The glucose clamp technique has become the most important tool for the assessment of the pharmacodynamic properties of antidiabetic agents. It allows an unbiased determination of their blood glucose lowering effect, without interferences such as hypoglycaemic counterregulation.

The author, Lutz Heinemann, PhD, Profil Institute for Metabolic Research, Neuss, Germany, has worked for more than a decade with this sophisticated technique and is internationally renowned for his systematic investigation into new forms of insulin therapy. Here, he summarizes the most important results of these investigations—of the time-action profiles of different insulin preparations, novel insulin analogues, as well as other routes of insulin administration (e.g., inhaled insulin), by means of a standardized glucose clamp technique.

In particular, the clinical impact of an optimization of insulin therapy by using insulin analogues with favourable pharmacodynamic properties is shown by the results of a clinical study investigating postprandial blood glucose control after a carbohydrate rich meal.

Several investigations address the intra-patient variability in insulin action, which is one of the most critical drawbacks in current insulin therapy. Lutz Heinemann presents studies about the key factors that influence insulin absorption, the understanding of which is an important first step to overcome this problem of diabetes treatment.

Thus, this overview is an important source of information for both scientists and physicians interested in modern insulin therapy.
Time-Action Profiles of Insulin Preparations

Lutz Heinemann
The cover illustration is a portrayal of the insulin molecule.
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<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ANOVA</td>
<td>ANalysis Of VAriance</td>
</tr>
<tr>
<td>AUC</td>
<td>Area Under the Curve</td>
</tr>
<tr>
<td>$C_{\text{basal}}$</td>
<td>basal serum or plasma insulin concentration</td>
</tr>
<tr>
<td>$C_{\text{max}}$</td>
<td>maximal serum or plasma insulin concentration</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of Variation</td>
</tr>
<tr>
<td>GIR</td>
<td>Glucose Infusion Rate</td>
</tr>
<tr>
<td>GIR$_{\text{max}}$</td>
<td>maximum glucose infusion rate</td>
</tr>
<tr>
<td>N.S.</td>
<td>not significantly different</td>
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<tr>
<td>NPH insulin</td>
<td>Neutral Protamine insulin Hagedorn</td>
</tr>
<tr>
<td>NPL insulin</td>
<td>Neutral Protamine insulin formulated with insulin lispro</td>
</tr>
<tr>
<td>$P$</td>
<td>Probability</td>
</tr>
<tr>
<td>$t_{\text{early 50%}}$</td>
<td>time needed to achieve 50% of maximal activity or concentration before maximal activity or concentration occurs</td>
</tr>
<tr>
<td>$t_{\text{late 50%}}$</td>
<td>time needed to return to 50% of maximal activity or concentration after maximal activity or concentration has occurred</td>
</tr>
<tr>
<td>$t_{\text{max}}$</td>
<td>time needed to reach maximal activity or concentration</td>
</tr>
<tr>
<td>U or IU</td>
<td>Unit or International Unit (measure of insulin strength)</td>
</tr>
<tr>
<td>$\bar{x}$</td>
<td>mean value</td>
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I would like to express my deepest gratitude to all those who have assisted and supported me and my work over the years, and without whom all the research presented in this book would not have been possible.
1 Introduction

Under physiological conditions insulin is secreted in low amounts and rather constantly between meals (basal insulin secretion). During meals, a stimulation of insulin secretion takes place, which is highly variable in terms of quantity and duration and depends on the individual need (prandial insulin secretion). In intensive insulin therapy, insulin-dependent patients with diabetes mellitus try to imitate the kinetics of this complex endogenous insulin secretion by subcutaneous injection of short-acting insulin before meals and of long-acting insulin in the morning and at bedtime. The insulin preparations currently available, especially with regard to the blood glucose-lowering effect over time (time-action profile), aggravate the implementation of intensive insulin therapy. Consequently, clinical diabetologists have called for the development of insulin preparations with more adequate time-action profiles.

The considerable variability of insulin action after subcutaneous injection impedes the practicability of any insulin therapy; therefore, there is a demand for a more reproducible insulin action. One possibility to obtain insulin preparations with such properties lies in modifications of the insulin molecule itself. These approaches are based on the finding that the individual insulin molecules contained in the pharmaceutical insulin preparations will hexamerise in a process of self-association depending on the insulin concentration. Since the absorption of these hexamers through the pores of the capillary walls is poor, a time-consuming dilution of the insulin solution in the subcutaneous adipose tissue must precede. Thereupon, the hexamers will dissociate into smaller dimers and monomers, which are rather quickly absorbed into the blood stream. By means of genetic engineering, amino acids can be exchanged at any position of the insulin molecule, enabling the generation of insulin analogues, i.e., of molecules whose primary structure differs slightly from that of human insulin. Depending on which amino acids are exchanged, either the insulin monomers do not associate into hexamers any longer or the cohesional forces of the hexamers are so reduced that they rapidly dissociate after subcutaneous injection. It is, however, also possible to generate insulin analogues in which the cohesional forces of the hexamers are increased. In so doing, the absorption of insulin analogues after subcutaneous injection is either faster than that of short-acting insulin preparations or even slower than that of long-acting insulin preparations.

Before implementing subcutaneous insulin therapy, it is necessary to become familiar with the time-action profiles of short and long-acting insulin preparations, and the factors which influence them. The only way to find out the required quantitative information on the pharmacodynamic properties of insulin preparations is to use the euglycaemic glucose clamp technique.

Early investigational approaches were either limited to determining the pharmacokinetic properties of the insulin preparations, or simply used the quantitative decrease in blood glucose as an indication of their pharmacodynamic properties—rendering their results invalid.
Recent refinements of the glucose clamp technique, however, have enabled the accurate quantitative study of the pharmacodynamic properties of insulin preparations. Using this method, the following seven investigations were carried out in order to obtain important information for the implementation of insulin replacement therapy:

1. A comparative study and description of the time-action profiles of the most important insulin preparations.
2. A characterisation of the time-action profiles of rapid and long-acting insulin analogues as compared to their non-analogue counterparts.
3. The investigation of the time-action profiles of premixed insulin preparations formulated with rapid-acting insulin analogues.
4. Analyses of factors with influence on the absorption rate of short-acting insulin preparations from the subcutaneous insulin depot.
5. A description of the intra- and interindividual variability of insulin action that may occur after multiple subcutaneous injections of identical doses of a short-acting insulin preparation or a rapid-acting insulin analogue.
6. The pattern of the time-action profiles resulting from three different kinds of insulin administration: inhalative, subcutaneous and intravenous administration.
7. The reduction of postprandial hyperglycaemia after a meal, which is rich in carbohydrates, using a rapid-acting insulin analogue for prandial insulin substitution in patients with type 1 diabetes mellitus, compared with the administration of a short-acting insulin preparation.
2 Methodology
Measuring the concentration of a drug and its metabolites in the body over time allows the description of its pharmacokinetic properties in quantitative terms. The time course is determined by the processes involved in the absorption of a drug, its distribution, biotransformation and finally its elimination. Evaluating the receptor interactions and the subsequent biological processes of a drug, allows the quantification of its pharmacodynamic properties. The purpose of this is to derive statements concerning the time-action profiles of the blood glucose-lowering effect of insulin preparations.

![Temporal shift between the course of the serum insulin concentrations (pharmacokinetics) and the glucose infusion rate profile (pharmacodynamics) after subcutaneous injection of 12 U of a short-acting insulin preparation (U-40).](image)

Figure 2.1
Temporal shift between the course of the serum insulin concentrations (pharmacokinetics) and the glucose infusion rate profile (pharmacodynamics) after subcutaneous injection of 12 U of a short-acting insulin preparation (U-40).9

2.1 Temporal relationship between pharmacokinetics and pharmacodynamics
The blood glucose-lowering action of insulin—one of the most important metabolic effects—is closely correlated to the course of the insulin concentration in the blood, but the action occurs with a certain delay (Fig. 2.1). In this case, action is defined as the glucose infusion rate (GIR) required in euglycaemic glucose clamp experiments to keep the blood glucose concentration constant. This shift between serum insulin concentration and time-action profile (hysteresis) was already observed in the first glucose clamp studies and described by other authors.10-12
The extent of this temporal shift depends on, among other things, the absorption rate of insulin. Thus, the shift is most pronounced after intravenous administration of insulin, and metabolic activity can still be noticed after the blood insulin concentration has already returned to basal values (see pg. 112). In the case of delayed insulin absorption the hysteresis becomes smaller, since the transport of insulin to the insulin receptors occurs almost simultaneously with the delayed insulin absorption.

The mere investigation into the pharmacokinetic properties of insulin preparations would ignore this hysteresis, which is based on a series of events occurring between the increase of the intravascular insulin concentration and the resulting metabolic activity. The metabolic activity (increase of glucose utilisation) of insulin administered by subcutaneous injection depends on:

- the fraction of the dose which is absorbed,
- the rate of absorption,
- the rate of elimination,
- the fraction which passes the endothelium of the capillaries and is transported into the interstitial space (transcapillary insulin transport),
- the time required for insulin to reach the cell surface,
- the binding to the insulin receptors,
- the activation of tyrosine kinase at the intracellular receptor part,
- the subsequent translocation of glucose transporters from the inner cell to the cell membrane (and/or transporter activation),
- the current glucose transport,
- the glucose phosphorylisation and
- the subsequent metabolic processes.

The shift between changes in the blood insulin level and changes in the glucose requirement is caused by the time required to complete the cascade of steps making up the cellular mediation of the insulin action. Once the insulin has been absorbed into the blood stream, this time is more or less constant. Furthermore, this cascade explains why insulin action can still be detected after the blood insulin level has returned to basal values. The possibilities to describe the temporal shift between the changes of the insulin concentration and of the glucose requirement include the calculation of an insulin/GIR ratio or a figure showing the insulin concentrations against the GIR values at the different time points during an experiment.

The endothelial barrier between the vascular and interstitial spaces, present in the peripheral tissues but not in the liver, does not seem to be the only factor in determining the delay between absorption and action, but also has a strong influence on the extent of the insulin transport into the interstitial space (Fig. 2.2). When interstitial fluid was taken from the subcutaneous tissue by means of the microdialysis technique, the insulin concentrations in that fluid were 50-60% lower than the plasma concentrations prevailing at the same time—the latter having been increased to
200 µU/ml during the euglycaemic glucose clamp experiments. The infusion of inulin (a polysaccharide whose molecular weight is similar to that of insulin), on the other hand, did not reveal any concentration differences. A sharp increase in plasma insulin concentrations by doubling the insulin infusion rate only managed to raise the insulin concentration in the interstitial fluid after 20 min. Rapid changes and increases of the blood insulin concentrations only cause a delayed and minimal modification of the concentration in the interstitial fluid. It is still under dispute whether the transendothelial transport of insulin is mediated by receptors or not.
2.2 Previous approaches to determine the properties of insulin preparations

The quantitative measurement of the pharmacokinetic and pharmacodynamic properties of insulin preparations is based on so-called ‘indirect’ and ‘direct’ methods.24 Direct methods are those measuring the occurrence of insulin in the blood or the insulin action. Indirect methods, on the other hand, use the measurement of signals of substances coupled to insulin.

2.2.1 The indirect method

With the indirect method, the pharmacokinetic properties of an insulin preparation are studied as follows: the subjects receive subcutaneous injections of labelled insulin (\(^{125}\text{I}\text{}\text{isotope, gamma emitter}\)). The attenuation of the gamma radiation above the skin area with the insulin depot is then measured by an external scintillation counter.25 It is assumed that a decline in the radiation activity measured above this skin area is due to the absorption of labelled insulin. The result of the first measurement after insulin injection is defined as 100%, and the relative decline in radioactivity is plotted as a percentage of the arbitrary baseline value in a semilogarithmic representation over time. The reduction of radioactivity is said to indicate the absorption rate of the injected insulin into the bloodstream.26

Many studies of pharmacokinetic properties of insulin preparations have been and are still being performed using this indirect method, since it can be carried out rather quickly and easily, and does not require any taking of blood samples or other invasive measures. However, the absorption rate can be determined from the rate of decay of radioactivity of the injected insulin only if the following three conditions are met:25

- the labelled insulin has the same absorption kinetics as the native hormone;
- the insulin molecule has to be absorbed unchanged, i.e., intact, into the blood stream; and
- the measured radioactivity is proportional to the amount of unabsorbed insulin.

It is still under dispute whether the first condition can really be fulfilled, in spite of the development of moniodinised insulin preparations which are also now available for NPH insulin preparations.27 As for the second condition, experiments have shown that a significant portion of the subcutaneously injected insulin is immediately degraded and deactivated by proteolytic enzymes.25,28,29 In addition to the degradation of insulin, there is an irregular distribution of the subcutaneously injected insulin in the subcutaneous fatty tissue. This distribution is neither symmetric nor concentric, but irregular, and influenced by the structure of the subcutaneous fatty tissue. Under these conditions, the activities registered externally are unlikely to really reflect accurately the total activity of the \(^{125}\text{I}\text{}\text{-labelled insulin below the injection site (third condition).}
The complex influences on the measurement geometry and of the constancy the scintillation counter (‘quenching’) have an unforeseeable impact on the results of the measurement. Depending on the counting characteristics of the gamma counter, the radioactivity of insulin molecules close to the surface is detected using a higher counter efficiency than that of molecules which have penetrated into deep tissue layers, where they may have been absorbed more quickly. The total efficiency of a scintillation counter can be defined as the ratio of the counting rate and the rate of decay. If and how many of the radioactive decays are registered depends on a series of factors. The influence of the counter efficiency on the result of the measurement as one of these factors (i.e., the ratio of the energy emitted as light and the energy released as radiation) is so great that an inter-observation variability occurs, which has to be standardised prior to each measurement. These factors, the local degradation of insulin, the importance of the measurement geometry, the efficiency of the scintillation counter as well as the irregular distribution of the hormone administered by subcutaneous injection, make the results obtained with this indirect method doubtful.

With the indirect method, lower absorption rates (longer half-lives) of the applied insulin preparations were measured than with other methods. Thus, a half-life of approximately 170 min following injection into the abdominal skin was reported for short-acting insulin.25,30,31 This half-life of nearly 3 h, is considerably different to the measured plasma insulin profiles and the metabolic activities of such insulin preparations: maximal plasma insulin levels were observed after 90 to 120 min, and 30 to 60 min after subcutaneous injection of short-acting insulin a marked reduction of the blood glucose concentration was observed. During this time, however, up to 80% of the insulin injected by the indirect method was left unabsorbed.

The slow dispersion of radioactive insulin from the injection site as against the more rapid rise of maximal plasma insulin concentrations may be explained by the fact that insulin absorbed into the blood is eliminated quickly, but the maximum absorption rate of insulin (in percent per hour of the injected dose) determines the maximal plasma insulin concentrations. These concentrations are reached earlier than the registered half-life of the subcutaneously injected labelled insulin.32

The degradation of insulin molecules by macrophages in the subcutaneous depot seems to be a logical explanation for the differences in the pharmacokinetic measuring of results in the case of long-acting insulin preparations (see pg. 38). But the metabolic activity is not the only outcome which calls the absorption rates determined by the indirect method into question. The absorption rates determined using the indirect method exhibited a linear course for up to 5 h. The linearity of absorption is in contradiction with the exponential course of the absorption rates determined with \(^{131}\)I and with the absorption kinetics calculated for subcutaneously injected substances.33 This mathematically-determined absorption rate could be confirmed in its monoexponential course in animal experiments using insulin labelled with \(^{3}\)H, the local insulin degradation in the tissue having been taken into account.28
Since the premises required to evaluate the absorption kinetics of subcutaneously injected insulin using the indirect method are not met in a satisfying manner, the indirect method is not suited for the quantitative investigation of the pharmacokinetic properties of insulin.

2.2.2 Direct methods
Direct methods can determine either the pharmacokinetic properties of an insulin preparation alone or can measure the pharmacokinetic and the pharmacodynamic properties simultaneously. The advantage of the direct methods—compared with the indirect method—lies in the fact that the measurement is restricted to the insulin that has currently been absorbed into the blood or to the current metabolic activity of the insulin. Unfortunately, the use of different methods and variations of these methods in the many pharmacokinetic and pharmacodynamic studies of insulin preparations impedes comparisons between the published results. Thus, the information given for an identical insulin preparation can vary considerably: the time to maximal action of subcutaneously injected short-acting insulin is, for instance, reported to be between 0.75 and 4 h. Various efforts are presently being undertaken to standardise directives for the implementation of pharmacodynamic investigations of insulin preparations (see below). One of the direct methods consists of measuring the plasma or serum insulin concentrations occurring after insulin injection (while the blood glucose concentrations decline) and determining the pharmacokinetic properties from these measurements. The insulin concentrations reflect the bioavailability of each insulin preparation. The bioavailability is not only determined by the insulin absorption, but also by the distribution and degradation of insulin in the subcutaneous tissue. The distribution and metabolism of insulin in the blood have a marked influence, too.

To clarify which proportion of the insulin concentrations measured in healthy subjects originates from the exogenous insulin supply and which proportion is due to the endogenous insulin production, blood samples are taken for the measurement of the serum C-peptide concentration parallel to the blood samples taken for the insulin determination. A decline in the serum C-peptide concentrations after subcutaneous insulin injection indicates the degree to which the endogenous insulin secretion is suppressed.

To estimate the pharmacokinetic summary measures, the insulin concentrations are determined by means of antibodies which are able to recognise specific amino acid sequences (epitopes) of the insulin molecules. By labelling the antibodies with radioactive isotopes or with enzymes, it is possible to develop measuring systems which enable the quantitative determination of insulin in small plasma or serum amounts with a high specificity. Such radio immunoassays or enzyme linked immunosorbent assays are commercially available. The antibodies used in the current radio immunoassays do recognise certain epitopes of the insulin antigen specifically, but they do not recognise whether the C-peptide is still linked to one of the two chains of the insulin molecule (in the case of the intermediate products of the conversion from proinsulin to insulin) or to...
both chains (in the case of proinsulin). Since these epitopes are identical for endogenous insulin and for exogenously-administered insulin, it is not possible to differentiate between these two insulin preparations. Thus, the antisera used for these determinations (mixture of polyclonal antibodies) also detect, in addition to insulin, the proinsulin or the intermediate products circulating in the blood (cross reactivity). The extent of this overestimation depends on the concentration of the insulin precursors in blood and on the extent of cross reactivity.

For the measurements of the circulating insulin analogue concentrations, the same radio immunoassay as in preparations formulated with human insulin was used. Due to the changes in the amino acid sequence, the antibodies may bind less avidly to these insulin molecules, depending on the epitope which the specific antibody recognises. However, assays employing specific antibodies for the quantitative measurement of the rapid-acting insulin analogues insulin lispro and insulin aspart are currently available.

If blood samples are used for insulin measurements and for measures of blood glucose concentrations at regular intervals, the latter shows evidence of the pharmacodynamic effects of the insulin preparations under study. The following parameters serve to describe the effects: the time to the earliest measurable blood glucose-lowering effect (‘time of onset of action’), the time to maximum decline of the blood glucose concentration (‘time to peak’), the blood glucose concentration at that time (‘peak’) and the time to return to baseline blood glucose concentration (‘time of action’). However, the decline of blood glucose levels occurring in this experimental design leads to hypoglycaemic values (<3.5 mmol/L), and thus to the stimulation of a counterregulatory reaction of the human body. The secretion of counterregulatory hormones induces an increase in the blood glucose concentration back to baseline values. This increase of blood glucose causes an underestimation of the duration of action of the insulin preparations (Fig. 2.3). In spite of this difficulty, which distorts the interpretation of the pharmacodynamic data obtained, all short- and long-acting insulin preparations have been investigated by means of this method, due to the rather easy experimental procedure.35

To prevent a counterregulatory reaction by insulin-induced hypoglycaemia, the subjects take in carbohydrates at regular intervals in the Gerritzen’s test (biscuits or mashed potatoes; 10 g carbohydrates/h).36 It is true that this measure prevents hypoglycaemia, but it may stimulate the endogenous insulin production, thus impeding the interpretation of the insulin concentrations measured in these experiments. In any case, this test is limited to the investigation of pharmacokinetic properties of insulin preparations, since the blood glucose concentration varies constantly.

### 2.3 The euglycaemic glucose clamp technique

During an euglycaemic glucose clamp, the infusion rate of an intravenous glucose infusion varies according to the measurements of the blood glucose concentrations in order to keep the blood glucose level constant. The amount of glucose infused reflects the metabolic
activity of the injected insulin (Fig. 2.3). This experimental approach is able to quantify the pharmacodynamic properties of insulin preparations. In the postabsorptive state (i.e., quite some time after a meal), the blood glucose concentrations are rather constant, i.e., the body’s glucose production rate ($R_a$) corresponds to the glucose utilisation rate ($R_d$). If the plasma insulin concentration is increased to a certain level by a continuous insulin infusion, there will be an increase in $R_d$ whereas $R_a$ decreases, i.e., $R_d > R_a$. This imbalance between glucose utilisation and endogenous glucose production would lead to a decrease in the blood glucose concentration if this were not prevented by an exogenous glucose infusion. The GIR is calculated and varied so as to allow the blood glucose concentration to remain constant at, for example, euglycaemic values of 4.0-5.0 mmol/L (euglycaemic glucose clamp experiments). The registered GIR then constitutes a measure of the net effect of the insulin, in that it describes the resulting sum of the decrease of hepatic glucose production and the increase of glucose utilisation. By keeping the blood glucose concentration constant, thanks to the glucose clamp technique, a counterregulatory reaction is prevented. When the glucose production rate is quantified by tracer methods, the ratio between plasma insulin concentration and glucose utilisation or hepatic glucose production can also be quantified. When insulin is just infused constantly by intravenous administration in glucose clamp experiments, the GIRs recorded after some time can be used as a quantitative measure of the insulin-mediated glucose uptake by the organism, which depends on the insulin sensitivity of the body. When higher insulin infusion rates are used (e.g., 1 mU/kg/min), the blood insulin concentration established in that way will lead to a complete suppression of the hepatic glucose production. Under these conditions, the amount of glucose infused corresponds to the glucose uptake into the insulin-sensitive tissues and reflects their sensitivity for exogenously administered insulin. Since in that case 85 to 90% of the glucose is taken up by the muscle tissue. This method mainly measures the insulin sensitivity of the muscle cells. The insulin sensitivity is subject to great intra- and interindividual variations depending on physical activity, nutrition, age and a series of other factors. The refinement of the glucose clamp technique we pursued allows a valid quantitative measurement of the pharmacodynamic properties of subcutaneously-injected insulin preparations. The serum insulin concentrations were raised to comparable values by a constant low dose intravenous insulin infusion (0.15 mU/min per kg body weight) in the different subjects and on the different study days. Thus, a comparable suppression of the endogenous insulin production was achieved without provoking a peripheral hyperinsulinaemia. The serum C-peptide concentrations fell by 20 to 30% prior to the subcutaneous insulin injection. After administering the subcutaneous insulin, the values fell by an additional 10 to 20% and remained constant at these low levels during the experiments or increased slowly towards the end of the experiments. Therefore, it can been assumed that the changes observed in the serum insulin concentrations measured
Principle of the euglycaemic glucose clamp technique. After subcutaneous insulin injection, the blood glucose concentration decreases. After some time, the metabolic activity of the administered insulin decreases again and the blood glucose level rises again. When the blood glucose level falls below the threshold at which a hormone secretion causing the blood glucose level to rise again is elicited (counterregulation), the blood glucose level increases more rapidly than one would expect from the time-action profile of the insulin preparation (dashed line). When a decrease of the blood glucose level is prevented by an intravenous glucose infusion, i.e., the blood glucose level is kept almost constant at baseline or a given target level (pearled line), the amount of glucose infused reflects the metabolic activity of the injected insulin at a given point of time, and the plotting of the glucose requirement over time reflects the time-action profile of the insulin preparation.
after insulin injection were predominately due to the absorption of the exogenously applied insulin. The registered GIRs were those required after the subcutaneous insulin injection over time, to compensate for the blood glucose-lowering effect of the applied insulin preparation. Depending on the pharmacokinetic properties of these insulin preparations, the glucose requirement changed with a variable delay and to a variable extent. The GIR thus constitutes the quantitative parameter which reflects the metabolic activity of these insulin preparations over time.

