monomeric insulins, in addition to what has already been mentioned, are likely to have a great impact on elucidating the structure of insulin in solution by NMR studies. Future studies aimed at increasing understanding of the structure-function relationship of the hormone, including possible differential effects and postreceptor degradation, are also likely to benefit from the availability of many different insulin analogues.

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Insulin glulisine: a faster onset of action compared with insulin lispro

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Aim: This randomized, single-centre, double-blind, crossover study compared the pharmacodynamic and pharmacokinetic properties of two different doses of insulin glulisine (glulisine) and insulin lispro (lispro) in lean to obese subjects.

Methods: Eighty subjects without diabetes, stratified into four body mass index (BMI) classes (<25, ≥ 25 to <30, ≥ 30 to <35 and ≥ 35 kg/m^2), were randomized to receive single injections of glulisine and lispro (0.2 and 0.4 U/kg) on four study days under glucose clamp conditions. Glucose infusion rates (GIR) and insulin (INS) concentrations were assessed for 10 h postdose.

Results: Glulisine showed a greater early metabolic action than lispro [GIR-area under the curve (GIR-AUC) between 0 and 1 h (0.2 U/kg: 102.3 ± 75.1 vs. 83.1 ± 72.8 mg/kg, $p < 0.05$; 0.4 U/kg: 158.0 ± 100.0 vs. 112.3 ± 70.8 mg/kg, $p < 0.001$)], with an earlier time to 10% of total GIR-AUC (0.2 U/kg: 1.4 ± 0.4 vs. 1.5 ± 0.4 h; 0.4 U/kg: 1.4 ± 0.3 vs. 1.5 ± 0.3 h, $p < 0.05$). The total metabolic effect was not different between the two insulins. In accordance with these findings, the time to 10% of total INS-AUC was faster with glulisine compared with lispro at either dose (0.2 U/kg: 0.7 ± 0.2 vs. 0.8 ± 0.2 h; 0.4 U/kg: 0.8 ± 0.2 vs. 0.9 ± 0.2 h, $p < 0.001$). The faster rise in insulin concentrations and the earlier onset of activity of glulisine vs. lispro was consistently observed in each individual BMI class.

Conclusions: Glulisine shows a faster onset of action than lispro, independent of BMI and dose.

Keywords: glucose clamp, insulin analogues, pharmacodynamics, pharmacokinetics
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Introduction

The therapeutic concept of 'intensified insulin therapy' aims at substituting the complex pattern of endogenous insulin secretion in people with diabetes. The aim of subcutaneous (s.c.) injections of short-acting insulin before meals is to mirror prandial insulin secretion, while the aim of retarded insulin preparations is to substitute basal insulin secretion [1,2]. Unfortunately, the time–action profile of s.c. injected regular human insulin (RHI) shows a slow onset of action (with a peak metabolic effect approximately 3 h postdosing [3]) and a prolonged duration of action beyond 8 h [4], which impedes the attainment of good postprandial blood glucose (BG) control without suffering from late postprandial hypoglycaemia [5]. Consequently, insulin products comprising of human insulin analogues with a faster onset of action and a shorter duration of action than RHI were developed and are now widely used. These insulins, used in intensified basal-bolus insulin

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regimens, enable achievement of tighter postprandial BG control, potentially resulting in improved metabolic control [6].

Insulin glulisine (glulisine) is a new, fast-acting recombinant human insulin analogue. It differs from RHI by the replacement of asparagine at position B3 by lysine, and lysine at position B29 by glutamic acid (Lys[B3], Glu[B29] human insulin). Glulisine, like other rapid-acting insulin analogues, displays a more rapid onset of action and a shorter duration of action vs. RHI [7], leading to improved postprandial BG concentrations [8] and better overall diabetes control [9].

Time–action profiles of currently available s.c. insulin products are prolonged with higher doses, and attenuated and delayed in obese subjects [10,11], which is unwanted. This phenomenon is most pronounced with RHI, which has a substantially longer duration of action with higher doses [4] and is particularly evident in subjects with a high body weight. These subjects not only have to inject higher insulin doses to obtain the same amount of insulin units per kilogram body weight, but also have to compensate for the insulin resistance associated with obesity. Fast-acting insulin analogues such as insulin aspart (aspart) and insulin lispro (lispro) also last longer when injected at higher doses [4,12], although for substantially less time compared with RHI. In a recent manual euglycaemic clamp study, glulisine was shown to have shorter times to onset of activity compared with lispro in non-diabetic, obese [body mass index (BMI) 30–40 kg/m^2] subjects [13]. Indeed, in that study, lispro displayed a delayed action profile compared with glulisine, as indicated by smaller fractional areas under the glucose infusion rate curve (GIR-AUCs) and longer time to 20% of total glucose disposal (GIR-$t_{20\%}$) ($p = 0.025$ at 2 h). In view of the potential clinical importance of this finding, this single-centre, randomized, double-blind, four-way, crossover study was carried out to characterize the observed differences in the pharmacokinetic (PK) and pharmacodynamic (PD) properties of glulisine and lispro in a population with a wider range of BMIs. This Biostator-supported euglycaemic clamp study focussed on early exposure and action with a standard dose of 0.2 U/kg and with 0.4 U/kg as a high dose.

Methods

The study was conducted from 13 April 2004 to 21 October 2004 in accordance with the ethical principles of the Declaration of Helsinki and of Good Clinical Practice. The clinical study protocol, informed consent documents and other appropriate study-related documents were reviewed and approved by an independent ethics committee, and all subjects provided written informed consent.

The study was performed in a single centre, in male and female subjects without diabetes, aged 18–65 years, with haemoglobin A_{1c} levels in the normal range. Subjects were stratified by BMI as follows: <25 kg/m^2 (lean), ≥ 25 to <30 kg/m^2 (overweight), ≥ 30 to <35 kg/m^2 (moderately obese) and ≥ 35 kg/m^2 (severely obese). Subjects were not receiving any regular concomitant treatment with prescribed drugs on entry of the study and in the 4 weeks before screening, with the exception of oral contraceptive agents in female subjects. Subjects received either 0.2 or 0.4 U/kg of glulisine or lispro, in a randomized, double-blind order, on four separate treatment days under euglycaemic clamp conditions. The commercial products of glulisine and lispro were supplied by Aventis Pharma Deutschland GmbH (Bad Soden, Germany). A randomization schedule (generated under the directive of the Department of Biometrics and Data Management, Aventis Pharma Deutschland GmbH) linked sequential subject numbers to treatment sequence codes allocated at random.

Subjects fasted overnight prior to the day of receiving study treatment. In the morning of each of the trial days, subjects were admitted to the research institute and connected to a Biostator [glucose-controlled insulin infusion system; MTB Medizintechnik, Ulm, Germany]. After a baseline period of 90 min, the study medication was administered by s.c. injection into the periumbilical region of the abdomen using a standardized skinfold technique and a 1 ml syringe with a needle length of 12 mm. Injection sides were changed between 5 cm left and 5 cm right of the umbilicus from experiment to experiment. The study medication was administered preferably by the same physician (only in exceptional case by a substitute) at all treatment sessions.

The Biostator measured BG continuously and automatically adjusted the infusion rate of a 20% glucose solution every minute to maintain BG levels at 10% below the individual fasting BG concentrations (determined as the mean of the three BG values measured 60, 30 and 5 min before study drug administration). The Biostator also automatically initiated and calculated GIR. The glucose clamp lasted for 10 h postdosing. Venous blood samples for determination of insulin glulisine and insulin lispro concentrations in serum were collected at the following times: $-90, -60, -30, 0, 10, 20, 30, 40, 50, 60, 90, 120, 150$, 180, 210, 240, 270, 300, 360, 420, 480, 540 and 600 min. Additionally, blood samples were taken at intervals of ≤ 30 min for BG measurements with a laboratory device using the glucose-oxidase method (Super GL Ambulance

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glucose analyser; Hitado Diagnostic Systems, Möhnesee, Germany) to readjust the Biostator BG measurements, if necessary. Subjects remained fasted during the entire glucose clamp period.

Venous blood samples for determination of serum C-peptide concentrations were collected at the following times: −90, 0, 60, 120, 180, 240, 300, 360, 420, 480, 540 and 600 min. A conventional radioimmunoassay (RIA) was used to measure serum C-peptide concentrations (Immulite C-Peptide; EURO/DPC, Llanberis, UK).

RIAs specific for glulisine and lispro (competitive-binding RIA; supplied by Linco Research, St Charles, MO, USA) were used to determine the concentrations in serum. Duplicate measurements were performed using a Cobra™ II series 5010 multidetector auto-gamma counting device (Packard, Meriden, CT, USA). Interbatch accuracy ranged from 94 to 112% for glulisine and from 93 to 108% for lispro. The interbatch precisions were 3.1–8.8 CV% (glulisine) and 2.4–7.2 CV% (lispro). For glulisine, the lower limit of quantification (LLOQ) was set at 5.0 μU/ml, the upper limit of quantification (ULOQ) at 150 μU/ml. The respective values for lispro were 10.0 μU/ml (LLOQ) and 175 μU/ml (ULOQ).

Statistical Methods

PD parameters were derived from the individual GIR profiles, and PK parameters from the serum lispro and glulisine concentrations (INS). AUCs were calculated from untransformed data with the trapezoidal rule. Maximum insulin concentration (INS_{max}) and the time to INS_{max} ($INS\text{-}T_{max}$) were taken as observed, while maximum metabolic activity (GIR_{max}) and the time to GIR_{max} ($GIR\text{-}T_{max}$) were taken from GIR profiles smoothed with a weighted regression technique (procedure LOESS in SAS, SAS Institute, Cary, NC, USA). All PD parameters pertaining to GIR-AUCs as well as GIR_{max}, and all PK parameters pertaining to INS-AUCs as well as INS_{max}, were analysed (PK parameters after a natural log-transformation) using an analysis of variance model, which included insulin type, dose regimen, BMI group, period and sequence as main factors, a nested factor for subjects and interaction terms, to allow the estimation of least square (LS) means of interest.

For treatment comparisons, based on the LS means from this model, point estimates and corresponding 95% CI were calculated for either differences between parameters (PD) or ratios of parameters (PK). All time-related parameters [$INS\text{-}T_{max}$, $GIR\text{-}T_{max}$, 10% of total INS ($INS\text{-}t_{10\%}$), $GIR\text{-}t_{10\%}$] were subject to distribution-free (non-parametric) analyses (Wilcoxon signed-rank test). Point estimates (median) with corresponding 95% CI were calculated for the differences between treatment parameters.

The sample size in this study was based on the results of a previous trial [13] investigating the PD and PK properties of glulisine in obese, non-diabetic subjects with a BMI >30 kg/m^2 (mean BMI 34.7 kg/m^2). A sample size of 18 subjects per BMI group in that trial was estimated to give the study a power of >80% to detect a clinically significant difference between BMI groups for glulisine in onset of action at a significance level of $\alpha < 0.05$ in a double-sided comparison. Therefore, assuming a drop-out rate of approximately 10% per group, a sample size of 20 subjects per BMI group was chosen for this current study. Dropouts were only to be replaced if there were more than two dropouts in one BMI group. This sample size was larger than usual for PD/PK trials to ensure that even small differences in the PD/PK properties of glulisine between subjects with different BMIs were captured.

Results

Subjects

A total of 114 subjects were screened. Of these, 83 subjects met the inclusion criteria, were randomized, received at least one dose of study medication and were included in the safety analyses. Three subjects discontinued before study completion: one after receiving 0.4 U/kg glulisine because of adverse events possibly related to study medication (eyelid and peripheral oedema), one because of a protocol violation and one because of the person's own decision. According to the protocol, these subjects were replaced by three substitutes who received the same treatment sequence as the replaced subjects. In total, 80 subjects, distributed evenly between the BMI groups (20 subjects per group), were included in the PK and PD analyses. There were no relevant differences between the BMI groups with respect to age and gender distribution (table 1). The overall mean baseline BG value for the entire study population was 84 ± 7 mg/dl; baseline BG values were similar for all administered treatment sequences, with no major differences between the BMI groups.

Pharmacodynamics

Both analogues showed comparable overall glucodynamic efficacy ($GIR\text{-}AUC_{0-10\ h}$) (figure 1) and GIR_{max} at either dose (table 2). While $GIR\text{-}T_{max}$ was comparable between the analogues, the onset of action was significantly faster for glulisine, as indicated by the significantly less time to achieve 10% of $GIR\text{-}AUC_{0-10\ h}$ ($GIR\text{-}t_{10\%}$) with glulisine, thus showing higher efficacy

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Table 1 Baseline demographics

	BMI (kg/m^2)				
Variable	**All**	**<25**	**≥25 to <30**	**≥30 to <35**	**>35**
Male, n (%)	42 (52.5)	8 (40)	12 (60)	12 (60)	10 (50)
Female, n (%)	38 (47.5)	12 (60)	8 (40)	8 (40)	10 (50)
Age (years)	38.8 ± 9.8	37.6 ± 9.8	39.0 ± 9.4	39.7 ± 12.0	39.0 ± 8.4
Height (cm)	173.5 ± 8.9	171.5 ± 10.0	175.2 ± 6.9	175.1 ± 9.0	172.5 ± 9.2
Weight (kg)	91.6 ± 21.3	68.4 ± 11.9	83.2 ± 6.8	98.2 ± 9.8	116.6 ± 15.9
BMI (kg/m^2)	30.3 ± 6.4	23.1 ± 2.1	27.1 ± 1.3	32.0 ± 1.1	39.1 ± 3.5

Data are given as mean ± s.d., except for gender distributions.
BMI, body mass index.

in the first hour postdosing (GIR-$AUC_{0–1\ h}$; table 2). Correspondingly, the significantly greater ratio of GIR-$AUC_{0–1\ h}$/GIR-$AUC_{0–10\ h}$ with glulisine showed a significantly higher proportion of total metabolic activity occurring in the first hour postdosing for glulisine when compared with lispro (figure 2).