The graphical representation of the registered GIRs describes the time-action profile of these insulin preparations. The insulin concentrations were determined from additional blood samples obtained during the experiments, and the pharmacokinetic properties of the subcutaneously-injected insulin preparations were determined from the shapes of the insulin concentration-time curves.

This direct method is currently the most suitable in investigating the pharmacokinetic and pharmacodynamic properties of insulin preparations.
3 Practical implementation of the glucose clamp technique

Glucose clamp experiments can be performed manually or using a Biostator (Fig. 3.1). The Biostator continuously measures the blood glucose concentration and calculates the infusion rate of an intravenous glucose infusion by means of a direct negative feedback algorithm based on the deviations of the measuring results from a given target value, so that the blood glucose concentration is constantly kept as close as possible to the target value. With the manual clamp technique blood glucose is measured at regular intervals (this can vary between 3 to 15 min depending on the individual study), and then the GIR is modified. The Biostator, on the other hand, adapts to the GIR every minute. The Biostator is also referred to as the ‘Glucose Controlled Insulin Infusion System’ (GCIIS).

This capability of the Biostator to infuse insulin, however, is not generally used, since the insulin infusion is not sufficiently accurate, and the high temperatures within the infusion pump may degrade the insulin. Accordingly, a more correct name for the Biostator in glucose clamp experiments would be ‘Glucose Controlled Dextrose Infusion System’ (GCDIS).

In our glucose clamp studies, the subjects were connected to a Biostator and an euglycaemic glucose clamp was established. To achieve this, an intravenous needle was inserted retrogradely into a larger vein of the left hand, and a double-lumen catheter was introduced into the needle in order to enable a continuous withdrawal of blood. One of the two tubes of the double-lumen catheter was used to pump a heparin-saline solution (50 IU heparin per ml of an isotonic NaCl solution) into the intravenous needle. The other tube was used to continuously drain the same amount of venous blood, mixed with the heparin solution. The mixture with heparin prevented blood coagulation and subsequent obstructions in the blood-draining tube. An arterialisation of the blood withdrawn via the double-lumen catheter was achieved by leaving the subject’s left hand in a so-called ‘hot-box’ during the entire duration of the experiment. A thermostat switch ensured an air temperature of 55°C in a wood case by activating and inactivating a 100 W light bulb. The warming of the hand caused the arteriovenous anastomoses to open, with the consequence that a part of the arterial blood flew directly into the venous system. These efforts in taking blood samples for continuous blood glucose measuring were undertaken because—depending on the prevailing insulin concentrations and the degree of activity of the musculature—the glucose concentration in the venous blood is lower than in the arterial blood.

The arterial blood glucose concentrations mirror the processes in the central compartment, which reflect the degree of glucose utilisation of all tissues or the glucose supply by the hepatic glucose production or by glucose absorption in the splanchnic bed without being influenced by local conditions. The blood glucose concentrations in the arterialised venous blood are comparable with those of the arterial blood.

The drained blood was pumped to the glucose sensor of the Biostator after dilution with a buffer (1 part blood/10 parts buffer, v/v). The sensor contains a removable membrane in which enzyme glucose oxidase is fixed. The glucose molecules contained in the blood-
Figure 3.1
Euglycaemic glucose clamp technique using a Biostator. The subject’s hand was left in a wooden case with an air temperature of 55°C. Blood was continuously pumped from the hand vein to the Biostator. The blood was made unclottable by mixing it with a heparin solution. The intravenous glucose infusion given into the forearm of the contra-lateral arm was continuously adjusted to the current blood glucose concentration. Saline solution was continuously infused with a low flow rate in order to keep the venous access patent. A constant low intravenous insulin infusion was administered into the vein of the same arm to establish comparable baseline insulin concentrations and to (partly) suppress the endogenous insulin production. Blood samples for blood glucose determinations and subsequent hormone measurements were taken from a cubital vein of the left arm at regular intervals. The results printed by the Biostator were, at the same time, stored in a computer for subsequent data analysis.
buffer mixture diffused into the membrane where they were oxidised. In this reaction $\text{H}_2\text{O}_2$ was formed, which was oxidised by a voltage of 700 mV applied to a platinum and a silver electrode of the sensor. The electrons released during that process led to a very low current (<40 nA) between the two polarised electrodes. The flow was directly proportional to the glucose concentration in the blood and was converted into a blood glucose concentration according to the results of a previous calibration. The continuously measured blood glucose concentration was indicated on a small display and internally transmitted to the computer incorporated in the Biostator. The computer calculated the glucose infusion rates required for maintaining the blood glucose concentrations and automatically actuated the roller infusion pump of the Biostator to infuse the appropriate amount of glucose. The glucose infusion was administered via an intravenous catheter (Braunüle®, V-Viggo, 1.2 mm diameter, Braun-Melsungen, Melsungen, Germany) placed into a vein of the right forearm. In some situations, depending on the induced glucose requirement, the maximum GIR of the Biostator was not sufficient to maintain the blood glucose concentration at the target value. Then an additional glucose infusion by means of an external pump (Infusomat, Braun-Melsungen, Melsungen, Germany) was started with a constant infusion rate via the same intravenous catheter. The time of the beginning of the additional glucose infusion and the infusion rate were recorded in a test protocol in order to take the amount of glucose administered into account for data analysis. Usually the infusion rate of this glucose infusion was modified in longer time intervals only. Blood samples for hormone and plasma glucose measurements were taken via an intravenous catheter (Braunüle®, V-Viggo, 1.4 mm diameter) from the cubital vein of the left arm at regular intervals. The needle was kept patent by an instillation of isotonic NaCl solution. The total blood loss by blood withdrawal did not exceed 500 ml in any of the studies. Due to the blood loss, the experiments performed in the same subject were separated by at least five days.

The blood glucose measurements by the Biostator were verified at regular intervals (30 min max.) by additional measurements of the plasma glucose concentration in venous blood samples using a different laboratory method (Beckman Glucose Analyser, Beckman, Munich, Germany). The measurements of the Biostator were adjusted when necessary. The quality of the blood glucose measurement of the Biostator could not be held stable, due to the deposition of blood constituents at the membrane surface of the sensor and due to a change in the enzyme activity immobilised in the membrane. In a detailed test protocol the following items were recorded:

- information concerning the experimental procedure and the participants,
- the setting of the perfusor for the baseline intravenous insulin infusion,
- the exact times when blood samples were taken,
- the results of the glucose measurements with the laboratory device and the corresponding results measured by the Biostator,
- the resulting adjustments of the settings of the Biostator,
The investigations were performed using the glucose clamp algorithm (Mode 9). This was developed especially for the Biostator. The algorithm calculates the necessary GIR from the current blood glucose concentration as well as from the variation in blood glucose during the preceding minutes. This GIR attempts to maintain the blood glucose concentration as close as possible to the defined target value. Since the Biostator does not offer any possibility to store and analyse data, the data required for analysis were transmitted to a computer and stored using a computer program especially developed for this purpose.45

The participants were fasting when they came to the clinic at 7 a.m. to 8 a.m. They had taken their last meal at least 12 h before the start of the experiment. The participants were lying in bed throughout the experiment and received mineral water ad libitum but remained otherwise fasting. After a baseline phase of 2 h during which the baseline glucose requirement was determined, the experimental intervention (insulin injection) takes place. The injections were performed using insulin syringes with a small dead volume (Micro-Fine IV or IV+, Becton-Dickinson, Heidelberg, Germany) with a volume of 0.3 or 1.0 ml for U-40 or U-100 insulin. To ensure that the injection was given subcutaneously, the insulin was injected into a fold of skin lifted from the abdomen 5 cm to the left of the umbilicus in all experiments. Care was taken to keep the modalities of the injection technique constant (injection angle, depth of injection and velocity of injection). As in the baseline period, the GIR required to keep the blood glucose concentration constant was recorded until the end of the experiments 6 to 24 h thereafter.

All experiments used human insulin preparations or insulin preparations modified by genetic engineering. Long-acting insulin preparations are suspensions. After removal from the refrigerator a good mixture of the precipitate with the clear supernatant was achieved by rolling the insulin vials between the hands several times before drawing up the required dose. More details of the experimental procedure are described elsewhere.44

The following description of our own investigations only contains a short outline of any deviations from the general procedure described above as well as information on the number, age and body mass index of the subjects or patients with diabetes mellitus. At the time of investigation, the patients were free from acute or chronic diseases and none of them received any pharmacotherapy. Overweight subjects (body mass index >26 kg/m²) were not examined, since such individuals often are insulin resistant. With the help of rules, the subjects had to follow prior to the study days, it was attempted to achieve a comparable insulin sensitivity, even intraindividually, at the various study days. The subjects were told to avoid unusual physical exercise and excessive alcohol consumption the day before the experiments. In the morning of the study day, the patients were instructed not to use the bicycle to get to the clinic, not to smoke, and not to drink tea or coffee.

All participants and patients received a detailed explanation of the study protocol prior to the start of the experiments. They were informed about possible risks and signed a
consent form. All investigations were performed in compliance with the Declaration of Helsinki. The study protocols had been approved by the ethics committee of the Medical Faculty of the Heinrich-Heine University of Düsseldorf.

The results of the studies, as presented below, are reported as an arithmetic mean including a measure of dispersion to describe the variability of the measurements (standard deviation in the tables, standard error in the figures). In the figures, the error bars are indicated either in upper and lower direction (±1 standard error) or in just one of the directions, to aid transparency.

3.1 Critical discussion of the glucose clamp technique

In our investigations, the pharmacokinetic and pharmacodynamic properties of various insulin preparations were determined. The method used was criticised with the argument that the endogenous glucose production was only partly suppressed and that the extent of suppression changed as a function of the prevailing insulin concentrations.46,47

It has been said that the recording of the GIRs required to maintain euglycaemia only presents a gross evaluation of the glucose metabolism but no quantitative determination. This criticism is based on the fact that the hepatic glucose production is suppressed to a certain extent depending on the established blood insulin level.48 The basal hepatic glucose production amounts to 2.0 to 2.5 mg/kg/min in fasting adults.48,49

In our own experiments, a GIR of 1.0 to 2.5 mg/kg/min was required during the baseline period (with serum insulin concentrations of 10 to 15 µU/ml) to keep the blood glucose concentration constant. Assuming that the exogenous glucose infusion replaces the endogenous glucose production, it can be concluded that the hepatic glucose production was almost completely suppressed during this phase of the experiment. This corresponds to reports in the literature, according to which serum insulin concentrations of as low as 10 to 15 µU/ml can be expected to cause a suppression of the hepatic glucose production by more than 30% (50% suppression at approx. 30 µU/ml).37,48,49

In our experiments, after subcutaneous injections of a short-acting insulin preparation, the serum insulin concentrations increased to 40 to 60 µU/ml. These concentrations led to an accordingly stronger, almost complete suppression of the hepatic glucose production. This suppression eventually ceased once the serum insulin levels returned to baseline values. The variable suppression of the hepatic glucose production during the experiments is part of the processes that occur in patients with diabetes mellitus as a physiological response to the exogenous insulin administration. The registered GIR compensates for the difference of the peripheral glucose utilisation rate and the hepatic glucose production resulting from the prevailing blood insulin level at the specific times after subcutaneous insulin injection.

The glucose clamp technique used determines this very glucose requirement of the body. The objective of our methodological approach was to determine the time-action profile of subcutaneously injected insulin preparations. A complete suppression of the hepatic
glucose production—for example, by a higher baseline blood insulin level—is neither necessary nor desirable, the aim being a situation as physiological as possible. If required, information about the exact contribution of the hepatic glucose production and of the peripheral glucose metabolism to these time-action profiles can be obtained, for instance, by measurements with stable radioactive isotopes. In the healthy subjects, the experimental method used allowed us to prevent a stimulation of the endogenous insulin secretion and to suppress the hepatic glucose production by the infused insulin. Methodological alternatives to our experimental set-up—as used in other studies—are:

1. the injection of high insulin doses with up to 0.5 U insulin/kg body weight,
2. intravenous infusion of high amounts of insulin to establish a high baseline blood insulin level, and
3. maintenance of the blood glucose concentration at 3.5 mmol/L.

In the methodological alternatives (1) and (2), it is true that the endogenous insulin production and the hepatic glucose production are further suppressed. However, due to the resulting non-physiological hyperinsulinaemia, there may be a stimulation of the peripheral glucose utilisation (see below). In the methodological alternative (3), there may be a secretion of counterregulatory hormones if the blood glucose falls below a threshold, which is subject to interindividual variations, even with blood glucose concentrations around 3.5 mmol/L. The secretion of cortisol and adrenaline leads to a rapid and considerable worsening of insulin sensitivity. Therefore, these methodological alternatives cannot be used for pharmacological investigations of insulin preparations under conditions reflecting reality as far as possible. In the case of longer-lasting glucose clamp experiments (>5 h) with constantly increased blood insulin levels, the peripheral glucose utilisation has been reported to increase, due to an alteration of the insulin sensitivity during the experiments. During these experiments non-physiologically constant plasma insulin concentrations of 100 µU/ml were established, which were two to 10 times higher than those achieved temporarily in our investigations. A complete suppression of the endogenous insulin production can also be achieved by a continuous infusion of somatostatin. However, this method does not only result in a total suppression of beta-cell activity, but also in a simultaneous suppression of alpha-cell activity. Consequently, in order to restore a normal hormone situation, glucagon—and other hormones—must be replaced. Moreover, somatostatin provokes a suppression of the perfusion of the splanchnic bed, slowing down both the emptying of the stomach and absorption from the intestine. For pharmacological investigations of insulin, it is important that somatostatin reduces the insulin clearance by 20%, the duration of insulin action thus being prolonged artificially.

An increase of the intravascular insulin concentration elicited by meals or by exogenous administration leads to a decrease of the potassium concentration in the extracellular space. This reduction is caused by a transport of potassium ions from the extracellular space.
into the intracellular space, which is triggered at the muscle tissue (and probably in the liver, too) by an insulin-induced stimulation of Na/K-ATPase. Hypokalaemia or a potassium depletion, in turn, constitutes a stimulus for the renin-angiotensin system. Following subcutaneous injection of insulin during an euglycaemic glucose clamp experiment, rising insulin concentrations may activate the renin-angiotensin system due to a decrease in potassium concentration in the extracellular space. An increase in the systemic angiotensin II concentration would, in turn, result in a redistribution of the cardiac output from insulin-insensitive tissues to insulin-sensitive musculature. An increased recruitment of muscle capillaries, mediated by angiotensin II, could lead to an increase of perfusion and thus to an increase of an insulin-dependent glucose utilisation during a glucose clamp. This hypothesis was verified in a study with 20 healthy subjects. In euglycaemic two-step glucose clamp experiments (intravenous infusion of 0.25 and 1.0 mU insulin/kg/min) either a KCl solution or a physiologic saline solution was infused in randomised order. On one study day, the potassium infusion was adjusted so as to keep the plasma potassium concentration constantly at baseline. On the other study day, however, a decrease of the plasma potassium by 0.35±0.05 mmol/L was observed after 120 min. There were, however, no differences in the GIRs on the two study days, i.e., under these study conditions the decrease of the potassium concentration in the extracellular space did not result in differences in glucose utilisation.

3.2 Proposal for a standardised glucose clamp technique

The use of a standardised glucose clamp technique to conduct studies aiming to estimate the pharmacodynamic properties of insulin preparations, should lead to a better comparison of the results than in the past. The summary measures obtained by using a uniform method can help insulin-producing companies to generate a comparable presentation form to describe their insulin preparations. This would enable physicians and their patients with diabetes mellitus to compare the properties of different insulin preparations. The following procedure is proposed for the implementation of a standardised glucose clamp technique:

3.2.1 Study design

If possible, all studies should use a double-blind design in order to facilitate the evaluation and comparison of study results. In all studies with novel insulin formulations or insulin application techniques, conventional insulin preparations or application techniques should also be studied for comparison. The double-blind design is also required to exclude the problem of an arbitrary or unconscious influencing of the study results.

3.2.2 Implementation of glucose clamp experiments

Glucose clamp experiments can be performed manually or in an automated manner using a Biostator. When using the manual glucose clamp technique, the glucose
concentration is determined from blood samples taken at regular intervals, and the GIR is varied according to the results of the measurements. This technique provides similar results to the automated method, as long as there are no rapid changes in the glucose requirement.\textsuperscript{58} If such rapid changes occur, they might not be recognised in time, because of the length of the interval between the blood glucose measurements during the experiment. The sampling frequency required to adequately recognise and describe the signal has to be high enough in order that the rapid changes of the glucose requirements after injection of rapid-acting insulin analogues\textsuperscript{58} is detected, thereby ensuring that the study results have not been influenced by the investigator.

For these reasons, comparative studies of insulin preparations are better performed using an automated glucose clamp technique.

### 3.2.3 Subjects

It is advisable to examine healthy subjects of normal weight in the range of 18-45 years with a body mass index of <27 kg/m\(^2\). All subjects must initially undergo a thorough medical examination, including an ECG, not only to ensure the subject’s safety in view of the stress related with the investigations, but also to exclude impacts on the insulin sensitivity by other diseases (such as by thyroid disease or infections) as far as possible. Especially clinical chemistry parameters, serving to determine liver and kidney function, should lie within the reference range, since a physiological function of these organs is crucial for insulin degradation and elimination. The aim of these inclusion and exclusion criteria is to obtain a relatively homogeneous group of participants.

### 3.2.4 Gender of the subjects

Until now, women have not normally participated in glucose clamp studies. Nevertheless, this would be reasonable in order to evaluate whether the substances under study act comparably in both sexes. There are several reasons why this did not happen in the past. When new drugs (e.g., insulin analogues) are studied, there are often no data available regarding the possible harmful effects on the unborn during the phase of the implementation of early studies (phase I studies). Should, however, women participate in such studies, an adequate contraception has to be ensured.

It still remains uncertain whether a woman’s insulin sensitivity varies to such an extent that relevant changes of the experimental results may occur, depending on the point of time in her menstrual cycle. The results of the studies performed in order to elucidate this question are contradictory\textsuperscript{59-66} If the menstrual cycle had a relevant influence on insulin sensitivity, the investigations should ideally always be performed at the same time of the cycle. Since there always has to be at least some days between the experiments, the most unfavourable consequence would be that only one experiment per cycle could be performed. Should several experiments be performed, this would mean a long study
duration. During this time, other effects influencing insulin sensitivity may occur such as differences in physical activity, depending on the season. In spite of these problems, women should participate as subjects in future studies, so that relevant information can be obtained, as early as possible, about the properties of the substance under investigation for both genders.

3.2.5 Insulin dose

The insulin doses studied should not considerably exceed the therapeutically reasonable range which is commonly used in clinical routine, i.e., >0.2 U/kg body weight for short-acting insulin preparations or >0.3 U/kg for long-acting insulin preparations. Depending on the metabolic effect of each insulin preparation, equimolar dosages may not necessarily yield comparable effects. Thus, the determination of an equipotent dose for insulin analogues or other routes of insulin administration is difficult and can only be approximated, since the preliminary data from animal experiments or small pilot studies provide only limited information for humans. When high insulin doses are applied, high amounts of glucose (amounts of liquid) have to be infused in order to keep the blood glucose concentration constant, with the consequence that the technical feasibility of the studies will be aggravated by frequent interruptions because the subjects have to void urine or go to the lavatory. This can adversely affect the results of the glucose clamp experiments.

3.2.6 Site and mode of injection

To delay insulin absorption, long-acting insulin preparations are frequently injected into the thigh. Experimental results obtained with short-acting and long-acting insulin preparations are only comparable when the same injection site is always used, because otherwise the lag effect associated with the injection site, which is difficult to estimate, has to be taken into account. In the case of abdominal subcutaneous injection, the injection should be given into a lifted fold of skin 5 to 10 cm to the left or right of the umbilicus in the direction of the iliac crest. All injections should preferably be performed by an experienced investigator and always with the same injection technique in order to ensure a reproducible subcutaneous injection.

3.2.7 Endogenous insulin production

During the experiments the endogenous insulin production should remain partly suppressed, otherwise it cannot be excluded that the registered glucose requirement is not only induced by the subcutaneously applied insulin but also, at least partly, by endogenously secreted insulin. The endogenous insulin secretion may be stimulated by the intravenous glucose infusion. Suppression of the endogenous insulin secretion can be determined by measuring the serum C-peptide concentration. It can also be shown that the insulin secretion remained suppressed during the experiments.
3.2.8 Blood glucose target value
The target value of blood glucose should be identical in all experiments, since the glucose requirement is influenced by that target value. Therefore, the individual basal blood glucose should not be used as a target value. In order to get a uniform (and thereby comparable) blood glucose level this was kept constant at 5.0 mmol/L during the experiments in most of our own studies.

3.2.9 Quality of the glucose clamp implementation
There are no definite criteria, which, in the manner a ‘quality control’, would allow to decide whether a glucose clamp was successful or not. If there are prolonged technical problems during the course of an experiment (loss of results of measurements >10% of the duration of the experiment) associated with a major divergence of the current blood glucose level from the target value, the experiment should be interrupted and repeated at a later time point. If the blood glucose falls to hypoglycaemic values (<3.0 mmol/L), the experiment should also be discontinued and repeated as well since, in that case, the insulin sensitivity cannot be expected to be comparable on different study days. One measure to describe the quality of glucose clamp experiments is to state the coefficient of variation (CV=(standard deviation/\bar{X})*100) of the registered blood glucose concentrations. The CV provides information about the variability of a measurement, irrespective of the order of magnitude of the values. Usually, a CV of the blood glucose concentration of <5% is considered an adequate criterion for a glucose clamp of sufficient quality. However, this interpretation ignores the fact that it directly depends on the time-action profile of the insulin preparation under study in how far the glucose concentration can be kept constant. If drastic changes in the glucose requirement occur, it is more difficult to keep the blood glucose concentration constant. Another possibility to describe the uniformity of the blood glucose level over time (this dimension is missing in the CV and in frequency diagrams) is the CUSUM representation.67

3.3 Characterisation of time-action profiles

3.3.1 Variability of the recorded glucose infusion rates
The adaptation of the GIR to the current glucose requirement is based on the blood glucose concentration measured either every minute (Biostator), or at varying intervals (manual clamp). Due to the regulation times in the closed loop, a change of the GIR does not immediately induce a change in the blood glucose concentration. Depending on the regulation characteristics of each system, the blood glucose concentrations during a clamp do not usually match the target value exactly. In response to that, variations of the GIR occur. These variations (noise) interfere with the analysis of the GIR profile (signal). Depending on the amount of noise, a determination of summary measures (see below) from the original data may be difficult or cannot be performed reasonably, due to the correlated confounding
factors. Thus, a single massive variation in the GIR profile might be interpreted as maximal metabolic activity, although this does not correspond to the global shape of the curve.

The reasons for such large variations in the GIRs, which may be randomly distributed over the duration of the experiment, can be of a technical nature, such as malfunctions in the continuous blood glucose measurement.

A rapid adaptation of the GIR to a changing glucose requirement (with an imminent decrease of blood glucose) presupposes that the regulation algorithm even responds to a rather small decrease of the blood glucose level by an increase of the GIR. The extent of the delay occurring with the glucose clamp algorithm used by the Biostator is approximately 5-10 min, although the feedback algorithm takes the velocity of the change in the blood glucose concentration into account. The slowness of this control impedes a timely adaptation of the GIR in the case of rapid increases in the glucose requirement, leading to a short-term decline of the current glucose concentration below the target value. Thus, in our experiment, a few minutes after the injection of rapid-acting insulin analogues, a deviation by <0.3 mmol/L from the target value of 5.0 mmol/L was observed over a period of about 10 min. In the development of the successor model of the Biostator, attempts are being made to shorten the feedback time by locating the site of measurement directly at the sampling site.

The noise of a registered signal can be reduced by various measures. With the available smoothing techniques it must be taken into account that they may lead to a distortion of the current signal. The use of moving averages introduces a shift of the time-action profile to the right on the time x-axis, since increasing GIR values provoke an increase of the mean value only after several arithmetic steps. By fitting a mathematical function to the registered original signal (see below), the noise in the GIR profiles can be suppressed if all available data are taken into account. The variations in the GIR profiles induced by short-lived artefacts are not interpreted as measurement signals when fitting a function to the total time-action profile. In our own investigations each individual GIR profile including the fitted function underwent a visual inspection to check the quality of the fitting.

3.3.2 Fitting of a function to the time-action profiles
The recorded GIRs describe the metabolic activity of the administered insulin preparation (time-action profiles). To characterise unimodal time-action profiles, as they are typically obtained with insulin preparations, the following measures (‘summary measures’) appear to be the most relevant, since they characterise the time-action profiles which are most important for insulin therapy in clinical practice: onset of action, maximal action, time of maximal action and duration of action (Fig. 3.2). Depending on the shape of the registered profiles, various summary measures may be useful.

The use of these summary measures has been recommended in the literature for the description of results from serial measurements in order to adequately describe the most important properties of such curves and to be able to apply suitable methods of
The purpose of the determination of summary measures is to describe comprehensible and interpretable characteristics of the time-action profiles using as few parameters as possible, these parameters having been determined for each individual participant or for each individual experiment. The raw data themselves (in this case the originally registered time-action profiles) do not undergo statistical analysis, but in this two-stage approach the summary measures are determined during the first step and then analysed by appropriate statistical procedures in the second step (see opposite and overleaf). The advantage of such summary measures is that the big number of measuring results is reduced considerably, without losing important information which is collected in extensive studies.

The summary measures can be determined either directly from the registered time-action profiles or after application of adequate smoothing methods to such profiles. One possibility is to fit a polynomial function of a sufficiently high order to the data. This can easily be done by means of standard table calculation programs. Another possibility is the fitting of appropriate non-linear models to the time-action profiles. By means of a non-linear regression model, the

Figure 3.2
An individual time-action profile with certain summary measures: GIR$_{\text{max}}$ represents the maximal GIR required to maintain the blood glucose concentration at the desired target value, considering the variations of the original values during the experiments. The time point after subcutaneous injection at which GIR$_{\text{max}}$ was achieved is described by $t_{\text{max}}$. The time points at which 50% of the GIR$_{\text{max}}$ occur before ($t_{\text{early 50%}}$) and after ($t_{\text{late 50%}}$) the GIR$_{\text{max}}$ determine the rate of increase/decrease of action. The area under the time-action profile (AUC) can be calculated either for the entire time-action profile, i.e., until the latter returns to the zero line (AUC$_{0-\infty}$), or just for the period of the experiment or for shorter intervals (AUC$_{0-T}$).
following lognormal function with overlaid autocorrelated confounding factors can be fitted to
the registered time-action profiles: 
\[ f(t) = a t^{-1} e^{-b [\log(t) - c]^2} \quad (t>0, \ a,b,c >0) \]

The time-action profiles registered during glucose clamp experiments must have the following
characteristics to allow the successful fitting of the proposed function: a relatively rapid
increase followed by a slower decrease after the single peak with many measurement points.
The signal can be overlaid by correlated confounding factors. Usually the mean baseline GIR
is subtracted from all GIR values registered after the experimental intervention before the
function is fitted. In order to fit the function, all available GIR values are used, i.e., up to 1,440
values for experiments lasting 24 h. The fitting of the function is performed using the PROC
MODEL, which is a special program of the SAS statistics program package.
The advantage of such a fitting of a function is that the entire relevant information on the
time-action profile is summarised in the three function parameters a, b and c. Although
this lognormal function has only three parameters, it can be adapted to a great number
of unimodal curves of various shapes.
This function is much more flexible than, for example, the Bateman function. It allows a point
of inflection before the peak, which is of major importance in the time-action profiles of insulin
preparations in order to obtain a good fitting of the function to the original GIR profile.
Another advantage of this analytical approach is that the underlying model considers the serial
correlation of the data values, which can be put down to the feedback algorithm used by the
Biostator for glucose clamp experiments, by applying an autoregressive error term.
The summary measures used to describe the time-action profiles (Fig. 3.2) can simply and also
definitely be calculated by means of the three parameters a, b and c of the regression function:

\[
GIR_{\text{max}} = a \exp \left( \frac{1}{4b} - c \right)
\]

\[
t_{\text{max}} = \exp \left( c - \frac{1}{2b} \right)
\]

\[
t_{\text{early 50%}} = \exp \left( c - \frac{1}{2b} - \frac{\log(2)}{b} \right)
\]

\[
t_{\text{late 50%}} = \exp \left( c - \frac{1}{2b} + \frac{\log(2)}{b} \right)
\]
A (complex) statistical approach to the analysis of original time-action profiles (without fitting of a function), known as the analysis of variance (ANOVA) for repeated measurements, is quite frequently used when measurements of the same parameters are to be performed in the same subject over a certain period of time. This method, however, cannot be reasonably used if:

- the number of measurement times is high;
- the number of subjects is comparably small; and
- several ‘within-subject’ factors are present.

These conditions were almost always given in our own studies with multiple administration of different insulin preparations or dosages on different study days and with long durations of experiments with many measurement values.

In the ANOVA analysis for repeated measurements (MANOVA with $\Phi$ adjustment of the degrees of freedom) the number of degrees of freedom is insufficient under these conditions, i.e., no meaningful statistical analysis can be performed. Another inconvenience of this approach is that an interpretation of the results is difficult and that medical questions cannot be answered in a satisfactory manner. These methods were originally developed for the analysis of studies using only 3 to 20 different measurement times—not for several hundred measurements during one experiment like with time-action profiles. Since the ANOVA approaches were not usable for the analysis of time-action profiles (long time series), the parameters of the fitted function and/or the summary measures were used for statistical analysis.

The questions (study hypotheses) underlying our own studies were analysed with the help of the determined summary measures using simpler methods of ANOVA (such as the two-factorial ANOVA for a randomised block design). If the F-values obtained showed significant differences between the insulin preparations (treatment block), a test for multiple comparisons was used for the further analysis of differences between the individual insulin preparations. Moreover, it was analysed whether significant differences between the subjects (subject block) were present for the various parameters under analysis. In the statistical analysis a difference was considered significant if the $P$ value in a two-sided test was $<0.05$. 

\[
\text{AUC}_{0-\infty} = a \left( \frac{\pi}{\sqrt{b}} \right)
\]

\[
\text{AUC}_{0-T} = a \left( \frac{\pi}{\sqrt{b}} \Phi(\sqrt{2b(\log(T) - c)}) \right)
\]
3.3.4 Accuracy in the determination of summary measures

The fitting of functions with subsequent determination of summary measures and statistical analysis is only adequate when the functions describe the shape of the original curves so well that the summary measures can be determined with sufficient precision. The advantage of the fitting of functions on a non-linear model compared with other procedures used to reduce signal variations, is that the precision with which the measures are determined can be estimated based from the model used. Since the measures represent non-linear functions of lognormal parameters, the calculation of confidence intervals is complex, but they serve as a good control for the reliability of the individual measures. The calculation of confidence intervals indicates the precision with which the measures can be reported for individual profiles in statistical analyses. The combination of confounding factors caused by the feedback algorithm of the Biostator and other disturbances which may occur, as well as in particular by the variability of insulin effects, reduces the precision in determining the summary measures.

3.3.5 Group average and treatment average

The results of a study are a series of individual time-action profiles with fitted functions. Usually the arithmetic mean of the time-action profiles is then calculated for one insulin preparation and plotted, including a measure of dispersion, usually the standard error, in the figures. Now, the question is in how far the resulting mean curve describes the typical shape of the individual time-action profiles. The arithmetic mean is calculated from the GIR values, recorded at a specific point of time after insulin injection. The mean values of the various points of time, the so-called ‘treatment mean values’, are plotted against the time and describe the time-action profile of that insulin preparation. However, the resulting curve shape does not necessarily reflect the typical shape of the individual curves. As shown in Fig. 3.3a with hypothetical data, the mean value curve resulting from the four individual curves (reaching their peaks at different times) is flatter and broader than the individual curves. If a function is fitted to the individual curves and the summary measures of this function are averaged, the shape represented in Fig. 3.3b is obtained. This curve describes the basic shape of the individual curves better than the arithmetic mean curve. An example of this is provided in Fig. 3.4.

The studies presented here were evaluated by fitting a non-linear model to the individual time-action profiles and then calculating the summary measures. Their arithmetic mean, a ‘group mean’, is indicated in the text and in the tables including a measure of dispersion (standard deviation) to describe the results obtained with each insulin preparation obtained in the individual experiments. Numerically, this group mean value is not identical to the treatment mean value, as read from the arithmetic mean value curve. Since the individual summary measures are obtained at various times, a measure of dispersion can be given for the group mean value (e.g., for $t_{\text{max}}$), whereas this is not possible for the corresponding treatment mean value.
3.3.6 Problems with the time-action profiles of long-acting insulin preparations

When recording the time-action profile of long-acting insulin preparations it is difficult to assess, in the case of slowly declining insulin action, whether the glucose requirement registered at a specific point of time must still be put down to the effect of the s.c. applied insulin or to the baseline intravenous insulin infusion. Due to the variations of the GIR, it is often difficult to fix a definite point of time for the end of the insulin action without fitting a function. Therefore, information about the duration of action of long-acting insulin preparations are obtained with poor precision. In contrast,
the time at which the insulin action has fallen to 50% (or 25%) of the maximal action can be determined with a rather high precision; this time point is described by the $t_{\text{late 50\%}}$ parameter.

![Time-action profile of the rapid-acting insulin analogue insulin lispro as obtained from arithmetic averaging of 30 individual time-action profiles (line with error bars ($\pm$1 standard error)) and the curve resulting as a mean from the functions fitted to the individual curves (solid line).](image)

**Figure 3.4**

*Time-action profile of the rapid-acting insulin analogue insulin lispro as obtained from arithmetic averaging of 30 individual time-action profiles (line with error bars ($\pm$1 standard error)) and the curve resulting as a mean from the functions fitted to the individual curves (solid line).*\(^{73}\)

The assumption that in glucose clamp studies the GIR returns to baseline values when the increase of the blood insulin level following the administration of insulin preparations returns to baseline values, is based on the hypothesis that the baseline glucose requirement remains constant over a period of time. When recording the time-action profiles of long-acting insulin preparations in our own studies, a trend towards a new increase of the GIR was observed at the end of the experiment after 24 h. In single cases, i.e., in the morning hours the glucose requirement was slightly above the baseline rate and was thus higher than in the preceding hours. One cause of this may be the change of the baseline glucose requirement over time.

It is known that, physiologically, fasting over a period of 36 h leads to an increase in insulin sensitivity. The identical baseline blood insulin level, as a consequence of the constant intravenous insulin infusion, may then lead to a higher metabolic activity. It is
difficult to assess if in glucose clamp studies with a duration of 24 h the metabolic situation (insulin sensitivity) is comparable at the start of the experiment (after 12 h without intake of solid food) and at the end of the experiment (after 36 h without intake of solid food).

During the experiments, however, the participants were not fasting in the true meaning of the word, because they were receiving intravenous infusions of up to a few hundred grams of glucose per experiment, depending on the insulin preparation and dose under investigation. During the experiments the blood glucose concentration did not fall as it would normally happen during such a duration of fasting. With respect to carbohydrates, the subjects were not in a state of fasting. However, with respect to proteins and fats, which would otherwise have been taken in with food, they were. Since the glucose clamp experiments were conducted over a period of 24 h from one morning to the next morning, it was necessary to take into account the influence endogenous chronobiological cycles have on insulin sensitivity. Insulin sensitivity is known to be reduced in the early morning hours, especially due to an increased secretion of growth hormone (‘dawn phenomenon’). With a given blood insulin level, this leads to a lower glucose consumption in the early morning hours. Since in most investigations with long-acting insulin preparations a return to baseline GIRs was recorded, insulin sensitivity did not seem to change much during the experiments. Other authors did not report an increase in glucose consumption at the end of their experiments conducted over a period of 40 h either.53,74

The implementation of a control experiment, in which no insulin is injected subcutaneously, did not provoke a comparable metabolic situation during the glucose clamp experiments, since under this condition much less glucose is infused. It is not possible to accurately define a constant metabolic situation from a constant GIR (baseline rate), as observed in our own study with a mere baseline intravenous insulin infusion over 19 h.53,74

The fitting of a non-linear model to the time-action profiles obtained with long-acting insulin preparations is often difficult because such profiles do not show a unimodal shape, which is the prerequisite for a successful fitting (see above). In the description of the time-action profile of long-acting insulin preparations and long-acting insulin analogues there is the problem that it is difficult and doesn’t make sense to indicate the time and the degree of the maximal action in a more or less flat time-action profile. With long-acting insulin analogues, which exhibit an almost constant action over time (rectangular profile), information about the maximal action does not make sense at all.

In the face of the problems described for the investigation of long-acting insulin preparations, coming along with the considerable variability of the insulin action of long-acting insulin preparations, the detection of statistically significant differences in the duration of action or of other summary measures between various long-acting insulin preparations would require an unrealistically large sample size of participants.
3.3.7 Areas under the curves
The area under the curves (AUC) of concentration-time profiles (pharmacokinetic summary measures) or time-action profiles (pharmacodynamic summary measures) can be calculated for the entire area below the profiles from 0 to infinity (AUC$_{0-\infty}$), or for a certain interval $[0,T]$, $T$ being any point of time, for example, the entire duration of the experiment or a specific period of time after the subcutaneous insulin injection. These partial AUCs$_{0-T}$ can be calculated by integrating the fitted function (see pg. 26) or for the recorded original profiles using the trapezoidal rule. The calculation of the AUCs over various periods of time after insulin application can be useful to evaluate how rapid blood insulin levels and thereby glucose requirements change with different insulin preparations or application techniques.

Since the determination of the time of onset of insulin action is relatively arbitrary, the definition of thresholds may be useful. Waldhäusl suggested that the onset of action should be defined as the point of time at which—after subtraction of the mean baseline GIR rate—5% of the total AUC has been reached, and the end of action as the point of time at which 95% has been reached.$^{75}$ Another possibility would be to take a sum of the recorded GIRs over time. This method shows within which period of time after injection which amount of metabolic activity has been reached and when 100% of the activity has been achieved. The suggested limits also allow us to estimate and compare the duration of action of different insulin formulations.

The calculation of the AUC is an attempt to obtain a summary measure enabling a simple comparison between various concentration-time profiles or time-action profiles. Nevertheless, it has to be taken into account that identical AUCs may be obtained, although the shapes of the curves can differ considerably. On the other hand, differences in the baseline values may have such a great influence on the AUC that significant differences occur, although the shapes of the curves are identical.

3.3.8 Determination of pharmacokinetic summary measures
In our studies (most of them involving healthy subjects, see below) the serum insulin concentrations were determined using a radio immunoassay (Pharmacia Insulin RIA, Uppsala, Sweden). As patients with diabetes mellitus may have developed antibodies to insulin, which compete for binding sites with the antibodies of the radio immunoassays during measurement, the blood samples taken in the study performed in patients with type 1 diabetes underwent a polyethylene glycol extraction immediately after having been obtained (see pg. 122). This procedure enabled the antibodies to precipitate with bound insulin, and the detection was restricted to the free plasma insulin concentrations.$^{76}$

The following pharmacokinetic summary measures were determined from the concentration-time profiles after subtraction of the baseline rate either directly or after fitting of a function: basal concentrations ($C_{basal}$), maximal concentration ($C_{max}$), time at
which the maximal concentration was achieved ($t_{\text{max}}$), as well as time at which the early
and late half of the maximal concentration were obtained ($t_{\text{early } 50\%}$ and $t_{\text{late } 50\%}$). The
AUC was calculated for various time intervals using the trapezoidal rule.
The serum C-peptide concentrations were also determined by radio immunoassays
(RIAgnost HC-Peptide; Behring-Werke, Marburg; Human C-Peptide RIA Kit, Linco
Research, St. Charles, Montana, USA). The serum C-peptide concentration profiles were
then checked for constantly suppressed values during the experiments. During the
experiments, there were no concentration increases which had to be interpreted as a
relevant stimulation of the endogenous insulin production. This allows us to state that the
changes observed in serum insulin concentrations and GIRs measured after insulin
application are predominately due to the action of the applied insulin.
4 Time-action profiles of insulin preparations

The change in the metabolic activity of subcutaneously injected insulin preparations over a period of time characterises the two types of insulin preparations required for the implementation of an intensive insulin therapy: short-acting and long-acting insulin preparations. In view of the differing reports in the literature concerning the time-action profiles of short-acting insulin, NPH insulin, and a 30/70 premixed formulation of both, we investigated the metabolic activities of these insulin preparations under uniform study conditions. So far, no such investigation of the time-action profiles of these most commonly-used insulin preparations has been performed in one single study. The results of our study were, on the one hand, presented as examples of the time-action profiles of these insulin preparations and, on the other hand, presented in a direct comparison (see pg. 47).

Method

Double blind study design; total of 36 males made up of three groups of 12; duplicate investigation of one of three insulin preparations; subjects comparable in terms of age, weight and height; short-acting insulin: age 26±2 years; body mass index 22.9±1.3 kg/m²; NPH insulin: 26±4 years; 23.3±1.4 kg/m²; premixed formulation: 26±2 years; 23.4±1.4 kg/m²; insulin antibodies not detectable in any of the subjects; dose of 0.3 U/kg body weight; baseline period 120 min; duration of study: 12 h for short-acting insulin and 24 h for NPH insulin and premixed insulin preparation.

4.1 Short-acting insulin preparations

A number of synonyms exists for those insulin preparations which are used for covering the prandial insulin requirements, regular insulin being the most common term. Here, the term ‘short-acting insulin’ is used to describe the pharmacodynamic properties of this insulin preparation. The first insulin preparation after the discovery of insulin in 1921 was a short-acting insulin. Due to the high degree of impurities contained in it, particularly proteins, these insulin preparations had a delayed action compared with modern, highly purified short-acting insulin preparations. Thanks to the purification techniques developed in the following years, insulin could be produced with such a purity that different pharmaceutical preparations (see pg. 36) became possible. Up to the seventies, the short-acting insulin preparations, which were still impure, had been adjusted to a pH value of 2.8 to 3.5, rendering them more stable.

Nowadays, only highly purified short-acting insulin preparations with a neutral pH are used. Thus, skin irritations observed with acidic and impure preparations can now be largely prevented, and a stable mixture with neutral long-acting insulin preparations is also possible (see pg. 42). The insulin formulation Actrapid®, marketed in 1961, was the first short-acting insulin with a neutral pH. Similar formulations of other manufacturers were marketed only later.
Stable neutral preparations of short-acting insulin must contain 0.4% zinc as an additive to allow the insulin molecules to associate into hexamers, corresponding approximately to two zinc atoms per insulin hexamer. Moreover, the addition of zinc increases the physical stability of the preparations.

The duration of action of short-acting insulin is reported to be 6 to 8 h in textbooks, with an onset of action 15 to 30 min after injection and a maximal activity after 90 to 150 min. A review of the published information on short-acting insulin revealed considerable differences depending on the investigational method, dose and injection sites used. The strong dose-dependency of the time-action profile of short-acting insulin (see pg. 85) seems to be the main reason for these differences.

The time-action profile of short-acting insulin, as determined in the 24 experiments with the relatively high dose of 23±2 U (0.3 U/kg) (Fig. 4.1, opposite; Tab. 4.2, pg. 48), shows an increase of the glucose requirement within 30 min after the subcutaneous injection. After the onset of action, it took more than 2 h until the GIR_{max} was recorded—the maximal action remaining almost constant over a period of approximately 2 h (120 to 240 min). Whereas the t_{early 50%} was reached 1 h after the subcutaneous injection, it took almost 6 h for the t_{late 50%} to be reached. Only after 10 h, the glucose requirement returned to baseline values. The serum insulin concentrations reached maximal values 2 h after the subcutaneous injection (Fig. 4.1; Tab. 4.2). After 8 to 10 h, the serum insulin concentrations had returned to baseline.

For this dose, the time-action profile of short-acting insulin is characterised by the following:

- the onset of action does not occur until 15 to 30 min after the subcutaneous injection,
- the maximal activity is achieved only after 2 to 3 h, and
- the action is maintained over a period of up to 10 h.

### 4.2 Long-acting insulin preparations

Intensive insulin therapy with its basal/bolus regimen requires an insulin preparation covering the basal insulin requirement between meals and at night. Ideally, this would be an insulin absorbed with a constant low rate. During continuous subcutaneous insulin infusion the substitution of the basal insulin requirement by an infusion of short-acting insulin into the subcutaneous tissue is almost optimal.

For more than one decade after its discovery, insulin was only available as a (relatively) short-acting preparation, which was injected each time before the main meals. Immediately after the discovery of insulin several attempts were made to develop insulin preparations with a delayed absorption and thus a longer lasting effect, however, these attempts were not successful, due to the impurity of the insulin preparations. A series of substances have since been tested for their suitability in the formulation of long-acting insulin preparations (e.g., gum arabic, lecithin, insulin-oil suspensions, not specified proteins, cholesterol, alum, tannin, adrenaline/vasopressin, globin). Some of these substances were investigated because the prolonged duration of action of impure short-
Glucose infusion rates (top) and serum insulin concentrations (bottom) measured after subcutaneous injection of a short-acting insulin preparation in a dose of 0.3 U/kg at the time t=0 in 12 subjects on two study days. The baseline glucose infusion rates and serum insulin concentrations (determined previously as mean value of the baseline phase) were subtracted from all values obtained after injection⁴. 