The faster onset of action with glulisine was not limited to any specific BMI group or to one dose. As shown in table 2 and figure 2, the PD parameters for onset of action showed significant differences between treatments for both 0.2 and 0.4 U/kg, and in nearly all BMI groups, although not all differences in the individual BMI groups reached statistical significance. However, no statistically significant ($p > 0.1$) interaction between insulin type and BMI group was observed for any PD parameter; thus, the observed differences were consistent across BMI subgroups.

Pharmacokinetics

The PK parameters derived from the lispro and glulisine concentrations for the total study population are listed in table 2. Higher maximum serum analogue concentrations and greater total area under the concentration time curves were measured with glulisine compared with lispro (for INS-$AUC_{0–10\ h}$ by approximately 40%; figure 1). However, because the total metabolic responses were comparable between treatments and the absolute bioavailabilities of glulisine and lispro are similar (approximately 70% [14,15]), the differences in insulin

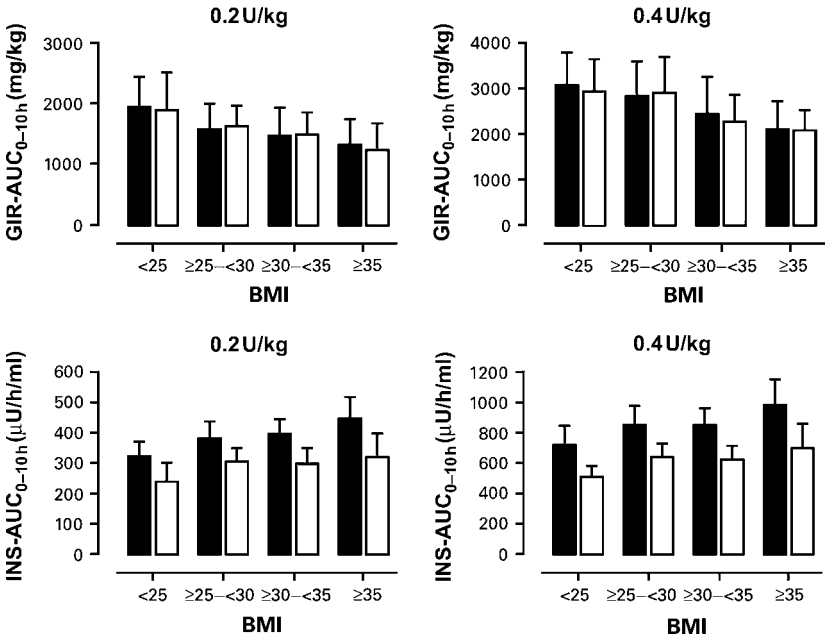

Fig. 1 Mean and s.d. for total glucose disposal (upper panel) and insulin exposure (lower panel) at 0.2 and 0.4 U/kg. Black bar = insulin glulisine; white bar = insulin lispro. BMI, body mass index; AUC, area under the curve; GIR, glucose infusion rate; INS, insulin.

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Table 2 Pharmacodynamic and pharmacokinetic results

Variable	BMI (kg/m^2)	Insulin glulisine (0.2 U/kg)	Insulin lispro (0.2 U/kg)	Insulin glulisine (0.4 U/kg)	Insulin lispro (0.4 U/kg)
Pharmacodynamics					
GIR-AUC$_{0–10\ h}$ (mg/kg)	All	1569 ± 521	1554 ± 512	2564 ± 811	2459 ± 760
GIR-AUC$_{0–1\ h}$ (mg/kg)	All	102 ± 75*	83 ± 73	158 ± 100†	112 ± 71
$\frac{\text{GIR-AUC}_{(0–1\ h)}}{\text{GIR-AUC}_{(0–10\ h)}}$ (%)	All	6.4 ± 3.9†	5.1 ± 3.9	6.1 ± 3.3†	4.5 ± 2.6
	<25	9.8 ± 3.9	9.2 ± 4.6	9.2 ± 3.4†	7.0 ± 2.9
	≥25 to <30	6.8 ± 2.3†	4.8 ± 2.7	5.7 ± 2.0	4.5 ± 1.1
	≥30 to <35	4.9 ± 3.7*	3.6 ± 2.5	5.7 ± 3.2†	3.3 ± 2.1
	≥35	4.0 ± 2.9*	2.7 ± 1.8	3.7 ± 1.7	3.1 ± 1.7
GIR-t$_{10\%}$ (min)	All	83 ± 26*	87 ± 23	85 ± 20*	88 ± 18
GIR$_{max}$ (mg/kg/min)	All	5.8 ± 2.1	5.9 ± 2.6	8.4 ± 2.9	8.3 ± 3.0
GIR-T$_{max}$ (min)	All	190 ± 75	171 ± 53	196 ± 73	198 ± 65
Pharmacokinetics					
INS-AUC$_{0–10\ h}$ (μU/h.ml)	All	385 ± 69†	281 ± 68	842 ± 158†	603 ± 129
INS-AUC$_{0–1\ h}$ (μU/h.ml)	All	70 ± 24†	47 ± 22	135 ± 56†	84 ± 34
$\frac{\text{INS-AUC}_{(0–1\ h)}}{\text{INS-AUC}_{(0–10\ h)}}$ (%)	All	18.8 ± 7.4*	17.4 ± 8.8	16.6 ± 7.8†	14.5 ± 7.0
	<25	26.4 ± 6.7	27.4 ± 9.0	25.4 ± 8.0	22.6 ± 6.7
	≥25 to <30	19.9 ± 5.8*	17.1 ± 5.7	17.0 ± 4.7*	13.4 ± 3.8
	≥30 to <35	15.6 ± 5.6	14.0 ± 5.2	12.9 ± 5.3*	11.3 ± 5.5
	≥35	13.2 ± 3.5*	11.3 ± 4.8	10.9 ± 3.1	10.8 ± 4.5
INS-t$_{10\%}$ (min)	All	44 ± 11†	50 ± 14	49 ± 14†	54 ± 12
INS$_{max}$ (μU/ml)	All	115.2 ± 27.8*	95.9 ± 28.4	234.8 ± 68.5*	185.0 ± 51.7
INS-T$_{max}$ (min)	All	94 ± 42	76 ± 39	100 ± 40	92 ± 38

Data are given as mean ± s.d.