Figure 4.1
acting insulins was put down to the presence of other proteins. In the long-acting insulin preparations developed in the following (Tab. 4.1) the solubility of insulin is reduced at physiological pH values in the subcutaneous insulin depot, i.e., the insulin absorption is delayed and thereby the duration of action is prolonged. The delayed absorption is obtained by the addition of vehicles which are as inert as possible and do not exhibit therapeutic properties themselves. After the addition of these substances insulin is precipitated in a salt-like manner or as a poorly soluble complex in crystals of various shape.81

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<td>Scott and Fischer</td>
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Table 4.1
Development of long-acting insulin preparations82

In long-acting insulin preparations the insulin is present in the precipitated form (suspension), which means that it must be mixed thoroughly before use (with the exception of the completely soluble surfen insulin preparations which are no longer in use today). After subcutaneous injection the crystals first must dissolve in the subcutaneous tissue and the previously bound hexamers must be diluted so as to allow them to dissociate into dimers or monomers. Only monomers or dimers can rapidly pass through the pores of the capillaries (diffusion) and thus be absorbed (see pg. 49). The long-acting insulin preparations can be grouped as follows:

a. Protamine zinc insulin
The first long-acting insulin which was stable in neutral solution was protamine zinc insulin. This formulation was an amorphous or crystalline preparation combining insulin, zinc and an excess of protamine (0.15 to 0.25 mg zinc and 1.2 mg protamine per 100 U insulin). The long duration of action of protamine zinc insulin (up to 72 h) led to an accumulation of the effect of the insulin subcutaneously administered on successive days.

b. NPH insulin
Isophane insulin preparations contain insulin and protamine in a stoichiometric ratio, i.e., neither insulin nor protamine are available in excess (molar ratio of protamine: insulin
1:6, 0.016 to 0.04 mg zinc and 0.4 mg protamine per 100 U insulin). NPH insulin preparations (Neutral Protamine insulin developed at the laboratory of Hagedorn) are neutral suspensions with a smaller amount of protamine and zinc than protamine zinc insulin.\textsuperscript{83,84} At a neutral pH value, insulin has a negative net charge, whereas protamine has a positive charge, i.e., protamine and insulin bind by interchain ionic bonding, forming poorly soluble crystals. In the NPH insulin preparations, insulin is available in the form of whitish, suspended tetragonal crystals. These crystals contain at least 2 zinc atoms and 20 molecules m-cresol per hexamer in addition to protamine. The neutral pH value of NPH insulin is achieved by the use of a phosphate buffer. Protamine is a generic name for a group of basic proteins with many positive charges. Protamine combine with DNA to form nucleoproteins in the nuclei of sperm cells. The alkaline character of protamine is based on its high content of arginine. Commercially used highly purified protamine is mainly obtained from fish sperm.

c. Insulin zinc suspensions

The first insulin preparation for which a delayed action was obtained by mixing insulin and zinc without the addition of proteins, which do not naturally occur in the body, was lente insulin. This development was made possible by the discovery that insulin effect can be delayed and kept at a neutral pH by the sole addition of zinc when an acetate buffer is used instead of a phosphate buffer. The amount of zinc added is 10 times as high as for NPH insulin or short-acting insulin. The extent of the retardation of the action of insulin zinc suspensions depends on the physical condition of the insulin. Amorphous insulin, for example, has a faster and shorter action than crystalline zinc insulin. Preparations with amorphous insulin (semilente) have a less delayed insulin action than those with crystalline zinc insulin (ultralente from bovine insulin, for instance, has a duration of action of 18 to 36 h). The amount and type of insulin crystals can be influenced by appropriate crystallisation methods. The individual amorphous particles have a size of 1 µm and form loosely connected aggregations. Semilente as an amorphous insulin suspension is reported to exhibit biphasic absorption kinetics, which means that an initial phase with a rather slow absorption is followed by a second phase with an apparently more rapid absorption. Ultralente insulin—as a crystalline suspension with larger insulin particles—is absorbed more slowly and has a long duration of action. Ultratard\textsuperscript{®,} formulated with human insulin, is a suspension of insulin-zinc crystals in a neutral, cloudy solution with a zinc content of 0.08 to 0.1 mg/ml.

Amorphous and crystalline zinc insulins are used to prepare a mixture resembling NPH insulin with respect to the course of action, known as lente insulin. At first, this kind of combination was only possible when the amorphous part consisted of pork insulin and the crystalline part of beef insulin. Later, a long-acting insulin, known as insulin monotard, was successfully developed containing amorphous and crystalline pork insulin in a ratio of 3:7. Its duration of action is longer than that of semilente insulin, but shorter than that of ultralente insulin.
4.2.1 Time-action profile of NPH insulin
Although a number of different long-acting insulin preparations is available, the most commonly used insulin in Germany is NPH. In a comparative study, the subcutaneous injection of an NPH insulin (Basal-H-Insulin Hoechst®, 23±3 U) provoked an increase in glucose requirement to half-maximal values within 70 min (Fig. 4.2, opposite; Tab. 4.2, pg. 48). The maximal metabolic action was achieved after 4 h. Afterwards, the glucose requirement fell slowly and steadily, and the half-maximal activity was achieved again after 10 h. Near-baseline GIRs were observed 18-20 h after injection. The insulin administration caused maximal insulin concentrations within 6 h (Fig. 4.2; Tab. 4.2). After 20 h, the serum insulin concentrations returned to approximate baseline concentrations. The time-action profile of NPH insulin is thus characterised by a marked maximal activity, achieved within some hours after the subcutaneous injection; consequently, the desired constant insulin action for a substitution of the basal insulin requirements is not achieved. The duration of action of NPH insulin is—in spite of the relatively high dose used here—not long enough to cover the insulin requirement over a period of 24 h. The use of a dose of 12 U NPH insulin allows a duration of action which provides sufficient basal insulin substitution over a period of 24 h when NPH insulin is injected twice daily at 12 h intervals. An injection of NPH insulin at the time of the evening meal does not yield sufficient blood insulin levels in the early morning hours, resulting in increased fasting glucose levels.

4.2.2 Modification of the time-action profile of NPH insulin
Several attempts have been made to modify the time-action profile of NPH insulin in such a way that the maximal action is less pronounced and that the action lasts longer. These tests focused on changes in the pharmaceutical formulation of NPH insulin, i.e., variation of the content of zinc and protamine, of the pH value and of the manufacturing process. One advantage of this approach is that it would not result in a new chemical substance, but only in another pharmaceutical formulation of a known substance. The disadvantage is that the product continues to be a suspension and that protamine is still necessary to delay the action. Moreover, it has yet to be tested whether a modified NPH insulin can be mixed with short-acting insulin without problems. So far, none of these approaches has led to a clinically usable NPH insulin preparation with a different time-action profile.

4.2.3 Tissue reactions after subcutaneous injection of NPH insulin
The information about the time-action profile of NPH insulin obtained in euglycaemic glucose clamp experiments differ from the results of pharmacokinetic studies in which labelled insulin was injected subcutaneously (see pg. 6). The data obtained in the pharmacokinetic studies suggest that NPH insulin remains markedly longer at the injection sites than has been observed during the measurement of the course of serum insulin
Figure 4.2
Glucose infusion rates (top) and serum insulin concentrations (bottom) determined after subcutaneous injection of 0.3 U/kg NPH insulin in 12 subjects on two study days."
concentrations after insulin injection and determination of the time-action profile in pharmacodynamic studies. Immuno-histochemical studies provide an explanation of these discrepant results. These studies show that, within a few hours after the subcutaneous injection of NPH insulin into the neck skin of pigs, a massive migration of macrophages into the area of the insulin depot takes place and that local tissue reactions occur. The macrophages engulf the NPH insulin crystals which are dispersed between fat cells and the interstitial space. They phagocytose the crystals and degrade the NPH insulin. Due to these local inflammatory reactions in the subcutaneous tissue, there is less insulin available for absorption after some time. On the other hand, the radioactive label attached to the insulin molecule is maintained in the macrophages in the tissue for quite some time and is interpreted as absorbable insulin during the pharmacokinetic measurements. These observations explain the described discrepancies in the pharmacological summary measurements of NPH insulin obtained with different experimental methods.

It is known for 20 years that a part of the injected insulin is lost by subcutaneous degradation when injecting short-acting insulin, whereas there is hardly any comparable investigation about this phenomenon with long-acting insulin. If the bioavailability of long-acting insulin is reduced by local tissue reactions, it can be assumed that the longer the long-acting insulin remains in the subcutaneous depot, the more insulin will be lost. This aspect might limit the maximal duration of action that can be achieved, which may be of importance for the development of long-acting insulin analogues (see pg. 68). In view of the long duration of action of bovine ultralente insulin preparations (up to 40 h), one wonders if crystals formulated with beef insulin are less attractive for macrophages. Should the strength of the tissue reactions after the subcutaneous injection of NPH insulin vary depending on the local conditions, this phenomenon may contribute to the high variability of action observed with long-acting insulin preparations.

### 4.2.4 Time-action profile of lente insulin preparations

There is hardly any study of the time-action profile of the semilente preparation and of the lente insulin preparation—only data about ultralente (Ultratard®, if formulated with human insulin) have been published (Fig. 4.4). Ultratard® is only used to a minor extent as a basal insulin in Germany. However, in other countries (for example, the US) it is used more frequently. In a study of six patients with type 1 diabetes, the subcutaneous injection of 0.8 U pork insulin in an ultralente formulation per kg body weight led to a slow increase of the plasma insulin concentration during the first 12 h, maximal levels after 16 to 20 h, with a return to baseline after 28 h. Multiple injections of pork ultralente in 24 h intervals led to a considerable accumulation of the insulin levels, i.e., a more or less constant basal insulinaemia at an elevated level. In another investigation the time-action profiles of ultralente formulations with beef insulin, pork insulin and human insulin after subcutaneous injection of 0.4 U/kg were investigated in subjects and
compared with placebo over a period of 40 h.\textsuperscript{53} Basically, the formulation with beef insulin did not show any peak (neither in the time-action profile nor in the insulin concentrations). The pork and the human insulin formulations, on the other hand, exhibited peaks after 20 to 24 h and 5 to 10 h respectively. In all formulations, the action was maintained over a period of 30 h—an unusually long duration of action, especially for human insulin formulation. This study suggests that in this kind of insulin preparation, the origin of the insulin (i.e., the species) is of considerable importance for the time-action profile of lente insulin preparations. For short-acting insulin preparations or other long-acting insulin preparations the observed differences in the time-action profile were less pronounced or even insignificant when comparing preparations formulated with human insulin to those formulated with animal insulin.\textsuperscript{91}

Since semilente is not available as a formulation with human insulin as yet, it is not possible to make any statement concerning a possible difference between its time-action profile and that of the formulation with pork insulin.

In view of its time-action profile, the ultralente formulation with beef insulin appears to be an ideal insulin for basal insulin substitution, however, the strong immunogenicity of that preparation has almost discontinued its use in industrialised countries. The absorption of ultralente is said to be highly variable, but there are almost no studies investigating this phenomenon.\textsuperscript{92,93}

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Figure 4.4
Comparison of time-action profiles of the NPH insulin Protaphane\textsuperscript{®,} (solid line) and of the ultralente insulin Ultratard\textsuperscript{®,} (broken line), as determined after subcutaneous injection of 12 U in six subjects.\textsuperscript{74}
4.3 Premixed insulin preparations

When short-acting insulin and long-acting insulin are drawn from their vials into one syringe one after the other in order to avoid two injections, pharmaceutical and chemical compatibility (e.g., identical pH, similar buffers) of the two components are prerequisite for the mixing, to avoid undesired alterations of the pharmacological properties of the preparations.\(^94,95\) For instance, if the short-acting insulin interacts with substances added to the long-acting insulin to obtain the delay of action, this may lead to a modification of the time-action profile of short-acting insulin. Therefore, protamine zinc insulin must not be mixed with short-acting insulin in one syringe, because unstable mixtures and/or transformations of short-acting insulin into long-acting insulin can occur due to the interaction of short-acting insulin with protamine, which is available in excessive amounts.

Pure crystalline zinc insulin (e.g., Ultratard\(^\oplus\)) must not be mixed with short-acting insulin either, since the excessive amounts of zinc will transform at least part of the available zinc into long-acting insulin.\(^96\) When mixing short-acting insulin with preparations having an amorphous insulin portion (semilente or lente insulin preparations), there will be a transformation of the short-acting insulin into amorphous insulin particles.\(^97\) Even if the mixture is injected immediately after mixing, the bioavailability of the short-acting insulin will be modified. Therefore, these long-acting insulin preparations cannot be used to prepare mixtures.

Due to the lack of free protamine or excessive zinc, NPH insulin can be mixed with short-acting insulin without leading to any modifications of the pharmacological properties of short-acting insulin in any of the mixing ratios of NPH insulin and short-acting insulin. The action profiles remain identical to those observed with separate injections of the two components.\(^94,98\) This allows the formulation of stable premixed insulin preparations. Premixed insulin preparations are commercially available preparations with a fixed ratio of short-acting insulin and NPH insulin. Mixtures are available in different ratios (from 10/90 to 50/50 with graduations of 10%), the 30/70 or 25/75 mixtures being the most commonly used.\(^11,99\)

4.3.1 Time-action profiles of premixed insulin preparations

The idea here, is that premixed insulin preparations exhibit a biphasic time-action profile, i.e., after subcutaneous injection of such an insulin preparation two peaks in the metabolic effect are observed. The first phase is expected to achieve a maximal activity, due to the short-acting insulin component (intended for prandial insulin substitution), and the second phase is expected to achieve a later, lower-than-maximal activity (to cover the basal insulin requirement). However, our comparative study with an investigation of the time-action profile of a 25/75 mixture (Depot-H-Insulin Hoechst\(^\oplus\); 24±2 U) revealed that, even though the courses of action of the two insulin components can still be recognised, these courses exhibit so much overlap that no biphasic time-action profile is obtained.
Due to the long duration of action of short-acting insulin and to the fast onset of action of NPH insulin, the effects of short-acting insulin and NPH insulin combine, forming a time-action profile with a unimodal shape (Fig. 4.6). As a consequence of the summing up of the effects of the two insulin preparations over time, the maximal effect is higher than it would be if short-acting insulin were administered alone. The half-maximal activity was achieved more than 1 h after subcutaneous injection and the maximal activity after 3 h (Tab. 4.2, pg. 48). Due to the NPH insulin component the return to half-maximal values was slower (achieved after 9 h), and only after 16 to 18 h the time-action profile returned to almost baseline values. Within 3 h after injection of the premixed insulin the serum insulin concentration had increased to maximal values of 110 pmol/L, baseline values were reached again after 17 to 20 h.

4.3.2 Computability of time-action profiles of premixed insulin preparations

In our comparative study, the time-action profiles of short-acting insulin, NPH insulin and a 27/75 premixed insulin preparation were determined under identical experimental conditions. Starting from the assumption that the time-action profile of the premixed insulin is identical to a situation in which the proportion of short-acting insulin and NPH insulin contained in the premixed insulin are injected separately, this gave the opportunity to check how far the registered time-action profile of the premixed insulin preparation can be calculated on the basis of the proportion of the two components used. Therefore, the GIR values determined in the individual experiments with short-acting insulin were multiplied by 0.25 and those determined with NPH insulin by 0.75. The mean values of the two insulin preparations were summed up. As shown in Fig. 4.6, this calculated time-action profile of the premixed insulin formulation corresponds largely to the registered time-action profile. There was also a rather good agreement for serum insulin concentrations, the calculation being performed as for the GIR values. Parallel to the investigation of the time-action profiles of the short-acting, NPH, and premixed insulin preparation, the time-action profiles of two other premixed insulin preparations (15/85 and 50/50; same insulin dose) of the same insulin manufacturer were studied by Prof. Dr. M. Dreyer at the Bethanien-Hospital in Hamburg, using a manual glucose clamp technique. In these mixing ratios the calculated time-action profiles were in agreement with the recorded time-action profiles, too (Fig. 4.7).

The computability of the time-action profile of premixed insulin preparations of short-acting insulin and NPH insulin allows us to check the plausibility of time-action profiles of premixed insulin preparations observed in glucose clamp studies. The comparability of the calculated time-action profiles of the mixtures with the measured time-action profiles demonstrate once more the reproducibility of the determination of time-action profiles obtained using the euglycaemic glucose clamp technique.
Figure 4.5
Glucose infusion rates (above) and serum insulin concentrations (below) after subcutaneous injection of 25/75 premixed insulin (24±2 U) in 12 subjects on two study days.⁴
Figure 4.6
Comparison of the glucose infusion rates (broken lines, top) and serum insulin concentrations (bottom) of 25/75 premixed insulin calculated from the results of short-acting insulin and NPH insulin with the results registered after subcutaneous injection of the premixed insulin (solid lines). The proportions of the time-action profiles of short-acting insulin and NPH insulin, on which the calculations are based, are represented by the dotted lines.
4.4 Time-action profiles of short-acting insulin, NPH insulin and 25/75 premixed insulin

The combined representation of the time-action profiles of short-acting insulin, NPH insulin and a 25/75 premixed insulin with an identical scaling of the axes allows us to compare the metabolic effects of these insulin preparations over time (Fig. 4.8 opposite; Tab. 4.2, pg. 48). The onset of action after subcutaneous injection occurs within 60 min with all preparations. The injection of short-acting insulin leads to a maximal activity that is more than twice as high as that of NPH insulin, but it is not reached until 2 h after injection. From the maximal activity the action slowly decreases over a period of a few hours and does not return to baseline values until 10 h after the subcutaneous injection of short-acting insulin. The injection of NPH insulin leads to a marked maximal metabolic activity within 4 to 5 h after injection. During the following 10 to 14 h the activity slowly decreases. According to the proportions of the two components, the 25/75 premixed insulin exhibits a stronger maximal activity than NPH insulin. The time of maximal activity is between that of short-acting insulin and that of NPH insulin. After 7 to 8 h the time-action profile of the premixed insulin corresponds to that of the NPH insulin. The serum insulin concentrations show an analogous course—the differences in the maximal serum insulin concentrations between short-acting insulin and NPH insulin being more pronounced than in the glucose infusion rates.
Figure 4.8
Comparative representation of glucose infusion rates (top) and serum insulin concentrations (bottom) registered after subcutaneous injection (0.3 U/kg) of short-acting insulin, NPH insulin and 25/75 premixed insulin in 12 subjects in each of two experiments with one preparation.4
### Table 4.2
Pharmacodynamic and pharmacokinetic summary measures determined after subcutaneous injection of short-acting insulin, NPH insulin and of a 25/75 premixed insulin in a dose of 0.3 U/kg body weight in 12 healthy subjects in each of two experiments with one preparation.

<table>
<thead>
<tr>
<th></th>
<th>short-acting</th>
<th>NPH insulin</th>
<th>25/75 premixed insulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>blood glucose (mmol/L)</td>
<td>5.0 ± 0.3</td>
<td>5.0 ± 0.2</td>
<td>5.0 ± 0.2</td>
</tr>
<tr>
<td>CV (%)</td>
<td>5.2 ± 0.9</td>
<td>3.7 ± 0.9</td>
<td>3.9 ± 1.3</td>
</tr>
<tr>
<td>baseline GIR (mg/kg/min)</td>
<td>1.3 ± 0.9</td>
<td>1.8 ± 1.1</td>
<td>1.5 ± 0.7</td>
</tr>
<tr>
<td>GIR(_{\text{max}}) (mg/kg/min)</td>
<td>11.3 ± 2.7</td>
<td>4.8 ± 1.8</td>
<td>6.4 ± 2.6</td>
</tr>
<tr>
<td>(t_{\text{max}}) (min)</td>
<td>148 ± 41</td>
<td>242 ± 108</td>
<td>189 ± 50</td>
</tr>
<tr>
<td>(t_{\text{early 50%}}) (min)</td>
<td>64 ± 22</td>
<td>74 ± 43</td>
<td>72 ± 35</td>
</tr>
<tr>
<td>(t_{\text{late 50%}}) (min)</td>
<td>344 ± 80</td>
<td>607 ± 240</td>
<td>533 ± 135</td>
</tr>
<tr>
<td>AUC (g/kg x 720-1440 min)</td>
<td>3.5 ± 0.7</td>
<td>3.1 ± 1.3</td>
<td>3.5 ± 1.4</td>
</tr>
<tr>
<td>basal blood insulin (pmol/L)</td>
<td>42 ± 14</td>
<td>66 ± 20</td>
<td>67 ± 15</td>
</tr>
<tr>
<td>(C_{\text{max}}) (pmol/L)</td>
<td>258 ± 69</td>
<td>72 ± 25</td>
<td>107 ± 29</td>
</tr>
<tr>
<td>(t_{\text{max}}) (min)</td>
<td>147 ± 30</td>
<td>358 ± 269</td>
<td>182 ± 68</td>
</tr>
<tr>
<td>(t_{\text{early 50%}}) (min)</td>
<td>45 ± 14</td>
<td>76 ± 45</td>
<td>48 ± 23</td>
</tr>
<tr>
<td>(t_{\text{late 50%}}) (min)</td>
<td>326 ± 72</td>
<td>772 ± 292</td>
<td>459 ± 180</td>
</tr>
<tr>
<td>AUC (nmol/L x 720-1440 min)</td>
<td>34.1 ± 7.1</td>
<td>48.5 ± 24.2</td>
<td>56.3 ± 18.1</td>
</tr>
</tbody>
</table>
5 Time-action profiles of insulin analogues

Insulin analogues are insulin molecules whose structure is largely homologous to human insulin, differing only by at least one amino acid. Insulin analogues have been developed in order to obtain insulin preparations with pharmacodynamic properties which are more suitable for insulin therapy than preparations formulated with human insulin or animal insulin preparations. To counter the disadvantages of the time-action profiles of the currently used insulin preparations, the ideal insulin analogues must meet the following requirements (adapted from\textsuperscript{100}):

\begin{center}
\begin{tabular}{|l|}
\hline
\textbf{Properties of ideal insulin analogues} \\
\hline
\textit{Time-action profile of rapid-acting insulin analogue} \\
- onset of action \(<0.5\) h after subcutaneous injection \\
- high maximal activity \\
- duration of action \(<4\) h \\
\hline
\textit{Time-action profile of long-acting insulin analogues} \\
- onset of action \(\geq 4\) h after subcutaneous injection \\
- duration of action 24 h (= one injection per day) \\
- no pronounced maximal activity \\
- almost regular action over time \\
\hline
\textit{General} \\
- small intraindividual variability of insulin action \\
- metabolic effect >> mitogenic effects \\
- no significant immunogenic effects \\
- chemically stable \\
- no problems with mixability \\
\hline
\end{tabular}
\end{center}

\textit{Table 5.1: Properties of ideal insulin analogues}

Should these insulin preparations meet these requirements, an adequate combination of rapid-acting and long-acting insulin analogues should be able to imitate the physiological insulin secretion pattern better—even after subcutaneous insulin administration—than it has been possible with common insulin preparations. However, a switch to the use of insulin analogues for insulin therapy is only reasonable if their use
leads to a measurable clinical benefit, for example, an improved quality of metabolic control, while acute metabolic disorders are avoided. The results of clinical studies with insulin analogues must match the results that can be achieved with current insulin preparations.\textsuperscript{101}

\textbf{Figure 5.1}
Primary structure of human insulin, with information about the modifications of the amino acid sequence in rapid-acting insulin analogues (above) and in long-acting insulin analogues (below).
More than 1,000 analogues have been studied, although only 20 have been tested for clinical usefulness in humans so far. In the last years five rapid-acting insulin analogues (B9AspB27Glu, B10Asp, insulin aspart (B28Asp); NOVO NORDISK, Bagsvaerd, Denmark and insulin lispro (B28LysB29Pro), ELI LILLY, Indianapolis, USA; insulin glulisine (B3LysB29Glu), AVENTIS, Frankfurt, Germany) (see opposite) and three long-acting insulin analogues (NovoSol, insulin glargine and insulin detemir (see opposite)) have been studied more intensively. In insulin lispro (the first insulin analogue approved as a drug under the trade name Humalog®) the amino acids proline and lysine have been reversed at the positions 28 and 29 of the B-chain, leading to a modification of the spatial structure of the terminal portion of the B-chain. In the rapid-acting insulin analogue insulin aspart (NovoRapid®) the amino acid proline has been substituted with asparaginic acid at position B28 of the B-chain, i.e., an amino acid with an alcoholic OH group had been substituted with an amino acid with a carboxyl group, which is charged negatively under physiological conditions.

Modifications at the C-terminal end of the B-chain of the insulin molecule result in far-reaching alterations in the biological and physicochemical properties of insulin. This can be put down to the association of insulin molecules into more complex structures. Insulin molecules are present as monomers (measures 2.5x2x3 nm) at physiological concentrations in the blood (<10⁻⁹ mol/L) or at low concentrations in neutral solutions (<10⁻⁷ mol/L or <0.1 g/L). In the case of higher insulin concentrations, these monomers spontaneously and reversibly bind together to form dimers (relative molecular mass 12,000) by non-covalent intermolecular hydrogen bonds between the side chains of the peptide groups at the positions B24 and B26 of two monomers. If zinc ions are present, three of such dimers aggregate to an annular hexamer which includes two superimposed Zn²⁺ (relative molecular mass 37,500; diameter 5 nm, height 3.5 nm). This arrangement of six insulin molecules to one hexamer represents a stable quaternary structure of the insulin protein, which can conserve pharmaceutical insulin preparations for several years in a cool place without further aggregation or precipitation of the insulin. Since insulin is also physiologically present at high concentrations in β-cells, and unbound zinc is also found in these cells, it follows that zinc-containing hexamers are incorporated in such cells as a storage form of insulin in the electron-dense granules of the β-cells.

During the secretion process the granules will fuse with the membrane wall and insulin will be secreted into the blood in the form of hexamers. In the blood, the hexamers will dissociate quickly into monomers. The association of insulin monomers to dimers and to larger aggregates in a particular environment is subject to a complex, dynamic balance, which depends on the pH, on the ionic strength and on the protein concentration in the solution. Due to its high concentration (approx. 10⁻³ mol/L), short-acting insulin is present in the insulin preparations as a balanced mixture of monomers, dimers and hexamers. Hexamers are reported to have a share of more than 75%, depending on the insulin concentration.
The balance between the association forms is influenced by the above-mentioned cofactors. It is difficult in practice, however, to find out the current portion of hexamers in a given insulin preparation and insulin concentration.

The ultrastructure of the capillaries of the skin has a closed inner layer of endothelial cells with a thickness of 0.1 to 0.3 µm, characterised by numerous intercellular channels with a pore radius of 4.0 to 4.5 nm. For insulin, as a water-soluble but not lipo-soluble protein, the capillary wall is relatively impermeable. When short-acting insulin is injected subcutaneously, the hexamers contained in it have a poor chance to permeate the pores of the capillary wall, due to their spatial measures. Therefore, hexamers have a lower absorption rate than the smaller monomers, which can permeate the transmural channels and are thus readily absorbed. In order to cause the hexamers to dissociate into dimers or monomers, the insulin must be diluted by a factor $10^2$ to $10^5$. Since the colloid osmotic pressure of the injected insulin is higher than that of tissue and plasma, the inflow of water into the subcutaneous insulin depot effects a dilution of the insulin solution and an increased diffusion of the insulin molecules in the interstitial space. The protracted absorption of insulin from the subcutaneous depot is thus determined by the dilution-dependent and therefore time-dependent dissociation of the hexamers. The absorption properties of insulin from the subcutaneous depot can plausibly be explained by these processes (see pg. 93).

When a more rapid absorption of insulin administered by subcutaneous injection is desired, in order to obtain a better prandial insulin substitution, the self-association of the insulin molecules into dimers or hexamers has to be prevented, or the cohesive forces of the insulin molecules in the hexamers has to be reduced. A reduction of the intermolecular bonding forces, responsible for self-association, can be achieved by modifying the primary structure in certain locations within the insulin molecules. Such modifications may be attained by genetic engineering (recombinant DNA technology) or by chemical methods, which allow the insulin molecule to be modified at almost any position in its amino acid sequence, thus producing insulin analogues with different properties. The manufacturing methods of insulin analogues are similar to those used to manufacture human insulin by genetic engineering.

The side chains of single amino acids at the C-terminal end of the B-chain play a particular role in the self-association of the insulin molecules. By reversing or removing certain amino acids in this area, the self-association of the insulin molecules can be hindered or prevented when, for example, at a neutral pH, uncharged amino acids are substituted with negatively-charged amino acids. Introducing negative charges to the monomer-to-monomer interface impedes the interaction between the insulin molecules, thereby impeding self-association.

This self-association is also impeded by the substitution of amino acids with fewer hydrophilic amino acids, leading to an enhancement of the hydrophobic properties in certain locations within the insulin molecule (Tab. 5.2).
In other insulin analogues, amino acids are substituted with amino acids of another spatial structure, leading to modifications of the conformation in that area of the insulin molecule. Selective deletions (e.g., of the amino acid at position B27) interfere with the fitting of the side chains of the amino acid at the end of the B-chain. Modifications of the amino acid sequence are predominantly made in those areas of the insulin molecule located outside the molecular areas of the B-chain which are involved in the receptor binding.

Since all the information on its properties is encoded in its primary structure (including the kind of folds leading to its three-dimensionality), any changes to the primary structure of insulin will also produce changes to the secondary and tertiary structure. Since the binding of the insulin molecule to the insulin receptor involves the interaction of highly specific spatial structures, the biological effects of the insulin molecule are closely linked to its spatial structure. Therefore, the substitution of one amino acid in a certain molecular area in the insulin molecule does not only influence its absorption rate, but its other biological properties as well. As stated above, the modifications of insulin analogues are therefore made in molecular areas located outside the area reacting with the receptor, so as to interfere with this interaction as little as possible.

Nevertheless, most insulin analogues exhibit different states of binding to the insulin receptor as compared to human insulin, and thus a different biological effect. There are areas of the insulin molecule, for example, in which the substitution of even more than one amino acid will only incur minor changes of the biological action. Thus, the metabolic effect of beef insulin hardly differs from the effect of human insulin, although the two molecules differ in some three amino acids. On the other hand, beef insulin and human insulin differ markedly as regards their immunological properties in humans. Since adverse reactions are linked with the properties (and thus the structure) of insulin analogues, the development of rapid-acting insulin analogues focuses on those which strongly resemble human insulin with respect to their in vivo and in vitro characteristics (i.e., insulin lispro and insulin aspart).

Apart from the possibility to produce insulin analogues by changing the primary structure of insulin, insulin preparations with different properties can also be obtained by other methods. The specific glycosylation of individual amino acids, the use of cobalt ions instead of zinc ions, or the coupling of fatty acids to amino acids at the terminal end of the B-chain result in insulin preparations with a long duration of action (see pg. 68).

In another (experimental) approach it was investigated whether there is a delay in the metabolic effect when one end of the C-peptide is coupled to the otherwise unoccupied C-terminal end of the A-chain, and the other end to the likewise-unoccupied N-terminal-end of the B-chain. This procedure results in the so-called A-C-B proinsulin, contrary to the natural order, which is B-C-A. This insulin analogue, however, has only a low metabolic activity. As the time-action profiles of the currently investigated insulin analogues...
are still not optimal, the insulin analogues investigated so far can be considered a first step in the development of further insulin analogues.

5.1 Rapid-acting insulin analogues

5.1.1 Properties of rapid-acting insulin analogues

The modification of the primary structure of insulin allows the production of insulin analogues whose absorption from the subcutaneous depot is increased two to three times that of short-acting insulin. This is due to a reduced propensity for self-association of the monomers and dimers to hexamers or to reduced cohesive forces of the hexamers. The degree of self-association of the insulin molecules negatively correlates with the absorption rate of these insulin analogues, i.e., the higher the degree of self-association is, the slower the insulin analogue is absorbed. The rapid-acting insulin analogues insulin lispro and insulin aspart, however, do associate to form hexamers in their pharmaceutical formulations but do not remain in the preparations as monomers or dimers, as is the case with B10Asp or B9AspB27Glu. The
reversal of lysine and proline at the C-terminal end of the B-chain in insulin lispro reduces the binding forces between the two insulin molecules of a dimer to such an extent that the dimerisation constant is 300 times less than that of human insulin.\textsuperscript{113} In spite of this strongly decreased propensity for self-association, insulin lispro can be stabilised as a hexamer in the pharmaceutical solution when sufficient amounts of zinc and phenol are added. An insulin lispro hexamer binds two zinc molecules and three phenol molecules, whereas human insulin is able to bind six zinc molecules under identical conditions. The fact that insulin lispro is a hexamer has advantages for the physical and chemical stability of the preparation. The rapid absorption of this insulin analogue from the subcutaneous tissue can be explained by the rapid uptake of phenol into the surrounding tissue after injection. The hexamers are thus destabilised and quickly dissociate into monomers, given the conditions in the subcutaneous tissue. Therefore, the absorption rate of insulin lispro corresponds to that of insulin monomers. In human insulin the stability of the hexamers is so much higher, that the diffusion of phenol from the hexamer does not lead to their immediate dissociation.

5.1.2 Time-action profiles of rapid-acting insulin analogues

For the time-action profile of rapid-acting insulin analogues their differences in \textit{in vitro} properties, receptor binding and pharmacokinetic summary measures seem to be of less importance than their reduced propensity for association. The combined representation of the time-action profiles of the three insulin analogues B9AspB27Glu, B10Asp and B28Asp (insulin aspart), which have been investigated in two studies (Fig. 5.2), shows that the subcutaneous injection of these three insulin analogues leads to a comparable time-action profile, even despite differences in a number of other properties.\textsuperscript{58,114,115} Compared with the short-acting insulin preparation (U-100 insulin), which was identical in both studies, the onset of the blood glucose-lowering action after subcutaneous injection was much earlier in the insulin analogues. Within the first hour after injection of the insulin analogues, the action increased to values higher than the half-maximal. The action remained at higher maximal values over a period of about 60 min and then decreased. The AUC under the GIR profiles—as a measure of the metabolic activity—was significantly greater in the first 60 to 90 min after injection of the insulin analogues than with the short-acting insulin preparation. The AUCs under the total time-action profiles, however, were comparable, suggesting an equipotent effect, when using equimolar doses of short-acting insulin preparations and insulin analogues. The subcutaneous injection of the short-acting insulin preparation resulted in the known protracted time-action profile, which was reproducible in both studies (see pg. 33).

The primary goal of these studies was to investigate the time-action profile of these rapid-acting insulin analogues in the first hours after subcutaneous injection in the abdominal wall, since this time is of crucial importance for the substitution of the prandial insulin requirements. Due to the duration of the experiment of 240 min, however, it was
not possible to describe the time-action profiles of these insulin analogues completely. This means, it was not possible to make any statement concerning the question as to whether the insulin analogues had a significantly shorter duration of action than a short-acting insulin preparation, because the differences did not reach statistical significance at the end of the experiment.

When insulin action drops within 4 to 5 h after injection to such an extent that the hepatic glucose production is no longer suppressed sufficiently, there will be a massive increase of blood glucose if the basal insulin requirements are not met.\(^{116}\) For routine insulin therapy with rapid-acting insulin analogues, it is therefore important to be aware of their complete time-action profile—including the duration of action. To this end, we repeated the investigation of the time-action profile of the rapid-acting insulin analogue, insulin aspart, but over a longer period of time.\(^{117}\)

![Time-action profiles of insulin analogues](image)

**Figure 5.2**
*Time-action profiles registered after subcutaneous injection of the rapid-acting insulin analogues B9AspB27Glu (dashed line), B10Asp (dashed/dotted line) and insulin aspart (solid line) and a short-acting insulin preparation (dotted lines) in subjects in two studies with a largely identical study design.*\(^{58}\)

**Methods**

The experimental protocol was nearly identical to that of the earlier study,\(^{115}\) except the higher insulin dose (0.2 vs. 0.15 U/kg body weight; administered dose 15.7±1.4 vs.
11.0±1.3 U), the higher number of subjects (n=24; age 26±3 years; body mass index 23.0±1.5 kg/m²), and the longer duration of the experiment (10 h).

**Results and discussion**

As in the first study, after injection of insulin aspart, the early half-maximal GIR and GIR\textsubscript{max} were achieved sooner as compared to the activity of short-acting insulin (t\textsubscript{early 50%} 44±12 vs. 65±16 min, p<0.001; t\textsubscript{max} 105±18 vs. 148±27 min, p<0.001), and the maximal activity was different (GIR\textsubscript{max} 10.2±2.3 vs. 8.4±2.0 mg/kg/min, p=0.001) (Fig. 5.3). In addition to the earlier onset of action as compared to short-acting insulin, the decrease of the GIR after maximal activity—indicating the attenuation of the insulin action—was more rapid for insulin aspart than for short-acting insulin. The late half-maximal activity was achieved 80 min earlier for insulin aspart compared to short-acting insulin (t\textsubscript{late 50%} 256±44 vs. 337±56 min, p<0.001). After subcutaneous injection of short-acting insulin, the action was 272±50 min above the half-maximal level and thus 60 min longer than with insulin aspart (213±42 min; p<0.001). The amount of glucose, which had to be infused to neutralise the blood glucose-lowering effect of the injected insulin, was greater with insulin aspart than with short-acting insulin in the first two hours (AUC\textsubscript{0-2 h} 0.76±0.24 vs. 0.45±0.19 g/kg/120 min; p<0.001) and lower in the following 8 h (AUC\textsubscript{2-10 h} 1.72±0.43 vs. 2.01±0.53 kg/kg/480 min; p<0.001). The total AUC in the two insulin preparations was comparable (AUC\textsubscript{0-10 h} 2.48±0.59 vs. 2.46±0.60 g/kg/600 min; p=0.480). After subcutaneous injection of insulin aspart, not only the onset of action was earlier than with short-acting insulin, but also the duration of action was shorter. In addition, the serum insulin concentrations increased more rapidly and reached higher maximal values than with short-acting insulin (t\textsubscript{max} 48±18 vs. 123±67 min, p<0.001; C\textsubscript{max} 414±19 vs. 239±10 pmol/L, p<0.001; Fig. 5.3), and the return to baseline values also happened earlier with the insulin analogue (343±57 vs. 496±69 min; p<0.001).

In the first 2 h after injection, the AUCs differed (AUC\textsubscript{0-2 h} 3.23±0.64 vs. 1.75±0.47 nmol/L/120 min; p<0.001), which is not true for the AUCs over the entire 10 h (AUC\textsubscript{0-10 h} 4.33±1.15 vs. 4.75±0.88 nmol/L/600 min; p=0.168).

Strictly speaking, these statements concerning the time-action profile of insulin aspart are only valid for the doses studied. Another study with insulin lispro on the influence of the dose on the time-action profiles showed that, when using various doses, the time-action profiles of this rapid-acting insulin analogues varied less compared with short-acting insulin (Tab. 5.3, pp. 59 and 85).\textsuperscript{118} This study showed that the duration of action of short-acting insulin increases steadily with increasing doses (e.g., an increase of the dose from 0.1 to 0.3 U/kg results in an increase in the duration of action by 235 min), whereas this effect is less pronounced with insulin lispro (increase by 70 min if the dose is increased in the same manner).

Nothing has been published as yet, in which the time-action profiles of insulin aspart and insulin lispro were investigated in one comparative study. The confrontation of the time-
Figure 5.3
Glucose infusion rates (top) and serum insulin concentrations (bottom) after subcutaneous injection of 0.2 U/kg body weight of the insulin analogue insulin aspart (solid line) and short-acting insulin preparation (dashed line) in 24 subjects (after subtraction of the baseline rates). For the glucose infusion rates the individual mean (±standard error) are plotted for the $t_{\text{early 50\%}}$ and $t_{\text{max}}$, $t_{\text{late 50\%}}$ and for GIR$_{\text{max}}$.
action profiles of insulin aspart and insulin lispro, as determined by us in two separate studies (see overleaf), showed that the profiles, pharmacodynamic properties (i.e., the onset of action), the time to maximal activity, and the duration of action were all similar (Fig. 5.4). Nevertheless, different maximal activities were observed in the two studies, which was probably due to the different insulin dosage (0.2 U/kg for insulin aspart and 0.3 U/kg for insulin lispro). The two studies were conducted with a similar study protocol and with comparable subject groups with respect to demographic parameters. The study with insulin lispro, however, included women.

Table 5.3

Duration of action of short-acting insulin and insulin lispro after subcutaneous injection of various dosages.118

<table>
<thead>
<tr>
<th>U/kg</th>
<th>0.05</th>
<th>0.1</th>
<th>0.2</th>
<th>0.3</th>
<th>0.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>short-acting insulin (min)</td>
<td>180</td>
<td>255</td>
<td>465</td>
<td>540</td>
<td>555</td>
</tr>
<tr>
<td>insulin lispro (min)</td>
<td>195</td>
<td>225</td>
<td>270</td>
<td>295</td>
<td>360</td>
</tr>
</tbody>
</table>

The comparability of the time-action profiles suggests that the introduction of the negatively charged amino acid asparagine to insulin aspart, or the reversal of the two amino acids in insulin lispro, does not lead to differences in the pharmacodynamic properties. The differences in the charge at the C-terminal end of the B-chain does not appear to have any significance for the time-action profiles of these insulin analogues.

In our study, the injection of insulin aspart and short-acting insulin led to a comparable total metabolic effect, i.e., under our experimental conditions they were equipotent. This equipotency was also reported for insulin lispro.17,119 In these reports insulin lispro showed a higher metabolic activity than short-acting insulin within a period of 60-90 min after subcutaneous injection. Since this period is crucial for the behaviour of the blood glucose after a meal, the subcutaneous administration of such a rapid-acting insulin analogue before a meal should result in a lower postprandial blood glucose profile (see pg. 120). Moreover, the shorter duration of action of rapid-acting insulin analogues should reduce the risk of late postprandial episodes of hypoglycaemia, which may occur as a consequence of the long duration of action of short-acting insulin. On the other hand—as described—the short duration of action may result in a decline of insulinaemia to levels that cause the blood glucose to rise before the next meal if the interval between meals are rather long.

Alterations in the basal insulin requirements must be made which reflect the different pharmacodynamic properties of the rapid-acting insulin analogues as compared to short-acting insulin. Non-observance of this necessity could explain why the first clinical studies with rapid-acting insulin analogues101 were unable to establish convincing evidence as to their benefits.
Figure 5.4
Time-action profiles of the rapid-acting insulin analogues insulin aspart (top) and insulin lispro (bottom), registered after subcutaneous injection of 0.2 and 0.3 U/kg body weight in 24 and 30 subjects in two different studies.⁷³,¹¹⁷
5.2 Premixed insulin preparations containing rapid-acting insulin analogues

After subcutaneous injection, a mixture of short-acting insulin and NPH insulin results in a time-action profile characterised by a single peak in metabolic activity after some hours and a slow decline of the metabolic activity (see pg. 42). Thus, the initial insulin action occurs too late for an optimum substitution of the prandial insulin requirement. A more adequate time-action profile to cover the prandial insulin requirement may possibly be achieved by using rapid-acting insulin analogues instead of short-acting insulin when producing the premixed insulin preparations. Subcutaneous injection of such premixed insulin preparations should result in both a faster onset of action and a reduced summation of the actions of the short-acting and long-acting insulin. Potentially, the use of a long-acting insulin analogue instead of NPH insulin would provide a better substitution of the basal insulin requirements. However, mixtures of a rapid-acting insulin analogue with a long-acting insulin analogue have not been studied thus far.

Regarding premixed insulin preparations formulated with rapid-acting insulin analogues, the NPH insulin should be formulated with an appropriate insulin analogue as well. This is done to avoid an exchange between the human insulin in the NPH insulin, commonly formulated with and the insulin analogue, in the solution after prolonged storage of the mixture (> a couple of weeks). After such a period of time, there would be a mixture of free rapid-acting insulin analogue and slower-acting short-acting human insulin in the solution and crystals with bound human insulin or insulin analogue. At least part of the advantage of using rapid-acting insulin analogues would be lost in such mixtures. However, it is not known whether a protamine-protracted insulin formulated with rapid-acting insulin analogues still has the same time-action profile as the NPH insulin formulated with human insulin.

The absorption of NPH insulin involves two processes: on the one hand, the release of the insulin from the crystal binding with protamine, and on the other hand, the absorption of the released insulin. In the case of NPH insulin, insulin is bound in the form of hexamers with protamine in the crystals, i.e., after disintegration of that bond, insulin is available in the form of hexamers. When using rapid-acting insulin analogues, the process of dissociation of the hexamers into quickly absorbable monomers and dimers should be more immediate than in the case of NPH insulin formulated with human insulin. This was confirmed in a study with eight subjects (0.4 U/kg), in that a faster increase of the insulin concentration and of the glucose requirement were observed with NPL insulin compared to NPH insulin.120

5.2.1 Time-action profiles of premixed insulin preparations containing insulin lispro

The formulation of NPH insulin with insulin lispro (NPL; insulin lispro protamine suspension) is an insulin formulation in which insulin lispro is co-crystallised with protamine to obtain
a preparation with a protracted action.\textsuperscript{121} This NPL insulin was developed to allow the production of stable premixed insulin preparations with insulin lispro. The time-action profiles of three mixtures formulated with insulin lispro and NPL insulin as well as the time-action profiles of insulin lispro and NPL insulin alone were investigated.\textsuperscript{73} The premixed insulin preparations investigated in this study contained insulin lispro and NPL insulin in a ratio of 25/75 (low-mixture (LM)), 50/50 (mid-mixture (MM)) and 75/25 (high-mixture (HM)).

**Method**
Open, randomised, balanced 5-way cross-over study; 30 healthy participants (12 women, 18 men; age 27±2 years, body mass index 23.0±2.3 kg/m\textsuperscript{2}); 0.3 U/kg body weight (U-100) LM, MM, HM, NPL insulin or insulin lispro on five study days; baseline phase 120 min, measurements over a period of 22 h after injection of premixed insulin preparations or NPL insulin, 10 h for insulin lispro.

**Results and discussion**
Irrespective of the proportion of dissolved insulin lispro in the three different mixtures, the maximal metabolic activity was observed 2 h after injection (Fig. 5.5; Tab. 5.4). If the percentage of insulin lispro was higher, a progressive, linear increase of the maximal activity was observed. But this increase was not proportional to the proportion of dissolved insulin lispro, i.e., an increase by 25% did not result in an increase of action by 25%. Higher proportions of insulin lispro led to a faster decrease of action to late half-maximal activity and to a larger area under the GIR profile in the first 360 min after injection. During the first 6 h after subcutaneous injection, the metabolic activity was predominantly determined by the proportion of dissolved insulin lispro. The glucose requirement in the following period of time, which was mainly determined by the different percentages of NPL insulin, varied depending on these proportions. However, the difference in the glucose requirements were not as pronounced as in the dissolved insulin lispro. After 22 h, the glucose requirements had not yet returned completely to baseline values. The requirements were different, depending on the percentage of NPL insulin in the premixed insulin preparations. The glucose requirements throughout the experiments were not different for the various insulin preparations (except for insulin lispro), i.e., the AUCs throughout the duration of the experiments of 1320 min were comparable.