Test statistics were performed using an ANOVA model for the normally distributed pharmacodynamic parameters: GIR-AUC$_{0–1\ h}$, GIR-AUC$_{0–10\ h}$ and GIR$_{max}$. The pharmacokinetic parameters INS-AUC$_{0–1\ h}$, INS-AUC$_{0–10\ h}$ and INS$_{max}$ were analysed with the same ANOVA model after a natural log-transformation. All time-related parameters (INS-T$_{max}$, GIR-T$_{max}$, INS-t$_{10\%}$, GIR-t$_{10\%}$) were tested with non-parametric analyses (Wilcoxon signed-rank test). Please refer to the Statistical Methods for further details.

ANOVA, analysis of variance; BMI, body mass index; AUC, area under the curve; GIR, glucose infusion rate; GIR-t$_{10\%}$, time to 10% of GIR-AUC$_{0–10\ h}$; GIR$_{max}$, maximum GIR; GIR-T$_{max}$, time to GIR$_{max}$; INS, insulin; INS-t$_{10\%}$, time to INS-AUC$_{0–10\ h}$; INS$_{max}$, maximum INS concentration; INS-T$_{max}$, time to INS$_{max}$.

*$p < 0.05$; †$p < 0.001$ vs. corresponding insulin lispro/BMI group.

exposure are considered artefactual and are because of differences in the cross-reactivity to human insulin between the analogue-specific kits used for analysis. Taking this into consideration, the PK parameters explain the PD findings. The absorption of glulisine was significantly faster than that of lispro in the total study population, indicated by the lesser time required to achieve early exposure with glulisine (INS-t10% approximately 5–6 min less), resulting in a greater INS-AUC0–1 h/INS-AUC0–10 h ratio (table 2; figure 2). The difference in INS-t$_{10\%}$ was statistically significant across the BMI ranges with both doses; except for 0.4 U/kg in morbidly obese subjects (figure 1). The difference in INS-t$_{20\%}$ also tended to be in favour of glulisine ($p = 0.058$ for 0.2 U/kg and $p = 0.151$ for 0.4 U/kg), although this did not translate into significant differences in GIR-t$_{20\%}$. Moreover, insulin exposure (INS-AUC$_{0–10\ h}$ and INS$_{max}$) increased as BMI increased, while glucose disposal (GIR-AUC$_{0–10\ h}$ and GIR$_{max}$) decreased with both insulin analogues (figure 1).

There were no significant differences in mean C-peptide concentrations between glulisine and lispro (data not shown). No relevant increases above baseline levels were observed in any of the clamps for this variable with either treatment, indicating that the study results were not influenced by changes in endogenous insulin secretion.

No relevant changes in the safety laboratory variables and no serious adverse events were observed with either treatment or dose, apart from decreases in erythrocyte, haemoglobin and haematocrit measurements, which were attributed to the frequent blood sampling during the study.

Discussion

This study compared the pharmacological properties of the two fast-acting insulin analogues, glulisine and lispro in subjects without diabetes, over a wide BMI range. Two different doses were used in this study, 0.2 U/kg as a standard dose and 0.4 U/kg as a high dose. Both analogues showed comparable overall glucodynamic efficacy

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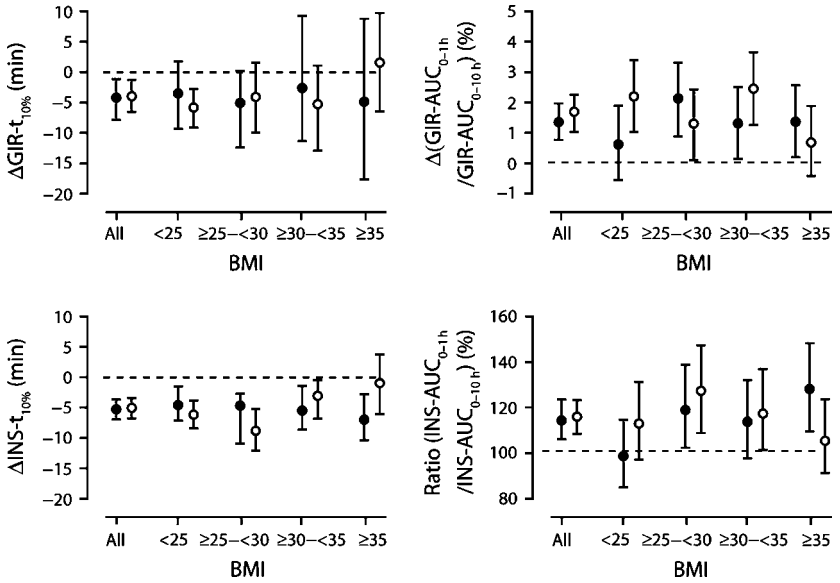

Fig. 2 Point estimates with 95% confidence limits for pharmacodynamic (upper panel) and pharmacokinetic (lower panel) parameters. Black circles = 0.2 U/kg; white circles = 0.4 U/kg. The plots show differences between insulin glulisine and insulin lispro or the ratio of insulin glulisine over insulin lispro. BMI, body mass index; AUC, area under the curve; GIR, glucose infusion rate; GIR-$t_{10\%}$, time to 10% of GIR-$AUC_{0–10\ h}$; INS, insulin; INS-$t_{10\%}$, time to INS-$AUC_{0–10\ h}$.

(GIR-$AUC_{0–10\ h}$), GIR-T_{max} and GIR_{max} at either dose. However, as shown by the greater ratio of GIR-$AUC_{0–1\ h}$/GIR-$AUC_{0–10\ h}$ with glulisine, a significantly higher proportion of total metabolic activity occurred in the first hour postdosing for glulisine compared with lispro (figure 2). This is also reflected in the higher efficacy in the first hour postdosing (GIR-$AUC_{0–1\ h}$) and accompanied by a faster onset in activity shown by significantly reduced GIR-$t_{10\%}$ with glulisine.

This finding confirms the observations of a previous glucose clamp study performed in obese subjects without diabetes, with a BMI of 30–40 kg/m^2, which also reported a faster rise in insulin concentration and a faster onset of action with glulisine than with lispro [13]. The present study with 320 euglycaemic glucose clamp experiments expands these findings to subjects with a BMI range of 20–40 kg/m^2. As no treatment by BMI interaction was shown for any PD parameter ($p > 0.1$), the statistical significance of these treatment differences established for the total study population can be generalized, i.e. the earlier onset of action of glulisine occurs in both lean and obese (and even morbidly obese) subjects.

Fast-acting insulin analogues have been compared for differences in PD and PK properties for clinical implications soon after their advent. For instance, Hedman *et al.* reported a faster rise and an earlier decline in insulin concentrations with lispro compared with aspart [16]. These differences in PK properties, observed in 14 patients with type 1 diabetes were not, however, accompanied by any differences in postprandial BG concentrations after a standard meal. Furthermore, other studies with more patients [17] or more complex methods [18] did not show any significant differences (in either PK or PD) between aspart and lispro. Thus, our confirmation of previous findings [13] of the faster onset of action of glulisine vs. lispro might be surprising, but may be because of the absorption processes of both insulins. The drug formulation of glulisine differs from those of lispro and aspart; glulisine is stable with polysorbate 20, whereas the other analogues need to be formulated with zinc [19]. Zinc is added to stabilize insulin molecules in hexamers (with two zinc atoms located in the centre of the hexamer) to achieve a practical shelf life [20]. Although lispro is more rapidly absorbed from pure monomeric solution compared with hexameric lispro (the prevalent form in the commercially available product), it lacks sufficient shelf life and in-use stability [15,21]. The oligomeric aggregates of glulisine molecules in solution are adequately stable without zinc, presumably because of the unaltered proline at position B28 allowing dimerization [22,23]. Thus, it is plausible to attribute the observed moderate disparity in early absorption and metabolic action between glulisine and lispro to differences in the association status of the insulin molecules. This is linked to the physicochemical properties of their formulations.

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As the difference between glulisine and other fast-acting analogues manifests in the zinc-free formulation of glulisine, the faster onset of action should be evident in all subjects (subjects without diabetes, subjects with type 1 or type 2 diabetes, lean or obese subjects). The fact that such a difference between glulisine and lispro was not observed in a previous study in subjects with type 2 diabetes [7] is probably because of the insufficient power of that study, which used an incomplete block design and thereby increased the variability between the treatment groups studied.

The imminent question regarding the clinical relevance of the observed faster onset of action of glulisine is a difficult one. While being statistically significant, the absolute difference, although small (e.g. INS-$t_{10\%}$ differed only by 5–6 min), afforded a 25–30% greater glucose disposal within the first hour after injection. In a previous study, the difference in the onset of action (expressed as the time to reach half-maximal activity) between aspart and RHI was reported to be not more than 13 min [24], indicating that the onset of action of glulisine might be meaningfully faster than that of the other fast-acting analogues. The clinical relevance of such findings has to be shown in adequately designed clinical studies. The only clinical study available so far with a head-to-head comparison between glulisine and lispro was conducted in patients with type 1 diabetes and did not show any difference in glycated haemoglobin or incidence of hypoglycaemic events between the analogues [25]. However, less basal insulin was required with glulisine as compared with lispro. This adds to the conclusion that improved PK/PD properties of new prandial insulins need to be accompanied by adaptations in the basal insulin regimen before leading to an improvement in overall metabolic control [6].

While the faster onset of action of glulisine was evident in all BMI subgroups in this study, it might be of highest clinical relevance in obese subjects. Previous findings report significantly delayed absorption in obese subjects [10,11], and a negative correlation of absorption and action with fat layer thickness for s.c. injection of RHI [26,27]. We observed a modest decrease in INS-$AUC_{0–1\ h}$/INS-$AUC_{0–10\ h}$ ratio (figure 2) at increasing total absorption, INS-$AUC_{0–10\ h}$ (figure 1), with increasing BMI. Nevertheless, insulin resistance, a characteristic feature of obesity [28], is closely associated with the amount of visceral fat [26,29,30], and leads to an attenuation of the metabolic activity of any insulin product, as also shown in this study for both glulisine and lispro.

Thus, both attenuated absorption and reduced metabolic activity have to be accounted for in obese people because the time–action profile of s.c. RHI is shifted to the right and shows less peak activity compared with lean subjects. For these patients, it may be of particular importance to use the insulin analogue with the most rapid onset of action to counteract the right-shift in the insulin time–action profiles.

In conclusion, our study confirms previous observations of a faster onset of action of glulisine as compared with lispro. This faster onset of action of glulisine, which is associated with the novel drug formulation, is evidently independent of the insulin dose and the subjects' BMI.

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A Comparison of the Steady-State Pharmacokinetics and Pharmacodynamics of a Novel Rapid-Acting Insulin Analog, Insulin Glulisine, and Regular Human Insulin in Healthy Volunteers Using the Euglycemic Clamp Technique

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Article

Abstract

Insulin glulisine is a new rapid-acting insulin analog. The aim of this study was to assess the glucodynamic efficacy of insulin glulisine compared with regular human insulin (RHI) using a manual euglycemic clamp technique. Steady-state pharmacokinetics of insulin glulisine, and its cardiac safety (ECG) and tolerability after intravenous administration, were also determined. This was a single center, randomized, open-label, two-way crossover study in healthy male subjects (n = 16). At the treatment visits subjects received an intravenous infusion of the study drug at a rate of 0.8 mU $kg^{-1} \cdot min^{-1}$ for 2 hours. Individual baseline glucose concentrations were targeted for euglycaemia and maintained with a manual adjusted 20% glucose solution over the clamp period of a maximum 6 hours. A glulisine-specific antibody was used to quantify glulisine concentrations by radioimmunoassay, while a non-specific insulin antibody and C-peptide based correction for endogenous insulin was used to estimate exogenous human insulin (RHI). At steady state (90 – 120 min), insulin glulisine and RHI had equivalent glucose utilization (GIR-AUC_{SS}, 214 $mg \cdot kg^{-1}$ for glulisine, 209 $mg \cdot kg^{-1}$ for RHI) and infusion rates (GIR_{SS}, 1050 and 995 $mg \cdot min^{-1} \cdot kg^{-1}$). Both insulins also presented equal total glucose disposal (GIR-$AUC_{0-clamp\ end}$, 1050 and 995 $mg \cdot kg^{-1}$) and onset of activity within 20 min. Insulin glulisine and RHI showed parallel time concentration profiles with similar distribution and elimination, but the different antibodies employed for radioimmunoassay impeded a quantitative comparison. There were no noteworthy individual or within-group changes in cardiac repolarisation parameters measured by 12-lead ECG during insulin glulisine infusion. In conclusion, insulin glulisine and RHI show similar distribution and elimination profiles and equivalent glucodynamic efficacy on a molar, unit-per-unit basis.

Key words
Insulin glulisine · regular human insulin · rapid-acting insulin analog · molar efficacy

Introduction

Tight glycemic control is the aim of diabetes treatment with basal–bolus insulin regimens (DCCT, 1993; UKPDS, 1998), and insulin analogs are increasingly accepted for both prandial and inter-prandial control of blood glucose (Owens et al., 2001).