The changes in the serum insulin concentrations were parallel to the metabolic reactions. The maximal serum insulin concentrations rose with a linear and proportional dependency on the proportion of dissolved insulin lispro (Fig. 5.5; Tab. 5.4). The points of time at which these values were achieved were comparable (except for NPL insulin). The areas under the serum insulin profiles were different among all preparations within the first 6 h after injection, but afterwards they became comparable for most preparations. The basal serum C-peptide concentrations and the degree of suppression of the endogenous insulin production by baseline intravenous insulin infusion and the subcutaneous insulin administration were comparable on all study days.
Figure 5.5
Time-action profiles (top) and serum insulin concentrations (bottom), recorded after subcutaneous injection of insulin lispro, NPL insulin and three mixtures thereof in 30 healthy subjects.\textsuperscript{73}
### Pharmacodynamic Summary Measures

<table>
<thead>
<tr>
<th></th>
<th>insulin lispro</th>
<th>high mixture</th>
<th>mid mixture</th>
<th>low mixture</th>
<th>NPL insulin</th>
<th>differences</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GIR&lt;sub&gt;max&lt;/sub&gt; (mg/kg/min)</td>
<td>12.7±3.0</td>
<td>10.2±2.7</td>
<td>9.0±2.8</td>
<td>7.3±2.6</td>
<td>4.9±2.3</td>
<td>IL HM MM LM</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>t&lt;sub&gt;max&lt;/sub&gt; (min)</td>
<td>107±21</td>
<td>120±25</td>
<td>121±22</td>
<td>141±36</td>
<td>252±64</td>
<td>IL HM MM LM</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>t&lt;sub&gt;early 50%&lt;/sub&gt; (min)</td>
<td>44±12</td>
<td>47±13</td>
<td>40±12</td>
<td>44±12</td>
<td>70±30</td>
<td>IL HM MM LM</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>t&lt;sub&gt;30%/100%&lt;/sub&gt; (min)</td>
<td>266±57</td>
<td>339±76</td>
<td>384±110</td>
<td>557±205</td>
<td>941±269</td>
<td>IL HM MM LM</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;0-360&lt;/sub&gt; (g/kg x 360 min)</td>
<td>2.73±0.58</td>
<td>2.45±0.52</td>
<td>2.19±0.58</td>
<td>1.85±0.61</td>
<td>1.24±0.68</td>
<td>IL HM MM LM</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;360-1320&lt;/sub&gt; (g/kg x 960 min)</td>
<td>(0.34±0.25)</td>
<td>1.29±0.60</td>
<td>1.62±0.82</td>
<td>2.20±1.05</td>
<td>2.53±1.38</td>
<td>HM MM LM NPL</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;0-1320&lt;/sub&gt; (g/kg x 1320 min)</td>
<td>3.07±0.71</td>
<td>3.73±0.94</td>
<td>3.79±1.16</td>
<td>4.03±1.51</td>
<td>3.75±1.96</td>
<td>IL HM MM LM</td>
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<tr>
<td>AUC&lt;sub&gt;0-360&lt;/sub&gt; NPL insulin = 100%</td>
<td>100</td>
<td>91.9±20.1</td>
<td>81.8±23.3</td>
<td>69.1±22.2</td>
<td>45.1±22.7</td>
<td>IL HM MM LM</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;360-1320&lt;/sub&gt; NPL insulin = 100%</td>
<td>(16.9±17.3)</td>
<td>57.0±29.1</td>
<td>66.7±28.7</td>
<td>82.6±29.7</td>
<td>100</td>
<td>HM MM LM NPL</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

### Pharmacokinetic Summary Measures – Serum Insulin

<table>
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<tr>
<th></th>
<th>basal concentrations (pmol/L)</th>
<th>C&lt;sub&gt;max&lt;/sub&gt; (pmol/L)</th>
<th>t&lt;sub&gt;max&lt;/sub&gt; (min)</th>
<th>AUC&lt;sub&gt;0-90&lt;/sub&gt; (nmol/L x 90 min)</th>
<th>AUC&lt;sub&gt;0-360&lt;/sub&gt; (nmol/L x 360 min)</th>
<th>AUC&lt;sub&gt;0-1320&lt;/sub&gt; (nmol/L x 960 min)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>84±16</td>
<td>79±19</td>
<td>79±22</td>
<td>78±17</td>
<td>128±72</td>
<td>200±162</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>C&lt;sub&gt;max&lt;/sub&gt; (pmol/L)</td>
<td>883±210</td>
<td>548±99</td>
<td>401±93</td>
<td>206±79</td>
<td>128±72</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>t&lt;sub&gt;max&lt;/sub&gt; (min)</td>
<td>71±17</td>
<td>82±20</td>
<td>81±23</td>
<td>94±45</td>
<td>200±162</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>AUC&lt;sub&gt;0-90&lt;/sub&gt; (nmol/L x 90 min)</td>
<td>58.1±15.1</td>
<td>33.±7.7</td>
<td>25.2±7.7</td>
<td>12.5±4.9</td>
<td>4.1±4.6</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>AUC&lt;sub&gt;0-360&lt;/sub&gt; (nmol/L x 360 min)</td>
<td>130.8±22.9</td>
<td>89.7±16.7</td>
<td>70.5±16.5</td>
<td>43.2±14.7</td>
<td>20.0±11.7</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>AUC&lt;sub&gt;0-1320&lt;/sub&gt; (nmol/L x 960 min)</td>
<td>(3.1±5.8)</td>
<td>12.8±15.7</td>
<td>34.4±36.0</td>
<td>35.1±18.1</td>
<td>42.6±20.0</td>
<td>&lt;0.0001</td>
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<tr>
<td></td>
<td>AUC&lt;sub&gt;0-1320&lt;/sub&gt; (nmol/L x 1320 min)</td>
<td>133.9±21.8</td>
<td>102.5±26.2</td>
<td>104.9±41.5</td>
<td>78.3±23.3</td>
<td>62.6±27.6</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

### Pharmacokinetic Summary Measures – Serum C-peptide

<table>
<thead>
<tr>
<th></th>
<th>basal concentrations (ng/ml)</th>
<th>mean values after injection (ng/ml)</th>
<th>% suppression until injection</th>
<th>% suppression after injection</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>1.45±0.69</td>
<td>0.75±0.23</td>
<td>67.6±22.8</td>
<td>56.1±19.5</td>
</tr>
<tr>
<td></td>
<td>1.38±0.45</td>
<td>0.74±0.25</td>
<td>66.3±18.2</td>
<td>55.2±13.6</td>
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<tr>
<td></td>
<td>1.52±0.81</td>
<td>0.71±0.23</td>
<td>56.3±15.2</td>
<td>50.0±13.8</td>
</tr>
<tr>
<td></td>
<td>1.47±0.43</td>
<td>0.74±0.24</td>
<td>63.0±25.6</td>
<td>52.4±17.6</td>
</tr>
<tr>
<td></td>
<td>1.30±0.43</td>
<td>0.72±0.24</td>
<td>70.2±23.5</td>
<td>57.6±18.7</td>
</tr>
<tr>
<td></td>
<td>0.394</td>
<td>0.911</td>
<td>0.099</td>
<td>0.234</td>
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</table>
Our study was the first one to investigate the pharmacokinetic and pharmacodynamic summary measures of fixed mixtures of insulin lispro and NPL insulin. The study showed the properties of insulin lispro in the mixtures are maintained, almost irrespective of the proportion of dissolved insulin lispro. The subcutaneous injection of the mixtures led to a rapid onset of the metabolic action and a maximal activity after similar times. However, the increase of maximal activity was proportional to the amount of dissolved insulin lispro. In the first 6 h after injection, significant differences were found for the GIR\textsubscript{max}, C\textsubscript{max} and AUCs with all mixtures. The metabolic activity, however, with all three mixtures was comparable throughout the experiment.

Our results correlate with those of an earlier manual glucose clamp study, in which the metabolic effects of identical mixture ratios and doses were investigated in 10 subjects\cite{120}. Contrary to our study, the mixtures used in that study were prepared each time immediately prior to use and injected, i.e., the study was not based on premixed insulin preparations. Moreover, not all subjects received all preparations (unbalanced study design). So far, neither premixed preparations nor preparations mixed immediately prior to injection have been investigated for a possible difference in their metabolic actions by means of separate injections into the same injection site.

One disadvantage of our investigation is that the time-action profile of NPL insulin was not compared with that of NPH insulin. The information on the time-action profile of NPL insulin corresponds, however, to the data obtained in a small comparative study conducted on eight subjects using a dose of 0.4 U/kg NPL insulin and NPH insulin\cite{120}. It remains to be investigated if the tendency to an earlier onset of action with NPL insulin observed in that study is a reproducible phenomenon.

### 5.2.2 Time-action profile of a premixed insulin containing insulin aspart

In another study with a similar design to that of our study on insulin lispro mixtures, the time-action profile of a premixed 30/70 mixture of insulin aspart and the pertaining protamine-protracted formulation (30/70 premixed insulin analogue) was investigated, compared with the corresponding premixed formulation with human insulin (30/70 premixed human insulin; Actraphane®, U-100; NOVO NORDISK).\cite{122}
**Method**
Twenty-four males (age 26±2 years; body mass index 23.7±1.7 kg/m²); two study days; 0.3 U/kg of 30/70 premixed insulin analogue or 30/70 premixed human insulin; baseline phase 2 h, duration of the experiment 24 h.

**Results and discussion**
The maximal metabolic activity after subcutaneous injection of the 30/70 premixed insulin analogue was higher and was reached earlier than in the 30/70 premixed human insulin (Fig. 5.6; Tab. 5.5). Moreover, the onset of action was faster. As a consequence, the glucose requirements (i.e., the AUCs under the GIR profiles) induced by the 30/70 premixed insulin analogue were higher in the first 4 h after injection (+37%). In the period from 8 h after injection until the end of the experiment, the AUCs for the 30/70 premixed insulin analogue were, however, lower than in the 30/70 premixed human insulin (\( \text{AUC}_{480-1440 \text{ min}} \) 1.42±0.86 vs. 2.11±0.93 g/kg x 960 min; \( p<0.02 \)). The total metabolic action, however, did not differ.

The serum insulin concentrations peaked earlier and were higher in the 30/70 premixed insulin analogue than in the 30/70 premixed human insulin (Fig. 5.6; Tab. 5.5). The AUCs under the serum insulin concentrations were different during the first 10 h after injection but similar over 24 h.

The results of our study showed that the pharmacodynamic properties of insulin aspart, namely a fast onset of action and a stronger action during the first hours after injection, are maintained in a premixed 30/70 combination. When the effect of the soluble proportion of insulin aspart in the 30/70 mixture diminishes after approximately 4 to 6 h, the time-action profile of the protamine-protracted insulin aspart is lower than that registered for the 30/70 premixed human insulin after the same period of time (corresponding to the differences in serum insulin levels).

Until now, the pharmacodynamic properties of pure protamine-protracted insulin aspart have not been investigated, neither per se nor compared to NPH insulin. It would also be interesting to compare the metabolic action obtained with a premixed combination to those obtained with separate injections of the two components.

The comparison of time-action profiles of pure dissolved insulin aspart and of short-acting insulin, as investigated in one of our own studies using a dosage of 0.2 U/kg (see pg. 55), and that of the two premixed insulin preparations in a dosage of 0.3 U/kg (with 70% of protamine-protracted insulin aspart or NPH insulin), showed that the course of the metabolic activity of the premixed insulin preparations during the first hours is predominantly determined by the dissolved insulin aspart or short-acting insulin (Fig. 5.7). Only then the action of the long-acting part was seen to postpone the decline of the metabolic action of premixed insulin preparations.
Figure 5.6
Time-action profiles (top) and serum insulin concentrations (bottom) after subcutaneous injection of a premixed 30/70 mixture of insulin aspart and the pertaining isophane protamine-protracted long-acting insulin and of a 30/70 mixture of short-acting insulin and NPH insulin in 24 male subjects.
Table 5.5
Pharmacodynamic and pharmacokinetic summary measures of a premixed 30/70 combination of insulin aspart and the isophane protamine-protracted long-acting insulin and of short-acting insulin and NPH insulin, determined in 24 healthy male subjects (difference with 95% confidence intervals).

<table>
<thead>
<tr>
<th></th>
<th>30/70 premixed insulin analogue</th>
<th>30/70 premixed human insulin</th>
<th>difference</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GIR&lt;sub&gt;max&lt;/sub&gt; (mg/kg/min)</td>
<td>9.7±2.3</td>
<td>7.4±1.7</td>
<td>2.3 (1.3, 3.2)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>t&lt;sub&gt;max&lt;/sub&gt; (min)</td>
<td>127±24</td>
<td>185±52</td>
<td>-57 (-81, -33)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>t&lt;sub&gt;early 50%&lt;/sub&gt; (min)</td>
<td>41±15</td>
<td>57±22</td>
<td>-15 (-26, -4)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>t&lt;sub&gt;late 50%&lt;/sub&gt; (min)</td>
<td>360±147</td>
<td>574±273</td>
<td>-220 (-321, -119)</td>
<td>&lt;0.0002</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;0-90&lt;/sub&gt; (g/kg x 90 min)</td>
<td>0.44±0.15</td>
<td>0.26±0.14</td>
<td>0.18 (0.09, 0.26)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;0-240&lt;/sub&gt; (g/kg x 240 min)</td>
<td>1.77±0.43</td>
<td>1.29±0.34</td>
<td>0.48 (0.28, 0.68)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;0-600&lt;/sub&gt; (g/kg x 600 min)</td>
<td>3.45±0.92</td>
<td>3.11±0.68</td>
<td>0.33 (-0.11, 0.77)</td>
<td>N.S.</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;0-1440&lt;/sub&gt; (g/kg x 1440 min)</td>
<td>4.49±1.52</td>
<td>4.74±1.29</td>
<td>-0.26 (-1.11, 0.59)</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>30/70 premixed insulin analogue</th>
<th>30/70 premixed human insulin</th>
<th>difference</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt; (pmol/L)</td>
<td>183±12</td>
<td>101±8</td>
<td>82 (57, 107)</td>
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<tr>
<td>t&lt;sub&gt;max&lt;/sub&gt; (min)</td>
<td>115±3</td>
<td>177±13</td>
<td>-63 (-89, -37)</td>
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<td>t&lt;sub&gt;early 50%&lt;/sub&gt; (min)</td>
<td>20±2</td>
<td>40±3</td>
<td>-20 (-26, -14)</td>
<td>&lt;0.0001</td>
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<tr>
<td>t&lt;sub&gt;late 50%&lt;/sub&gt; (min)</td>
<td>276±8</td>
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<td>&lt;0.0001</td>
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<td>AUC&lt;sub&gt;0-90&lt;/sub&gt; (nmol/L x 90 min)</td>
<td>12.5±5.3</td>
<td>7.8±2.8</td>
<td>4.7 (3.2, 6.2)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;0-240&lt;/sub&gt; (nmol/L x 240 min)</td>
<td>32.9±11.2</td>
<td>21.1±7.6</td>
<td>11.7 (7.7, 15.7)</td>
<td>&lt;0.0001</td>
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<tr>
<td>AUC&lt;sub&gt;0-600&lt;/sub&gt; (nmol/L x 600 min)</td>
<td>50.5±17.9</td>
<td>41.3±12.5</td>
<td>9.2 (2.0, 16.5)</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;0-1440&lt;/sub&gt; (nmol/L x 1440 min)</td>
<td>59.3±24.7</td>
<td>61.9±16.1</td>
<td>-2.6 (-13.3, 8.2)</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

5.3 Long-acting insulin analogues

5.3.1 Properties of long-acting insulin analogues
The objective of the development of long-acting insulin analogues is to obtain insulin preparations which provide a more adequate basal insulin substitution than that obtained with the preparations currently in use. An insulin preparation characterised by a time-
An action profile with a flat course and as little variability as possible and a duration of action of 12 or 24 h is what is required. A protracted mechanism of action can be achieved by using retarding mechanisms incorporated in the insulin structure itself, leaving it no longer necessary to supplement such preparations with substances foreign to the body (e.g., protamine in NPH insulin). Furthermore, with such clear insulin preparations, it would no longer be necessary to thoroughly mix the formulation before use, as is the case with the suspensions currently in use.

A basic question in the development of long-acting insulin analogues is that of the desired duration of action. For insulin therapy, several aspects must be taken into account. If the duration of action is longer than 24 h, there is the risk of an accumulation of the insulin action after several days with one daily injection. Since this accumulation produces a constantly raised blood insulin level, there is the risk of severe hypoglycaemic events when the insulin requirements of the body decrease—as is the case during exercise.

Figure 5.7
Time-action profiles of a premixed 30/70 mixture (0.3 U/kg body weight) of insulin aspart and the corresponding isophane protamine-protracted long-acting insulin (solid line) and a corresponding mixture of short-acting human insulin and NPH insulin (dotted line) as compared to the time-action profiles determined after subcutaneous injection of insulin aspart alone (solid line) and short-acting insulin alone (0.2 U/kg body weight, dotted lines).
This problem is known to occur with the extremely long-acting ultralente beef insulin and with protamine zinc insulin preparations, which have a duration of action of >30 h.

It is easier to adapt the insulin dose to the current insulin requirements if the duration of action does not exceed 24 h or if the duration of action is only 12 h. In the latter case, the disadvantage would be that the long-acting insulin would have to be injected twice daily. This would actually enable a more flexible insulin therapy.

In order to slow down the absorption of long-acting insulin analogues from the subcutaneous insulin depot and thus flatten their course of action, various mechanisms can be used. The isoelectric point of insulin, i.e., the pH at which insulin is least hydrosoluble) can be shifted (by the substitution of amino acids at the C-terminal portion of the B-chain) from 5.4 to a neutral pH of 7.4.

After injecting a slightly acid preparation of such an insulin analogue, there will be a precipitation of relatively small crystals, all similar in size, at the physiological pH in the subcutaneous tissue. Basic amino acids (i.e., those with positive charges) are incorporated to shift the isoelectric point, and there will be a neutralisation of carboxylic acids, such as in di-arginyl insulin (two arginine amino acids attached to the C-terminal end of the B-chain) or in NovoSol Basal® (with modifications at position A21, B27 and B30) (see pg. 50).

The clinical trials with NovoSol Basal revealed that the patients with type 1 diabetes mellitus who participated in the trials had to increase the dose of this insulin analogue considerably in order to achieve the same metabolic control (fasting blood glucose levels in the morning) as with Ultratard. This low bioavailability of NovoSol Basal compared with Ultratard (approximately 30%) is one of the main reasons for the discontinuation of this insulin analogue. Histological studies revealed that a massive migration of macrophages into the insulin depot after subcutaneous injection of NovoSol took place. The macrophages engulfed the insulin crystals dispersed in the tissue, and degraded the insulin, rendering a part of the administered insulin no longer available for absorption (see pg. 38).

In the insulin analogue HOE901 (insulin glargine (Lantus®)), the amino acid asparagine was substituted with glycine at position A21 (end of the A-chain) (Gly (A21)-Arg (B31, B32)-human insulin) to improve stability—different from di-arginyl insulin, previously under development. This introduces another retarding mechanism apart from the shifting of the isoelectric point (see above).

The intermolecular distances between the monomers forming a hexamer of insulin glargine are smaller than in human insulin, which results in an enhancement of the cohesional forces between the six insulin molecules of a hexamer (“crystal contact engineering”). Hence, the water content—as a measure of the packing density of the insulin molecules in a hexamer—is also decreased. In this situation, it is necessary to add greater amounts of zinc (0.5-2 µg/IU) to obtain a stable preparation. Disadvantages of
This long-acting insulin analogue lie in the acid pH of 4.0 and the excess zinc. Therefore, insulin glargine cannot be mixed with neutral short-acting insulin, as the time-action profile of the short-acting insulin would change immediately, due to the differences in the pH value and to the binding of zinc. Since insulin glargine is a clear, soluble preparation, there is no need for thorough mixing before use. With respect to receptor binding, activation of the initial insulin signalisation cascade and its mitogenic activity, insulin glargine behaved in one study like human insulin, but differently according to another study.130

The time-action profile of insulin glargine was investigated in a manual glucose clamp in 12 subjects.131 Two insulin glargine formulations, differing only in their zinc concentration (15 or 80 µg/ml) were studied. The time-action profiles of the two insulin glargine formulations showed almost no differences from each other, but differed considerably from the time-action profile of the NPH insulin, investigated for comparison. The profile of insulin glargine was flatter than that of NPH insulin: \( \text{GIR}_{\text{max}} 2.14\pm0.75 \) (15 µg zinc/ml) and 1.90\pm0.62 mg/kg/min (80 µg/ml) for insulin glargine vs. 4.02\pm2.22 mg/kg/min NPH insulin, \( t_{\text{max}} 12:08\pm3:46 \) h (15 µg zinc/ml) and 12:59\pm4:44 h (80 µg/ml) for insulin glargine vs. 6:27\pm2:55 h for NPH insulin (p<0.01). At the end of the experiment after 24 h the metabolic effect of insulin glargine had not yet returned to zero (duration of action of NPH insulin 16:22\pm1:45 h). For insulin glargine there was no evidence of a reduced bioavailability in clinical studies conducted over a period of four days.132

An important objective in the development of long-acting insulin analogues is a reduction of the great intra- and interindividual variability of the insulin action, as it is observed with the current long-acting insulin preparations. The results of the studies conducted so far, reveal at least a tendency towards achieving that goal (see below).92 The reason for the reduced variability is seen in the more regular absorption given by the smaller and more homogeneous size of the insulin crystals which are formed in the subcutaneous tissue. Another reason seems to be that the clear insulin analogue preparations can diffuse in a larger area of the subcutaneous tissue before precipitating as crystals, unlike the conventional preparations, in which the crystals remain restricted to a rather small area.133

Long-acting insulin analogues, showing a reduced in vitro sensitivity to chemical or enzymatic degradation, represent an interesting development, since such insulin analogues may exhibit an increased in vivo bioavailability.134

5.3.2 Time-action profile of des(64,65)-proinsulin

In the early eighties, human proinsulin was studied for its clinical applicability. This single-chain precursor of insulin was characterised by a delayed action after subcutaneous injection, the time-action profile being between that of short-acting insulin and that of NPH insulin.135 Since human proinsulin induces a relatively low metabolic effect compared
with human insulin, rather high amounts of the substance had to be injected to achieve an adequate blood glucose-lowering effect. In clinical studies, fatal cardiovascular events occurred in patients with diabetes mellitus treated with human proinsulin, which were put down to side effects of human proinsulin and led to a discontinuation of that substance. \textsuperscript{100,136}

One of the intermediate products known to occur naturally in \( \beta \)-cells during the bioconversion from proinsulin to insulin, is des(64,65)-proinsulin. \textsuperscript{136-138} Radio immunological determinations of the various circulating intermediate products using specific antibodies indicate that des(64,65)-proinsulin just comes to a small part of these products, whereas des(31,32)-proinsulin takes the highest portion in quantitative terms. \textsuperscript{136,139,140} This means that, among the two possible ways of conversion from proinsulin to insulin, the of opening of the single-chain, annular proinsulin molecule at position 32 and 33 of the B-chain and subsequent removal of the two basic amino acids at position B31 and B32 (des(31,32)-proinsulin) is the preferred one. Unlike proinsulin and des(31,32)-proinsulin, which are both characterised by a low insulin-like metabolic activity, des(64,65)-proinsulin showed a metabolic potency amounting to 55-100\% of that of insulin on a molar basis in animal experiments. \textsuperscript{136}

The higher metabolic activity of the intermediate product des(64,65)-proinsulin can be explained by the opening of the annular proinsulin at the junction between the A-chain and the C-peptide, resulting in a partial liberation of the biologically active centres of insulin. \textsuperscript{112,141} It may also be possible that a higher percentage of des(64,65)-proinsulin compared with des(31,32)-proinsulin is converted to insulin after subcutaneous administration (see below).

Animal experiments and pilot studies in humans have shown the pharmaceutical preparation of des(64,65)-proinsulin to have a time-action profile similar to that of NPH insulin, \textsuperscript{142} the variability of the insulin action after subcutaneous injection being lower than with short-acting insulin and with a lente insulin. \textsuperscript{142,143} These pharmacodynamic properties are based exclusively on the structure of des(64,65)-proinsulin (intrinsic depot activity) and not on the activity of any additives used to delay its action. Des(64,65)-proinsulin does not precipitate forming crystals after subcutaneous injection. The question of how the activity of des(64,65)-proinsulin is delayed, i.e., whether absorption is slowed down or whether the kinetics of the coupling of the des(64,65)-proinsulin molecules to the insulin receptors after absorption are modified compared with the normal insulin molecule, is still under investigation. Studies in rats have shown that after subcutaneous injection, des(64,65)-proinsulin is partly converted to insulin by local tissue enzymes or in the body organs (i.e., in their cells) (\textit{in vivo conversion}). \textsuperscript{144}

Des(64,65)-proinsulin can be prepared by recombinant DNA technology. \textsuperscript{143,145} The goal of one of our own studies was the comparative investigation of the time-action profiles of des(64,65)-proinsulin and NPH insulin. \textsuperscript{146}
Method
Nine subjects (age 26±3 years; body mass index 23.2±1.3 kg/m²); 6 study days; des(64,65)-proinsulin or NPH insulin in three different dosages (complete block design; Table 5.6); des(64,65)-proinsulin (ELI LILLY, Indianapolis, USA) contained per ml: 5.6 mg des(64,65)-proinsulin (molecular weight 9122), 16.0 mg glycerol, 2.8 mg phenol, 1.9 mg NaCl and 37.5 mg zinc; NPH insulin (Humulin N) contained per ml: 6.0 mmol human insulin, 0.14 mg protamine, 1.6 mg m-cresol and 0.65 mg phenol (both preparations with a neutral pH); baseline phase 90 min, duration of the experiment 1200 min.