Insulin glulisine (3 B-Lys-29 B-Glu-insulin) is a new insulin analog (Hennige et al., 1999) designed to provide the same total glucodynamic effect as regular human insulin (RHI), with a shorter duration of action when given subcutaneously (Becker et al., 2003). Insulin glulisine differs from RHI by the replacement of asparagine with lysine at position 3, and of lysine by glutamic

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acid at position 29, on the B-chain of the human insulin molecule. These alterations favor formation of stable monomers, which is key to the rapid absorption from subcutaneous tissue (Brange et al., 1990; Brange et al., 1988; Brems et al., 1992; Kang et al., 1991).

The aim of the present study was to assess the glucodynamic efficacy of insulin glulisine compared with RHI, during intravenous administration of equimolar doses to healthy volunteers using the manual euglycemic clamp technique. Additionally, steady-state pharmacokinetics was evaluated. Further, the safety and tolerability of insulin glulisine after intravenous administration were studied.

Materials and Methods

Study design

This study was a single center, randomized, open-label, two-way crossover design in healthy male subjects whose characteristics are shown in Table **1**.

Ethics

This study was conducted in accordance with Good Clinical Practice and conformed to the ethical principles of the Declaration of Helsinki. All study documentation was reviewed and approved by an independent Ethics Committee and the national health authority of South Africa. Prior to screening, and before admittance to the clinical study, all subjects gave written informed consent.

Study protocol

The study consisted of four trial periods: 0 (screening), 1 and 2 (treatment) separated by a minimum 7-day wash-out period, and 3 (follow-up). Trial period 1 took place within 28 days of period 0, and trial period 3 took place no more than 7 days after trial period 2.

The subjects fasted from the evenings prior to treatment visits after a carbohydrate-rich meal until the end of the clamp procedure. About 2 hours prior to study mediaction, subjects were prepared with a retrograde dorsal vein cannula in one hand, which was kept at about 55 °C with a thermostatic electric blanket to achieve arterialization of venous blood. This cannula was used to collect samples for determination of blood glucose, insulin, and C-peptide. A cannula in the contralateral forearm was used for infusion of glucose and study medication. From one hour prior to study medication, which comprised the pre-medication baseline period, until end of clamp, subjects remained in a semi-recumbent position.

Starting at about 08:00 a.m., subjects received a 2-hour continuous intravenous infusion of either insulin glulisine or RHI, at a rate of 0.8 mU/kg/min, as randomized for cross-over at subsequent visits.

Aventis Pharmaceuticals supplied insulin glulisine and RHI. Infusion solutions were prepared by diluting stock solutions (100 U/mL) in 114 mL isotonic saline solution (0.9%), to which 5 mL of the subjects own blood was added, such that each subject received 0.096 U/kg in 2 hours with a high precision pump (IVAC P2000, Basingstoke, UK).

Four arterialized venous blood samples were taken for the determination of baseline blood glucose, at 60, 30, 15 and 0 minutes prior to start of study medication infusion. Prompted by a 10% drop from its mean baseline blood glucose concentration in response to study medication infusion, a manual adjusted infusion of a 20% glucose solution was started to restore and maintain the subject's individual baseline blood glucose level. To this end, blood glucose was determined every 5 minutes throughout the clamp period, which lasted a maximum of 6 hours, or ended when the glucose infusion rate decreased to zero over a period of 15 minutes.

Further blood samples were taken prior to infusion of study medication and at 15-minute intervals during the entire clamp period in order to determine serum insulin and serum C-peptide concentrations. Blood samples for the determination of potassium concentration were taken immediately prior to the start of infusion of study medication, and again at 90 and 120 minutes thereafter.

Vital signs were assessed prior to, and immediately after, the euglycemic clamp period. Subjects received a meal, and were discharged from the study clinic after the investigator had ensured their safety, which had to be confirmed at a follow-up visit, 24 hours thereafter.

Pharmacodynamic assessments

Efficacies of insulin glulisine and RHI were assessed using glucose utilization at steady state, which was assumed for the period 90 – 120 minutes. The area under the GIR time curve at steady state (GIR-AUC_{SS}) was the primary measure, and glucose-infusion rate at steady state (GIR_{SS}) a secondary measure. The area under the entire GIR time curve (GIR-$AUC_{0-clamp\ end}$; total glucose disposal) was also assessed. All variables were adjusted by dividing values by the subject's body weight in kilograms.

For exploratory purposes, the maximum decreases in blood glucose concentration from baseline and the time to maximum decrease in blood glucose concentration after initiation of study drug infusion were also measured.

Pharmacokinetic assessments

The pharmacokinetic profiles of insulin glulisine and RHI were assessed by insulin exposure at steady state (INS-AUC_{SS}), which was determined as the area under the insulin concentration time curve from 90 – 120 minutes after start of the infusion of study medication, insulin concentration at steady-state (C_{SS}), total insulin exposure (INS-$AUC_{0-clamp\ end}$), total clearance (CL_{tot}), volume of distribution at steady-state (V_{SS}), terminal elimination half-life ($t_{½}$), and mean residence time (MRT).

Immunoreactive insulin concentrations after administration of RHI were corrected for endogenous insulin concentration, estimated from C-peptide concentration, to determine exogenous insulin levels. C-peptide samples were not required for assessment of insulin glulisine levels, due to the use of a specific antiserum to detect this analogue.

Safety assessments

Adverse events were defined as any unfavorable and unintended sign, symptom, syndrome, or illness developing, or worsening, during the observation period of the study. In addition physical examinations, including vital signs and assessments of hematology, clinical chemistry, and urine were carried out. Twelve-lead ECGs were recorded 30 min before and 1.5 hours and 6 hours after initiating the insulin infusions. Heart rate, PR interval, QRS complex, QT interval, QTc interval and RR interval were all assessed. These were analyzed by an independent cardiologist, blinded for study medication, for effects on repolarization.

Table **1** Demographics

Age (years)	22 (18, 32)
Weight (kg)	80 (71, 100)
Height (cm)	183 (172, 197)
BMI (kg/m²)	24 (20, 26)

Data are given as arithmetic mean (range)

Sample analysis

According to treatment, serum samples were analyzed for insulin glulisine with a radioimmunoassay specific for insulin glulisine (Linco Research Inc, St Charles; non commercial kit), with a lower limit of quantification (LLOQ) of 2.00 µIU·mL^{-1} and < 0.001 % cross-reactivity to human insulin, or as appropriate, for human insulin (RHI) and C-peptide. Human insulin was determined with a radioimmunoassay for immunoreactive serum insulin (Linco Research Inc, St Charles Missouri USA via Biotrend GmbH, Köln) with a LLOQ of 4.30 µIU·mL^{-1}, and C-peptide with the BIOSOURCE C-PEP-RIA-CT (Biosource Europe, Nivelles, Belgium) with a LLOQ of 0.070 nmol·L^{-1}. All analyses were performed by the Bioanalytical Services Division, FARMOVS PAREXEL (Pty) Ltd., Bloemfontein, South Africa. Blood glucose was determined with a Yellow Springs Instruments 2300 S glucose analyzer using the glucose oxidase method.