Results and discussion
The time-action profiles of des(64,65)-proinsulin and NPH insulin in the three different dosages are depicted in Fig. 5.8 together with the functions fitted to them. The GIRmax was different for the three dosages of des(64,65)-proinsulin (F=5.78, p=0.038; Tab. 5.6). The maximal activity rose with increasing doses of NPH insulin, but the differences did not reach statistical significance. The comparison between the two preparations showed that the maximum metabolic activity was higher after subcutaneous injection of des(64,65)-proinsulin than after NPH insulin (F=6.95, p=0.034). In the various dosages of des(64,65)-proinsulin and NPH insulin, the tearly 50% tended to be different (F=4.78, p=0.065), but the differences between the two preparations were the same.

In all dosages of des(64,65)-proinsulin, tmax was achieved earlier (3.5 h) than with NPH insulin (4.0 h; F=12.48, p=0.0096), but the tmax was comparable in all three doses of each preparation. After having reached the maximal activity, the metabolic activity of des(64,65)-proinsulin fell (tlate 50%) faster than that of NPH insulin (F=63.15, p<0.001). There were no differences between the three dosages of each insulin preparation. A comparison between the time-action profiles of des(64,65)-proinsulin and NPH insulin registered after subcutaneous injection of 0.2 U/kg—the latter being the only dose that was used for both insulin preparations—shows the differences in the metabolic activities of the two insulin preparations over time (Fig. 5.9).

The AUC under the GIR profiles—reflecting the entire metabolic activity of the injected insulin preparations—tended to be higher with NPH insulin than with des(64,65)-proinsulin (F=5.29, p=0.055). The subcutaneous injection of the three different doses of des(64,65)-proinsulin led to significant differences in the AUCs (F=6.84, p=0.009). The differences in the AUCs between the three doses of NPH insulin did not reach statistical significance, the same goes for the AUC above the half-maximal GIR. The variability of the metabolic responses tended to be lower with the clear insulin preparation (des(64,65)-proinsulin) than with the insulin suspension (NPH insulin), but this difference did no reach statistical significance (Tab. 5.6): the mean value of the CVs in the three dosages of the two preparations was calculated to be: GIRmax 31% vs. 38%, tearly 50% 30 vs. 28%, tmax 17 vs. 25%, tlate 50% 25 vs. 42%, AUC 33 vs. 39%.
Figure 5.8
Glucose infusion rates after subcutaneous injection of des(64,65)-proinsulin (left column) or NPH insulin (right column) in three different dosages in euglycaemic glucose clamp studies in nine subjects (solid line = arithmetic mean ±1 standard error (thin solid lines)). For statistical analysis log-normal functions were fitted to the individual profiles (thick solid lines).\textsuperscript{146}
The results of this study demonstrate that des(64,65)-proinsulin does not exhibit a time-action profile which would be more suitable than NPH insulin to replace the basal insulin requirement. Whereas the onset of action was comparable for the two insulin preparations, a higher maximal activity was achieved earlier with des(64,65)-proinsulin, and the duration of action was shorter than with NPH insulin. Des(64,65)-proinsulin is thus characterised by a time-action profile located between that of short-acting insulin and that of long-acting insulin. Thus, the time-action profile does not resemble that of NPH insulin determined in pilot studies, but that of human proinsulin.

Based on the results of a pilot glucose clamp study in six subjects and a similar study design, it had been assumed that des(64,65)-proinsulin had twice the metabolic potency of NPH insulin. The dosages of our own investigation were based on that assumption.

Table 5.6
Pharmacodynamic summary measures of des(64,65)-proinsulin and NPH insulin. The percentages indicate the coefficient of variation in each case.
Whereas the metabolic activity of 0.15 U/kg des(64,65)-proinsulin determined in our study was comparable with that of 0.3 U/kg NPH insulin (AUC 2.98 vs. 2.94 g/kg/20 h), the injection of des(64,65)-proinsulin in a dose of 0.2 U/kg led to similar results as NPH insulin in a dose of 0.2 U/kg (2.47 vs. 2.48 g/kg/20 h), and not as NPH insulin in dose of 0.4 U/kg (3.16 g/kg/20 h). Thus, it remains unclear whether the metabolic activity of des(64,65)-proinsulin is comparable with that of NPH insulin, or—as assumed when planning the study—twice as high as that of NPH insulin.

Similar problems with the definition of the potency of an insulin preparation were encountered in clinical studies with human proinsulin. Due to the influence of the pharmacodynamic properties of an insulin preparation on the result, when using the common methods to determine the potency of an insulin preparation, the use of euglycaemic glucose clamp experiments was proposed as an alternative for standardising the potency of long-acting insulin preparations and insulin analogues.

Due to its time-action profile, des(64,65)-proinsulin is unlikely to yield a homogeneous and constant basal insulin substitution during the day by two daily injections. The advantages of des(64,65)-proinsulin are spoilt by its time-action profile, which is inappropriate for basal insulin substitution.

Figure 5.9
Exponential functions fitted to the time-action profile of des(64,65)-proinsulin (solid line) and NPH insulin (broken line) in identical dosages (0.2 U/kg).
5.3.3 Time-action profile of a long-acting insulin analogue with a connected fatty acid

A novel approach to delay the action of insulin preparations was under clinical development, the insulin analogue insulin detemir (Levemir®, NOVO NORDISK). Another analogue using the same approach with the code W99-S32 (ELI LILLY) had been in development for a while, but this was stopped. E-LysB29-myristoyl, des(B30) human insulin (NN304 or insulin detemir) represents a long-acting soluble insulin analogue of this new class of insulin molecules modified in the following manner: an aliphatic fatty acid with a chain length of 14-C was acylated to the amino acid at position B29, and the amino acid at position B30 was removed. The coupling of this naturally occurring fatty acid to the ε-amino group of lysine at position B29 causes this insulin analogue to avidly bind to specific binding sites for free fatty acids of albumin in the blood and in the interstitial space at the injection site. The consequence of this binding to albumin is that insulin detemir shows no blood glucose-lowering effect after a relatively fast absorption into the bloodstream. An equilibrium between free insulin detemir and insulin detemir bound to albumin is established soon, however, more than 98% of insulin detemir are bound to this carrier protein in the blood. Insulin detemir is released from albumin in a delayed manner (insulin depot at the injection site and in the blood). Therefore, there is only a very small amount of free insulin detemir available in the blood. This free insulin detemir can cross the endothelial barrier, bind to the insulin receptor with a binding affinity of 48% (ref. human insulin), and induce a metabolic effect.

Albumin, as the most common protein in the extracellular fluid (0.6 mmol/L in the plasma and 0.4 mmol/L in the interstitial fluid), represents a multifunctional transport protein which binds many endogenous substances and drugs. Both endogenous substances and drugs become attached to the numerous free binding sites of albumin. The binding constant $K_a$ of insulin analogues with an acylated fatty acid to these binding sites is $10^4$ to $10^5$ M$^{-1}$, depending on the chain length (10 to 16 C atoms) and the kind of acylated fatty acid. The fatty acid used for insulin detemir has the highest binding affinity. The modifications of the insulin molecule do not affect its physical stability in pharmaceutical preparations. Insulin detemir is synthesised in three steps from a single-chain, biosynthetic precursor. The fatty acid makes insulin detemir lipophile, but the addition of zinc results in the formation of hexamers in the pharmaceutical preparation. The latter confer insulin detemir hydrophilicity and solubility.

The differences in the binding properties of insulin detemir to albumins of various species are noteworthy: the binding to porcine (20fold) and rabbit albumin (51fold) is considerably higher than that of human albumin. These measurements were made in a buffered solution. The determination of these values in serum revealed much smaller differences in the binding affinity (1.7fold and 2.5fold, respectively), suggesting that one or more serum components have an influence on the binding to the albumin of each species.
The insulin analogues of the two insulin manufacturers differed by the length of the attached fatty acid, but both manufacturers reported that the time-action profile did not show any peak in animal experiments. In a glucose clamp study with pigs, the subcutaneous injection of equimolar amounts of insulin detemir and NPH insulin led to the same total glucose consumption, which is interpreted as an indication of a comparable metabolic potency. With insulin detemir, the maximal activity was achieved after 6.4 h, with NPH insulin after 3.4 h. The course of the time-action profile was flatter, but the duration of action was not much longer than that of NPH insulin and was similar to that observed with studies of insulin glargine in humans (see above). The lower variability (better reproducibility of action) of insulin detemir observed in this study is put down to the fact that after subcutaneous injection of the clear and neutral solution, there is no crystallisation in the subcutaneous tissue (like with insulin glargine) and the solution can spread in a larger area in the subcutis. The histochemical analyses of skin biopsies suggest that the local reactions are less significant when injecting the soluble insulin detemir as compared to NPH insulin (see pg. 38).

So far, there have not been any data available on the time-action profile of insulin detemir from studies in humans. In one of our own studies, the metabolic effects of insulin detemir were investigated and compared with those of NPH insulin.

**Method**

Open, randomised cross-over study; 11 subjects (age 27±2 years; body mass index 24.1±1.7 kg/m²); five study days; three different dosages of insulin detemir (0.15, 0.3 or 0.6 U/kg body weight), on two other study days identical dosages of NPH insulin (0.3 U/kg; Protaphane®; all insulin preparations by NOVO NORDISK); insulin detemir is a clear, colourless preparation (pH value 7.5, concentration 600 nmol/ml (or 4 mg/ml), corresponding to 100 U/ml in other insulin preparations); baseline phase 120 min, duration of the experiment 24 h.

On the three study days with insulin detemir, samples were taken for the determination of the total plasma concentration of insulin detemir (at NOVO NORDISK) in addition to the samples taken to measure the serum insulin levels. Since virtually all insulin detemir is bound to albumin, the determination of the serum insulin concentration does not provide any information about the pharmacokinetic properties of insulin detemir. It is not possible to determine the small fraction of free and metabolically-active insulin detemir. However, the total insulin detemir concentrations—representing the sum of bound and free insulin detemir—must be determined by using an enzyme-linked immunosorbent assay, developed specifically for that purpose. As the injection of insulin detemir may lead to the development of specific antibodies, blood samples were taken on the first and last study day to determine the antibody titres, using a specific radio immunoassay (NOVO NORDISK).

**Results and discussion**

The subcutaneous injection of insulin detemir in the three different dosages led to an increase in the total insulin detemir concentration to maximal values after 4-6 h.
From these peaks, the total insulin detemir concentrations returned to baseline values within the duration of the experiment. The area under the total insulin detemir concentrations was characterised by a linear and proportional correlation with the administered dose. Compared to the metabolic effect of NPH insulin (mean of the results of the two study days obtained with that insulin preparation), the subcutaneous injection of insulin detemir resulted in a time-action profile characterised by a slower onset of action (Fig. 5.11; Tab. 5.7). Thus, in the first hours after subcutaneous injection, the AUC was larger with NPH insulin than with insulin detemir. From the maximal values achieved 6 to 8 h after the injection of insulin detemir, the activity slowly returned to baseline values. There were almost no significant differences between the three different dosages in any of the summary measures investigated. Only the AUC under the GIR profiles during the first 720 min with 0.15 U/kg was lower than with the two other doses. The area under the GIR profiles, reflecting the total induced metabolic activity, did not correlate with the administered dose (Fig. 5.15). The total metabolic activity induced by insulin detemir was lower than with NPH insulin. Starting from comparable serum insulin concentrations on all five study days, maximal serum insulin concentrations were measured 7-8 h after injection of NPH insulin (Fig. 5.12; Tab. 5.7). The variability of $C_{\text{max}}$ was lower than that of $t_{\text{max}}$ (CV 20 to 25% vs. 70 to 80%). The AUC exhibited a variability between 20% and 35%. The serum C-peptide concentrations fell from comparable basal values by 10 to 40% up to the time of insulin injection. Thereafter, the C-peptide levels fell by a further 10-20%.

The metabolic activity induced by the injection of NPH insulin on both study days peaked within 4 to 5 h after subcutaneous injection (Fig. 5.13; Tab. 5.7). The glucose consumption then decreased slowly, and after 24 h was comparable to the values obtained after insulin detemir administration. The time-action profile of NPH insulin was comparable on the two study days.

No side effects, which may have been attributed to insulin detemir, occurred during the study, i.e., none of the subjects complained about skin irritations at the injection site or other side effects of the insulin administration, and no development of specific insulin detemir antibodies could be observed.

This first investigation of the time-action profile of insulin detemir in humans showed that, compared with the time-action profile of NPH insulin, no pronounced peak in the metabolic effect takes place within some hours after injection. The time-action profile of insulin detemir is, however, not as flat and constant (‘square wave profile’) as it should be for an ideal long-acting insulin analogue.

The observed time-action profile of insulin detemir was comparable to that registered in glucose clamp experiments in pigs.88 The fast increase of the total insulin detemir concentration early after subcutaneous injection suggests that insulin detemir is absorbed rather quickly. The concentration profile shows a marked peak a few hours after injection. For a long-acting insulin analogue with a mechanism based on the binding to albumin, a
rapid loading of the albumin molecule, followed by a constant and slow release, should be ideal. Injections of insulin detemir in regular intervals possibly induce such a loading of the albumin molecules, so that the desired pharmacokinetic behaviour can be induced. 

As a basic problem it has to be considered that, even though the three different dosages of insulin detemir led to a proportional increase in the total insulin detemir concentrations, no corresponding proportional increase of the metabolic activity was induced. The fact that no dose-dependent metabolic activity was observed, may have a couple of reasons. The fraction of insulin detemir, which is released from the albumin binding sites and becomes metabolically active, may not be directly proportional to the loading of albumin with insulin detemir. The transendothelial transport of free insulin detemir may follow a different pattern to that of human insulin, i.e., it does not display linear transport kinetics. The measuring of the free insulin detemir concentrations in the blood or in the interstitial space may answer the question whether the transendothelial transport or other factors are the reasons for the lacking dose-response relationship. For methodical reasons, however, this has not been possible to date.

Given the differences in the binding of insulin detemir to albumins of different species and given the resulting differences in the pharmacokinetic properties, the results of animal studies and in vitro experiments can basically not be applied to humans. One consequence of the novel retarding principle of insulin detemir is that the total insulin detemir concentrations do not provide any information about the metabolic effects induced by them and that they cannot be compared with the pharmacokinetic data of, for instance, NPH insulin.

Until now, the place where retardation became effective was solely the subcutaneous tissue, whereas with insulin detemir, this retardation also occurs in the blood and the interstitial fluid in the insulin-sensitive tissues. This is of relevance, as insulin preparations that reside in the subcutaneous tissue for a long time, risk degradation (see pp. 36 and 37). This should make the development of long-acting insulin analogues with a delayed action based on a slowed absorption from the subcutaneous depot difficult. In insulin glargine, the study results did not reveal such problems, so there may be differences in the various insulin analogues with respect to subcutaneous degradation. Another advantage of the clear formulations of insulin detemir, is that there is no need to mix it thoroughly before injection.

As for any novel pharmacological principle, a number of open questions have to be clarified: the influence which variations in concentrations of albumin or free fatty acids has on the binding; the intra- and interindividual differences in albumin binding; the immunogenicity; and the displacement from the binding sites by other drugs. If the administration of other drugs leads to a comparably fast, undesired release of such long-acting insulin analogues from the albumin binding sites, this might be potentially dangerous—inducing episodes of hypoglycaemia. Investigations regarding this question showed that the binding sites of insulin detemir are different from those of most drugs and that frequently used drugs do not elicit any relevant displacement.
Figure 5.10
Total plasma insulin detemir concentrations after subcutaneous injection of 0.15, 0.3 and 0.6 U/kg in 11 subjects.\textsuperscript{157}

Figure 5.11
Glucose infusion rates recorded after subcutaneous injection of 0.15, 0.3 and 0.6 U/kg insulin detemir and 0.3 U/kg NPH insulin (thick solid line, mean of the two study days with NPH insulin) in 11 subjects.
Figure 5.12
Serum insulin concentrations after subcutaneous injection of 0.3 U/kg NPH insulin in 11 subjects on two study days.

Figure 5.13
Glucose infusion rates recorded after subcutaneous injection of 0.3 U/kg NPH insulin on two study days in 11 subjects.
Table 5.7
Pharmacokinetic and pharmacodynamic summary measures of the long-acting insulin analogue insulin detemir and of NPH insulin determined in 11 healthy subjects after subcutaneous injection.

### Glucose Intra-Subject Ranges - Pharmacodynamic Summary Measures

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<th>Parameter</th>
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<th>0.30</th>
<th>0.15</th>
<th>unit</th>
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</thead>
<tbody>
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<td>0.02 U/kg</td>
<td>unit</td>
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<tr>
<td>Insulin detemir (U/kg)</td>
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<td>mg/kg x 1440 min</td>
<td>mg/kg x 1440 min</td>
<td>AUC 0-1440 min</td>
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<tr>
<td>(CV (%))</td>
<td>5.0 ± 3.7</td>
<td>7.0 ± 6.7</td>
<td>9.0 ± 7.0</td>
<td>min</td>
</tr>
<tr>
<td>Mean day 1/2</td>
<td>3.0 ± 4.0</td>
<td>4.0 ± 5.0</td>
<td>5.0 ± 6.0</td>
<td>min</td>
</tr>
<tr>
<td>Tmax (min)</td>
<td>4.0 ± 5.0</td>
<td>5.0 ± 6.0</td>
<td>6.0 ± 7.0</td>
<td>min</td>
</tr>
<tr>
<td>Cmax (mg/kg/min)</td>
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<td>8.0 ± 9.0</td>
<td>9.0 ± 10.0</td>
<td>min</td>
</tr>
<tr>
<td>CIR (mg/kg/min)</td>
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<td>9.0 ± 10.0</td>
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<tr>
<td>Baseline CIR</td>
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### Glucose Intra-Subject Ranges - Pharmacokinetic Summary Measures

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<td>NPH insulin</td>
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<td>8.0 ± 9.0</td>
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<tr>
<td>Insulin detemir (U/kg)</td>
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<tr>
<td>(CV (%))</td>
<td>9.0 ± 10.0</td>
<td>11.0 ± 12.0</td>
<td>13.0 ± 14.0</td>
<td>min</td>
</tr>
<tr>
<td>Mean day 1/2</td>
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<td>1.5 ± 3.0</td>
<td>2.0 ± 4.0</td>
<td>min</td>
</tr>
<tr>
<td>Tmax (min)</td>
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<td>3.0 ± 4.0</td>
<td>4.0 ± 5.0</td>
<td>min</td>
</tr>
<tr>
<td>Cmax (mmol/l)</td>
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<td>4.0 ± 5.0</td>
<td>5.0 ± 6.0</td>
<td>min</td>
</tr>
<tr>
<td>Cmin (mmol/l)</td>
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<tr>
<td>AUC 0-1440 min</td>
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<td>9.0 ± 10.0</td>
<td>11.0 ± 12.0</td>
<td>min</td>
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Figure 5.14
Linear correlation of the areas under the total insulin detemir concentration profiles and the insulin detemir dose administered.

Figure 5.15
Linear correlation of the areas under the GIR profiles and the insulin detemir dose administered.
6 Influences on time-action profiles

The time-action profiles of insulin preparations are influenced by many factors. It cannot be assumed that the subcutaneous injection of a given insulin preparation will always result in homogenous metabolic activity—on the contrary, a considerable metabolic variability must be expected. Different blood insulin levels are established according to the dose of insulin administered. Insulin differs from other drugs through its relatively slow absorption rate (long absorption half-life), while the elimination rate is rather fast (short elimination half-life). Unlike most other drugs, insulin is absorbed from the subcutaneous depot into the blood slower than it is eliminated (flip-flop kinetics). Consequently, after subcutaneous injection of insulin, the increase of the plasma insulin concentrations is predominantly determined by the elimination rate and the decrease of the plasma insulin concentrations after the maximal levels are determined by the absorption rate.44,158

While there are some studies investigating the insulin levels as a function of the injected insulin dose, there are only a few studies on the changes of the time-action profile of insulin as a function of the injected dose. The subcutaneous injection of five different doses of short-acting insulin in normal weight adults (Fig. 6.1) results in an onset of action after approximately 30 min with all doses, but the further course of action differs depending on the dose administered.118 GIRmax, tmax, and the duration of action increase with increasing doses. In the two highest doses, however, the differences become smaller in the course of action.

The assumption that insulin has a linear and proportional dose-response relationship, is only true for a certain dose-range in short-acting insulin preparations. It has not yet been confirmed by experimental evidence for long-acting insulin preparations. Due to the dependency of the duration of action on the dose, an insulin action beyond the postprandial phase has to be taken into account when using short-acting insulin doses of 10 to 20 U (required for meals which are rich in carbohydrates). The dependency of the pharmacodynamic properties of insulin preparations on the dose, distorts the results of comparison of studies which use different doses.

6.1 Variability of insulin action

The first investigation aimed at describing the variability of insulin absorption in quantitative terms was carried out in 1959.159 In this study, as in most studies of the period following, it was not the variability of insulin action that was studied, but the variability of insulin absorption from the subcutaneous tissue (see pg. 6).25,160-164

The variability of insulin absorption cannot be considered equivalent to the variability of insulin action, because this would mean that, following absorption into the blood stream, the insulin molecules would always elicit a stereotypic effect in the body in terms of quantity and time. Depending on the individual insulin sensitivity, even identical plasma insulin concentrations (as they can be established by intravenous insulin infusion) induce different insulin effects among individuals (interindividual variability). Due to the fact
that the insulin sensitivity varies also within the same individual considerably, a substantial intraindividual variability is also evident. So far, only two studies have been conducted to quantify the variability of the metabolic activity of subcutaneously injected insulin under experimental conditions.\textsuperscript{10,165} We investigated the variability of the insulin action of a short-acting insulin preparation and of the rapid-acting insulin analogue, insulin aspart, in a study based on four subcutaneous injections of the same doses.\textsuperscript{166} According to analyses,\textsuperscript{167} and a clinical study with insulin lispro,\textsuperscript{168} there are some indications of a reduced variability of insulin absorption and insulin action when using rapid-acting insulin analogues.

**Method**

Twenty males (age 26±1 years; body mass index 22.8±1.9 kg/m\(^2\)) were given an abdominal subcutaneous insulin injection of 0.2 U/kg (mean insulin dose 14.4±1.6 U). Nine subjects received short-acting insulin on four study days (Actrapid HM, U-100), and 10 subjects received insulin aspart. Baseline phase 90 min; duration of experiment: 600 min. The intraindividual CVs were calculated from the results obtained for each subject in the four experiments; as were the mean interindividual CVs of all subjects receiving the same insulin preparation. An unpaired, two-sided t-test for the comparison of the intraindividual CVs was carried out; representation of the intraindividual variability according to Bland and Altman.\textsuperscript{169} The correlation coefficients amongst the subjects were calculated, with the repeated measurements of one subject being taken into account, by using a multiple regression method and a weighted correlation coefficient.\textsuperscript{170,171}
**Results and discussion**

During these glucose clamp experiments, the blood glucose concentrations were kept constant at identical levels (Tab. 6.1). The injection of short-acting insulin led to the already described time-action profile with a later onset of action, maximal activity and return to baseline values when compared with the activity of insulin aspart (Fig. 6.2; Tab. 6.1). The observed maximal activity did not differ between the two insulin preparations. The stronger activity of insulin aspart in the period immediately after subcutaneous injection led to a higher AUC in the first 3 h after injection, but the entire metabolic activity over a period of 10 h was comparable. The observed maximal activity did not differ between the two insulin preparations. The stronger activity of insulin aspart in the period immediately after subcutaneous injection led to a higher AUC in the first 3 h after injection, but the entire metabolic activity over a period of 10 h was comparable. The intraindividual variability of the summary measures of short-acting insulin and insulin aspart was not different (Fig. 6.3 and 6.4; Tab. 6.1)—only the t\textsubscript{late 50\%} had a lower intraindividual variability with insulin aspart than with short-acting insulin. The interindivdual CVs were 5 to 20\% higher than the intraindividual ones. For some summary measures (t\textsubscript{max}, t\textsubscript{late 50\%} and AUC within the first 120 min), the interindividual CV was lower for insulin aspart than for short-acting insulin (Tab. 6.1).