Statistics

The pharmacodynamic parameters GIR-AUC$_{SS}$, GIR$_{SS}$ and GIR-AUC$_{0-\text{clamp end}}$ of the glucose infusion rate were compared using a analysis of variance (ANOVA) model with adjustment for treatment, period, sequence and subject-within-sequence contribution (Schuirmann, 1987). Fieller's theorem (Vourinnen and Tuominen, 1994) method was used on the raw data, to generate the standard 90% confidence interval (CI) of the ratio of the treatment means.

The pharmacokinetic parameters are presented with descriptive statistics.

Results

Study conduct

All 16 subjects enrolled and randomized completed the study and were included in the pharmacokinetic, pharmacodynamic and safety analyses. Subjects were healthy male Caucasians whose demographics are presented in Table **1**. There were no protocol violations.

Clamp performance

The mean individual baseline blood glucose levels were similar at 82 mg·dL^{-1} (95% CI 79; 85 mg·dL^{-1}) prior to insulin glulisine administration, and 82 mg·dL^{-1} (95% CI 79; 84 mg·dL^{-1}) prior to RHI, without significant individual differences between clamp days [0.0 mg·dL^{-1} (95% C.I. – 2.7; 2.7 mg·dL^{-1})]. The actual mean blood glucose level at steady state was 86 mg·dL^{-1} (95% CI 85; 88 mg·dL^{-1}) for insulin glulisine, and 85 mg·dL^{-1} (95% CI 84; 87 mg·dL^{-1}) for RHI, without significant individual differences between clamp days [0.8 mg·dL^{-1} (95% C.I. – 0.9; 2.6 mg·dL^{-1})]. Average coefficients of variation for blood glucose at steady state (90 – 120 min) during clamp procedures were equal at 5% (95% CI 4; 6%) for both insulin glulisine and RHI, indicating reliably established euglycemia.

Glucodynamics

Glucose infusion had to commence on average 25 min (range 20; 50 min) after start of insulin glulisine infusion and was stopped at 200 min (range 175; 265 min). Similarly, 25 min (range 10; 35 min) passed after start of regular human insulin infusion before glucose infusion had to commence, which was stopped after 205 min (range 180; 295 min).

Maximum reduction in glucose concentration of on average – 11.3 mg·dL^{-1} (median, range – 21.9; – 6.0 mg·dL^{-1}) was to some extent delayed behind the start of glucose infusion, to 30 min (median, range 20; 50 min) after initiation of insulin glulisine infusion, and similar on average – 12.5 mg·dL^{-1} (median, range – 17.0: – 3.3 mg·dL^{-1}) after regular human insulin, also at 30 min (range 15; 85 min) (Fig. **1 A**).

The GIR profiles of both treatments were superimposable, indicating equal effectiveness for glucose disposal (Fig. **1 B**).

Thus, insulin glulisine and RHI were equally rapid in onset of activity and equally potent as shown both by equivalent glucose utilization (GIR-AUC$_{SS}$), and glucose infusion rates (GIR$_{SS}$) at steady state, as well as by equivalent total glucose disposal (GIR-AUC$_{0-\text{clamp end}}$) (Table **2**).

Pharmacokinetics

The concentration profiles of insulin glulisine and regular human insulin followed the same time course.

A distinct quantitative comparison of serum insulin levels in the two groups is inherently restricted due to the different antibodies used for detection of insulin glulisine and RHI (Fig. **1 C**). The insulin exposure under steady-state INS-AUC$_{SS}$ was measured as around 30% greater for insulin glulisine than for RHI. Also, insulin glulisine concentration at steady state, C$_{SS}$, and total insulin glulisine systemic exposure (INS-AUC$_{0-\text{clamp end}}$) were both measured as 21% higher than those for RHI (Table **3**).

Insulin concentration was 10 µU/mL (range 5; 20 µU/mL) at baseline prior to infusion of regular human insulin. Release of endog-

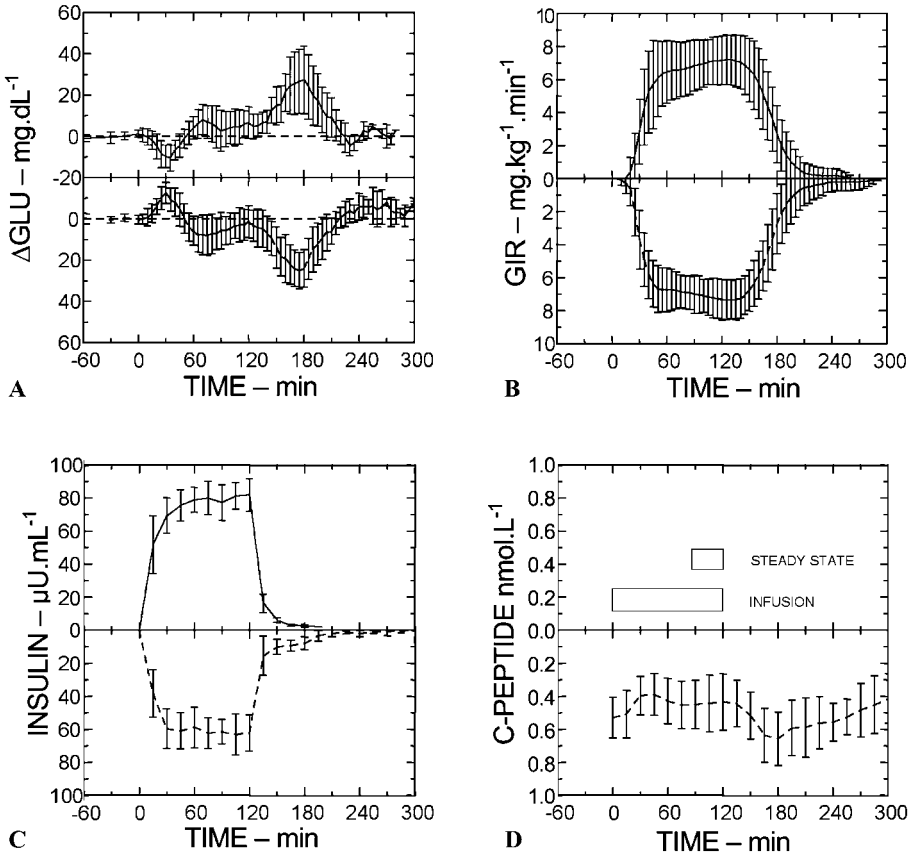

Fig. **1 A** to **D** **A** Change in blood glucose concentration (ΔGLU – mg · dL^{-1}) from baseline for insulin glulisine (upper panel) and regular human insulin (lower panel). **B** Glucose infusion rate (GIR – mg · kg^{-1} · min^{-1}) profiles. **C** Insulin concentration (INSULIN – µU · mL^{-1}) time profile. **D** C-peptide concentration (C-PEPTIDE nmol · L^{-1}) time profile (for RHI only). Data are given as arithmetic mean with standard deviation.

Table **2** Glucodynamics

	RHI	*GLU*	*Point estimate (90% CI) GLU/RHI*
GIR-AUC$_{SS}$ (mg · kg^{-1})	214	209	97.6 (88.4; 107.6)
GIR$_{SS}$ (mg · kg^{-1} · min^{-1})	7.2	7.0	98.4 (89.3; 108.5)
GIR-AUC$_{0-clamp\ end}$ (mg · kg^{-1})	1050	995	94.8 (84.5; 106.2)

GIR data are arithmetic means; PE based on raw scaled data; GLU = insulin glulisine

Table **3** Pharmacokinetics

	GLU	*RHI*
INS-AUC$_{SS}$ (µIU · min · ml^{-1})	2393 (2059; 2808)	1856 (1262; 2261)
C$_{SS}$ (µIU · ml^{-1})	70 (54; 95)	58 (35; 84)
INS-AUC$_{0-clamp\ end}$ (µIU · min · ml^{-1})	9262 (8083; 10751)	7652 (5274; 9790)
V$_{SS}$ (L)	13 (9; 17)	22 (13; 31)
T½ (min)	13 (9; 26)	17 (9; 26)
CL$_{tot}$ (mL · min^{-1})	927 (785; 1046)	1084 (864; 1606)
MRT (min)	14 (9; 17)	19 (12; 28)

INS data are geometric means (range); PE based on ln-scaled data; GLU = insulin glulisine

enous insulin was modestly reduced by about 20% during infusion as estimated from C-peptide concentrations (Fig. **1 D**).

Distribution and elimination of insulin glulisine and RHI were similar, as shown by similar V_{SS}, MRT, CL_{tot}, and $T_{½}$, with values of the latter reflecting fast elimination from the systemic circulation (Table **3**).

ECG readings

There were no noteworthy individual or within-group changes in conduction parameters (PR, QRS, QT, QTcB) from immediately before initiating the continuous i.v. infusion of insulin glulisine (0 minutes) until 90 minutes after the start of administration (at the time when steady-state levels of insulin glulisine were accomplished) or upon completion of the clamp (360 minutes) (Table **4**). Specifically, there were no statistically significant within-group changes from baseline in QTcB. Blinded over-reading of ECGs by a cardiologist did not detect any repolarization abnormalities associated with insulin glulisine administration. Serum potassium levels decreased on average by 0.2 mmol/L during insulin infusion.

Safety

There were no serious adverse events, no clinically relevant abnormalities with regard to clinical chemistry, and no change in insulin antibody binding, from baseline to the end of the study. Other safety assessments did not indicate any safety issue with insulin glulisine or RHI.

Table **4** ECG readings

	GLU		RHI	
ECG intervals	*Baseline*	*Change at steady state*	*Baseline*	*Change at steady state*
RR (ms)	1106	– 68 (– 291, 94)	1064	– 20 (– 179, 125)
PR (ms)	162	– 2 (– 20, 12)	158	6 (– 18, 32)
QRS (ms)	97	2 (– 10, 10)	97	4 (– 2, 22)
QT (ms)	423	– 9 (– 76, 34)	419	– 4 (– 40, 58)
QTcB (ms)*	403	3 (– 42, 35)	407	0 (– 19, 41)

* QT corrected according to Bazett; GLU = insulin glulisine; Data are given as arithmetic mean (range)

Discussion

The results of this euglycemic clamp study employing intravenous infusion of study medication show that insulin glulisine and RHI demonstrate equivalent efficacy on an administered molar dose basis, as evidenced by comparable glucose disposal at steady state, GIR-AUC_{SS} and GIR_{SS}. This is in agreement with previous reports of generally equal efficacy of insulin analogs (aspart, lispro, glargine) and RHI in humans (DiMarchi et al., 1994; Gillies et al., 2000; Simpson and Spencer, 1999; Wilde and McTavish, 1997).

The results are also in line with the finding, that regardless of differences in in vitro receptor binding affinities, insulin analogs have similar in vivo potency to RHI, as demonstrated in animal and human studies (Brange et al., 1988; Brange et al., 1990; Heinemann and Woodworth, 1998; Plum et al., 2000; Scholtz et al., 2003; Volund et al., 1991); this is attributable to receptor-mediated clearance. Notwithstanding the above, insulin glulisine has a similar insulin receptor binding affinity to RHI as well as equivalent in vitro potency (Hennige et al., 1999).

Though equivalence on a molar base may alleviate switching patients from RHI to insulin glulisine, it needs to be considered that improved post-prandial control with analogues such as insulin glulisine require prudent dose adaption, even when used in pump therapy (Anderson et al., 1997; Renner et al., 1999).

Insulin exposure in the steady state (INS-AUC_{SS}, C_{SS}) and total area under the insulin concentration time curve (INS-$AUC_{0-clamp\ end}$) were observed greater after insulin glulisine administration than after RHI administration. The apparent difference in exposure is unexplained since equimolar doses and hence the same total masses were infused, but may rest with the different antibodies employed for detection of insulin glulisine and RHI, which impede distinct quantitative comparisons. For perspective, following subcutaneous administration the absolute bioavailability of insulin glulisine and RHI (Becker et al., 2003; Frick et al., 2003) of about 70% is the same and similar to that of insulin lispro (Heinemann and Woodworth, 1998; Wilde and McTavish, 1997). Equivalent glucodynamic efficacy to regular human insulin at apparently different total systemic exposure is also reported for insulin lispro (Bott et al., 2004).

Distribution of insulin glulisine and RHI were similar and elimination was equally fast following intravenous administration, reflecting similar pathways. The more rapid elimination of insulin glulisine following subcutaneous injection is attributable to absorption-rate-limited elimination (Becker et al., 2003; Rave et al., 2004).

Insulin induced hypoglycaemia causes QT prolongation related to a decrease in potassium (Eckert and Agardh, 1998; Marques et al., 1997). All through euglycemia, insulin glulisine exposure did not affect cardiac conduction parameters as assessed by measurement of 12-lead ECGs. The absence of any effects of insulin glulisine on cardiac conduction under euglycaemia is reassuring as it attests to the absence of genuine effects of insulin and analogs on cardiac repolarization (Harris et al., 1999).

In conclusion, insulin glulisine and RHI show similar distribution and elimination profiles and equivalent glucodynamic efficacy on a molar, unit-per-unit basis.

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