The variability of the summary measures does not provide any information about the variability of insulin action over time. The calculation of the mean intraindividual CV of the GIR for each of the two subject groups shows that, while a certain metabolic activity was achieved (GIR >3 mg/kg/min), the CV of both insulin preparations was approximately 25\% (Fig. 6.4).

After subcutaneous injection of insulin aspart, the serum insulin concentrations increased more rapidly to higher values and returned faster to baseline values than with short-acting insulin (Fig. 6.2; Tab. 6.1). The AUCs under the serum insulin profiles for the first 180 min after injection were higher with insulin aspart than with short-acting insulin, whereas the AUCs were comparable over the entire duration of the study. The intraindividual variabilities of insulin absorption (i.e., of C\textsubscript{max} and t\textsubscript{max}) and of insulin action were comparable (Fig. 6.3 and 6.4), but the intraindividual variability was lower with insulin aspart than with short-acting insulin. The interindivdual CVs for C\textsubscript{max} and t\textsubscript{max} were also lower with insulin aspart than with short-acting insulin. Among the pharmacokinetic summary measures, the intra- and interindivdual variability for the AUCs was lower than with the pharmacodynamic summary measures.

This was the first study in which the variability of the insulin action was investigated after four administrations of insulin and a sufficiently long observation period to describe the time-action profile. Repeated subcutaneous injection of short-acting insulin in identical doses displayed an intraindividual variability of the induced metabolic action of 15 to 35\% and an interindivdual variability of 25 to 45\%. Only in some parameters the variability of the insulin action of insulin aspart was lower than that of short-acting insulin. The subcutaneous injection of insulin aspart provoked a more reproducible serum insulin profile than that of short-acting insulin.

Since most of the studies conducted so far have restricted the investigation of variability to insulin absorption, these studies can only provide an orientation as to the variability of...
To sum up, it can be concluded from the studies with labelled short-acting insulin that the time to attenuation of the activity to 50% of the basal value ($T_{50\%}$) has an intraindividual CV of approx. 15%, and an interindividual CV of approx. 30%. For long-acting insulin, 25% and 50%, respectively, were reported. After two subcutaneous injections of 0.2 U short-acting insulin, lente insulin, and NPH insulin into the upper arm of subjects, the serum insulin concentrations displayed an intraindividual variability of 64% and 107% (short-acting insulin), 28% and 34% (lente insulin) and 44% and 68% (NPH insulin), related to the maximal insulin concentration and the time after which this peak was achieved. In two investigations, attempts were made to quantify the degree of variability of insulin action. Using the euglycaemic glucose clamp technique, eight subjects received a subcutaneous injection of 0.4 U zinc insulin per kg body weight on two different days. In the unbalanced study design, four different zinc insulin preparations (one formulated with mixed beef and pork insulin and three with human insulin, in ultralente or lente formulations) were injected subcutaneously into the abdomen. The intraindividual CVs were 35% for the entire amount of glucose infused, 39% for $C_{\text{max}}$, 51% for $t_{\text{max}}$, and 44% for the AUC under the insulin profile. Another report on the same study provided information about the interindividual variability (for six subjects), which was reported to be 45% for the amount of glucose infused, 44% for $C_{\text{max}}$, 55% for $t_{\text{max}}$ and 46% for the AUC under the insulin profile.

The subjects examined in one of our own investigations after subcutaneous injection of four different NPH insulin preparations (the insulin preparations not being different as regards their time-action profiles) showed an interindividual variation of insulin sensitivity of 40%, whereas the intraindividual variability was only 15%. Ziel et al. investigated the variability of insulin absorption and insulin action in eight subjects after two injections of 0.15 U short-acting insulin per kg body weight using a similar study protocol as in our study. However, the duration of the experiment was only 360 min, i.e., the study did not investigate the total time-action profile of short-acting insulin. The intraindividual CV for the AUC under the insulin profile was 11%, thus being smaller than that of the insulin action (23%). The authors did not observe any correlation ($r=0.06$) between the AUC under the insulin profile and the glucose consumption, so that, according to their opinion, one cannot conclude from the blood insulin concentration as to insulin action. The interindividual variability of the total glucose infusion, amounting to 51%, was higher than that of the insulin AUC (18%). The authors conclude from their study that the daily variations of insulin sensitivity have a greater impact on the insulin action than modifications of insulin absorption. Note that the glucose clamp technique, used in that study and in our study, has a certain intrinsic variability, which, in addition to the biological variability of insulin action, contributes to the variability measured. The exact contribution of the measurement technique on the variability can only be determined if another independent measuring method is used to determine the variability of the measurement signal or if a defined signal, not subject to biological variations, is given.
Figure 6.2
Serum insulin concentrations and glucose infusion rates in 10 subjects after subcutaneous injection of insulin aspart (a. and b.) and in nine subjects after subcutaneous injection of short-acting insulin (c. and d.). Each individual curve represents the mean value of the four experiments conducted in one subject (= interindividual variability). The mean values of all individual profiles are depicted in the two lower figures (e. and f.).166
Figure 6.3
Representation of the standard error of the mean of pharmacokinetic and pharmacodynamic summary measures determined in quadruplicate (description of the intraindividual variability). Additionally, mean with standard deviation for the individual means and standard deviations.¹⁶⁹
Figure 6.4
Representation of the standard error of the mean of pharmacokinetic and pharmacodynamic summary measures determined in quadruplicate (description of the intraindividual variability; a. to d.). Additionally, mean with standard deviations for the individual means and standard deviation. In the figures e. and f. the mean values of the intraindividual coefficients of variation are represented for the serum insulin concentrations and for the glucose infusion rates for insulin aspart (solid line) and short-acting human insulin (dashed line) over time.
### Influences on time-action profiles

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unit</th>
<th>Intraindividual CV</th>
<th>Interindividual CV</th>
<th>Intraindividual CV</th>
<th>Interindividual CV</th>
<th>p value intra-individual CVs</th>
<th>p value between preparations</th>
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<td>61±11</td>
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<td>15±7</td>
<td>52.3±7.2</td>
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</table>
Even under the controlled experimental conditions of this study, the intraindividual variability of insulin action was considerable. Short-acting insulin and insulin aspart differed only in some summary measures with respect to the variability of the induced metabolic effect. Probably due to the shorter duration of action, the rapid-acting insulin analogue had a lower variability in its duration of action than short-acting insulin, which may facilitate the implementation of an insulin-replacement therapy. One study with a longer-acting insulin analogue, however, does indicate a lower absorption variability in comparison to Ultratard. 92

6.2 Insulin absorption

The absorption of insulin implies processes involved in the transport of insulin from the extracellular fluid depot in the subcutaneous tissue through the capillary system into the vascular system. The anatomical/physiological conditions in the subcutaneous tissue (i.e., the degree of capillarisation and perfusion) are crucial to both the absorption of insulin from the insulin depot, and to the absorption rate. The steps limiting the absorption rate of the injected insulin from the subcutaneous depot seem to be the distribution and dissolution in the interstitial fluid, the transport to the blood capillaries, the transport across the capillary membrane and the blood flow in the tissue. 174 Since the absorption of insulin is a prerequisite of insulin action, it is necessary to know the factors which influence the absorption rate (Fig. 6.5; Tab. 6.2). 41,94,172,175-178 In the following, the results of our study about the influence skin temperature and insulin concentration has on insulin absorption and insulin action are described. The role of other factors, such as local blood flow, thickness of the subcutaneous fat layer, injection site, injection technique, massage, muscle work, subcutaneous degradation, insulin species, influence of blood glucose and insulin antibodies for insulin absorption, are described elsewhere. 44,179

Influences of temperature

Activities that increase the subcutaneous blood flow in the area of the insulin depot (e.g., hot baths, hyperaemisation by massage, physical exercise, direct insolation of the injection site) or reduce it (local cooling or a cold bath), lead to corresponding changes in the insulin absorption rate. 41 Earlier studies showed that external influences on the skin temperature have a massive impact on insulin absorption. 94,174,176,180 So far, it has not been investigated whether normal skin temperature has an influence on insulin absorption. We studied the influence of skin temperature and the influence of a 2.5fold increase of the insulin concentration (from U-40 to U-100, see pg. 97) on insulin absorption from the subcutaneous insulin depot. 181
Figure 6.5
Schematic representation of the processes of insulin absorption, insulin action and insulin metabolism.41
Table 6.2
Factors influencing the absorption rate and action of subcutaneously injected insulin (↑⇑ = variable degree of influence on increase, ↓⇓ = variable degree of influence on decrease). Influences of insulin antibodies as well as differences in insulin sensitivity, which may rely on many factors, are not considered.

<table>
<thead>
<tr>
<th>Changes at the Injection Site</th>
<th>Differences Between Injection Sites</th>
<th>Insulin Preparation</th>
</tr>
</thead>
</table>

| ↑ Local Degradaion | ↑ age | ↑ Smoking |
| ↓ Ketodossa | ↑ hypoglycemia | ↑ hyperglycemia |
| ↑ Massagy | ↑ Presence of hyperglycemia | ↓ Thickness of subcutaneous adipose tissue |
| Physical state (dissolved or suspension) | Physical state (↓ with smaller dose) | Physical state (↓ colder) |
| Injection depth (↓ deeper) | Injection depth (↓ deeper) | Injection depth (↓ deeper) |
| Heat (↓ colder) | Heat (↓ colder) | Heat (↓ colder) |

6 Influences on time-action profiles
**Method**

Single-blind, randomised study, no glucose clamp study: 50 subjects, 21 female and 29 male (age 26±3 years; body mass index 22.5±1.8 kg/m²); 0.15 U short-acting insulin/kg; two study days; short-acting insulin in the form of U-40 insulin and U-100 insulin; baseline phase 30 min, duration of experiment 45 min; measurement of skin temperature in the area of the injection site using a calibrated electronic thermometer (Sekundenthermometer 1100, Testotherm, Lenzkirch, Germany; accuracy ±0.2°C) with each blood sampling (every 15 min); temperature in the testing room 22 to 24°C; determination of the thickness of the subcutaneous fat layer in the area of insulin injection by ultrasonic measurement (B-mode, Picker L-S 5000, Espelkamp, Germany).

**Results and discussion**

Starting from comparable basal serum insulin values (U-40 37±15 pmol/L, U-100 36±10 pmol/L), the injection of U-40 insulin led to a higher increase 30 min (140±46 pmol/L) and 45 min (164±42 pmol/L) after injection than U-100 insulin (116±37 and 128±35 pmol/L; ANOVA p<0.001) (Fig. 6.6). The differences in the serum insulin concentrations after 30 and 45 min were 25±39 and 37±30 pmol/L, respectively, and their frequency distribution resembled an unimodal normal distribution (N.S. according to the Kolmogorov-Smirnow test). The serum insulin concentrations 30 min after subcutaneous injection of U-40 insulin were higher than with U-100 insulin in 38 subjects, lower in 11 subjects and identical in one subject. After 45 min they were higher in 41 subjects and lower in seven subjects. Suppression of the endogenous insulin production, documented by decreasing serum C-peptide concentrations, occurred after 30 min. After 45 min the C-peptide values were identical with both insulin concentrations (U-40 71±24% vs. U-100 70±23% of the basal values). The blood glucose concentration began to fall from identical basal values 30 min after subcutaneous injection (U-40 4.9±0.5 mmol/L, U-100 4.9±0.4 mmol/L). A comparable decrease in blood glucose (U-40 1.2±0.5 mmol/L, U-100 1.2±0.6 mmol/L) led to similar absolute values after 45 min (U-40 3.7±0.7 mmol/L, U-100 3.8±0.8 mmol/L).

The thickness of the subcutaneous fat layer of the 50 subjects was 6±2 mm (range 2-10 mm) and showed a negative correlation with the serum insulin concentrations 45 min after injection of U-40 insulin (r=-0.40, p=0.004) and U-100-Insulin (r=-0.38, p=0.009). A step-wise multiple regression analysis of the results with U-40 and U-100 insulin revealed that the thickness of the fat layer and the skin temperature accounted for 47% (U-40) and 37% (U-100) of the variance of the serum insulin concentrations after 45 min. The influence of the total individual insulin dose injected was negligible (<0.1% of the variance) for both insulin concentrations.

This study demonstrated that the local skin temperature at the injection site has a significant, positive correlation with the serum insulin concentrations 45 min after injection of U-40 or U-100 insulin (Fig. 6.7). It also showed, for the first time, that normal skin temperature at the injection site has an impact on subcutaneous insulin absorption. In
agreement with earlier reports with labelled insulin (see above),\textsuperscript{32,178} this investigation demonstrates a negative correlation between insulin absorption and the thickness of the subcutaneous fat layer. The absorption of short-acting insulin in the U-40 concentration from the subcutaneous fatty tissue was faster than with U-100 insulin (see below). However, a change in blood glucose—as an indication of insulin action—did not differ in either of the U-40 or U-100 insulin concentrations.

### 6.3 Insulin concentration

In Germany, most insulin preparations have been available in a concentration of 40 U per ml solution (U-40; 1.5 mg/ml or 0.24 mmol/L) or 100 U per ml (U-100; 3.75 mg/ml or 0.59 mmol/L). Like in most other countries, the latter is currently the standard concentration. It is becoming recognised that the concentration and volume of an injected insulin formulation influence on the absorption rate of insulin in that the absorption rate decreases with increasing concentrations. This can possibly be explained by the proportion of hexamerised insulin molecules in the insulin preparations, since that proportion increases with rising insulin concentrations.\textsuperscript{104} The time required for dissociation of the injected hexamers into dimers and monomers by dilution of the injected insulin bolus in the subcutaneous tissue should be longer with U-100 insulin than with U-40 insulin. Hitherto, there are no publications on the current proportion of hexamers. This proportion should be higher in U-100 insulin, as its concentration is 2.5 times as high as that of U-40 insulin. Another reason for the faster absorption of a lower concentrated insulin preparation from the subcutaneous insulin depot is the higher injection volume. With higher volumes, the contact interface between the insulin bolus injected and the capillaries in the subcutaneous tissue is larger. On the other hand, higher concentrations increase the diffusion pressure through the capillary wall.

Differences in the absorption rates were found for highly different concentrations (U-10 vs. U-5000).\textsuperscript{172} For the smaller difference in concentration between U-40 and U-100 short-acting insulin, however, a faster initial absorption of U-40 short-acting insulin from the subcutaneous tissue, compared with U-100 short-acting insulin, was observed in some studies,\textsuperscript{9,182,183} but not in all.\textsuperscript{172,184-186}

In our own glucose clamp study the pharmacokinetic and pharmacodynamic differences after subcutaneous injection of short-acting insulin in a U-40 and a U-100 formulation were investigated. The injection of 12 U short-acting insulin in a U-40 formulation on one study day and the injection of the same dose in a U-100 formulation on the other study day led to higher serum insulin concentrations in eight subjects immediately after insulin injection (after 10, 15, and 20 min) when using U-40 than after administration of U-100 insulin.\textsuperscript{9} The differences observed in the serum insulin concentrations amounted to approximately 10% of the maximal insulin concentrations. The metabolic effects, however, were not different throughout this study, not even in the period immediately after insulin injection.
Figure 6.6
Serum insulin levels, serum C-peptide concentrations and blood glucose concentrations after subcutaneous injection of short-acting insulin (0.15 U/kg body weight) in the form of U-40 insulin (broken line) or U-100 insulin (solid line). Asterisks mark significant differences.181
Figure 6.7
Correlation between the serum insulin concentrations after injection of short-acting insulin (0.15 U/kg body weight) in two different concentrations (U-40 or U-100) and the local, uninfluenced skin temperature at the injection site (U-40 insulin: $y=3.05x-73.8$, $r=0.55$, $p<0.001$; U-100 insulin: $y=2.12x-48.7$, $r=0.47$, $p<0.001$).
In another study on the action of U-40 and U-100 insulin, with a similar design as ours, the authors, likewise, observed a faster insulin absorption after injection of U-40 short-acting insulin when compared with U-100 insulin, but no faster onset of action. In this study, long-acting insulin preparations were evaluated as well. With NPH insulin, the total amount of insulin absorbed was higher for the U-40 formulation than for the U-100 formulation. Accordingly, the total amount of glucose, which had to be infused to compensate for the insulin action, was higher with the U-40 formulation than with the U-100 formulation. With a 25/75 pre-mixed insulin, the absorption rate was lower for the U-100 formulation and the effects were higher with the U-40 formulation. Based on these results, the authors came to the conclusion that with short-acting insulin, the effects of the two concentrations are comparable, whereas with long-acting insulin, the effect of the U-100 formulation is delayed and weaker.

If the onset of action tends to be faster when using a lower insulin concentration, it remains to be seen in how far the time-action profile of a U-40 short-acting insulin preparation differs from that of a rapid-acting insulin analogue.

To this end, an analysis of our study results obtained with six subjects showed that the metabolic effects of the rapid-acting insulin analogue, insulin aspart, occurred faster than those obtained with both concentrations of short-acting insulin. However, the differences were smaller when compared to the U-40 formulation than those observed in comparison with the U-100 formulation. In order to answer this question with a larger sample size, the metabolic effects induced by subcutaneous injection of a U-40 vs. U-100 of a short-acting insulin formulation and of the same U-40 short-acting insulin formulation vs. the insulin analogue insulin aspart was investigated in another glucose clamp study (in the context of our investigation on the variability of insulin action see pg. 85).

**Method**

Nine subjects participated in the experiments with U-100 short-acting insulin and U-40 short-acting insulin (age 25±1 years; body mass index 22.6±2.1 kg/m²); Actrapid HM (NOVO NORDISK) 0.2 U/kg, 15±2 U. Eight subjects participated in the experiments with insulin aspart (U-100) and U-40 short-acting insulin (26±2 years; 22.8±2.1 kg/m²); insulin aspart (NOVO NORDISK) 0.2 U/kg, 14±2 U; baseline phase 90 min; duration of experiment: 600 min.

**Results and discussion**

Within 30 min after subcutaneous injection, short-acting insulin in the U-40 and U-100 formulation induced an increase in glucose requirements (Fig. 6.8; Tab. 6.3). After the onset of action, it took more than 2 h with both concentrations until the maximal insulin action was registered, with a comparable maximal action to be observed thereafter over a period of approximately 2 h. The half-maximal action before maximal activity was achieved 1 h after injection, but it was not until 6 h after injection that the action had
Figure 6.8
Glucose infusion rates (top) and serum insulin concentrations (bottom) after subcutaneous injection of short-acting insulin (0.2 U/kg body weight) in a U-40 (dotted line) or U-100 formulation (solid line) in nine subjects.\textsuperscript{188}
declined to half-maximal values again. It required 10 h after injection until the glucose requirement returned to baseline values. There were no significant differences in insulin action with both insulin concentrations, neither for the summary measures stated nor for the AUCs. The serum insulin concentrations reached a peak of 200 pmol/L 2 h after subcutaneous injection. After 8 to 9 h, the serum insulin concentrations had returned to baseline concentrations. A comparable serum insulin profile was achieved with the two concentrations of short-acting insulin. However, similar to the results obtained in a previous study (see above), serum insulin levels tended to be higher with the U-40 formulation in the first 2 h after injection (Fig. 6.8).

The subcutaneous injection of the rapid-acting insulin analogue insulin aspart resulted in an increase of glucose requirement within 15 min after injection (Fig. 6.9; Tab. 6.3), whereas after injection of U-40 short-acting insulin, the onset of action occurred after 30 min. The comparable maximal insulin action was registered with the insulin analogue after 110 min, whereas 150 min were required with U-40 short-acting insulin. The half-maximal action before maximal activity was achieved after 45 min with the insulin analogue, but after 60 min with U-40 short-acting insulin. With the insulin analogue, the return to half-maximal values after the maximal values was achieved 90 min earlier than with U-40 short-acting insulin. There were no differences in maximal activity and in the total area under the glucose infusion rates with insulin aspart and U-40 short-acting insulin. Higher maximal serum insulin concentrations were achieved 30 min earlier after injection of insulin aspart compared with U-40 short-acting insulin. With insulin aspart, the serum insulin concentrations had returned to baseline values 160 min earlier than with U-40 short-acting insulin. There were no differences between the two groups of subjects as to the pharmacokinetic and pharmacodynamic summary measures for U-40 short-acting insulin.

In agreement with the results of other clinical experimental studies, our study showed that the subcutaneous injection of short-acting insulin in the two most common commercially-available concentrations does not lead to different metabolic effects. The U-40 short-acting insulin also failed to produce a significantly faster increase of the serum insulin concentrations than the U-100 short-acting insulin. Compared with U-40 short-acting insulin, the injection of the rapid-acting insulin analogue insulin aspart—as described several times for the comparison with U-100 short-acting insulin—led to a faster onset and a shorter duration of action. Thus, the injection of U-40 short-acting insulin did not provoke a time-action profile which ranges between that of U-100 short-acting insulin and insulin aspart, but which coincides with that of U-100 short-acting insulin.

A shortcoming of this study has to be noted here: The three insulin preparations (U-40 short-acting insulin, U-100 short-acting insulin, insulin aspart) were not studied in all subjects. Also the sample size (statistical power) of the study was probably not sufficient to detect a significant difference. The results of this study also do not allow a statement concerning the importance of the insulin concentration in long-acting insulin preparations with respect to the induced metabolic effect.
Figure 6.9
Glucose infusion rates (top) and serum insulin concentrations (bottom) after subcutaneous injection of short-acting insulin (0.2 U/kg body weight) in a U-40 formulation (broken line) or of the rapid-acting insulin analogue insulin aspart (solid line) in eight subjects.188
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<th>U-100 SI</th>
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<td>111±35</td>
<td>129±36</td>
<td>0.268</td>
<td></td>
<td>97±21</td>
<td>71±11</td>
<td>0.005</td>
</tr>
<tr>
<td>t\text{basal} (min)</td>
<td>502±89</td>
<td>487±67</td>
<td>0.506</td>
<td></td>
<td>474±71</td>
<td>311±47</td>
<td>0.001</td>
</tr>
<tr>
<td>AUC 0-90 min (nmol/L x 90 min)</td>
<td>12.0±5.0</td>
<td>10.4±3.4</td>
<td>0.497</td>
<td></td>
<td>13.2±4.2</td>
<td>21.9±2.5</td>
<td>0.002</td>
</tr>
<tr>
<td>AUC 0-120 min (nmol/L x 120 min)</td>
<td>17.7±6.5</td>
<td>16.1±4.0</td>
<td>0.622</td>
<td></td>
<td>19.8±6.3</td>
<td>29.9±3.0</td>
<td>0.007</td>
</tr>
<tr>
<td>AUC 0-180 min (nmol/L x 180 min)</td>
<td>28.4±8.5</td>
<td>26.7±5.5</td>
<td>0.702</td>
<td></td>
<td>31.7±9.9</td>
<td>41.3±3.5</td>
<td>0.038</td>
</tr>
<tr>
<td>AUC 0-600 min (nmol/L x 600 min)</td>
<td>52.0±10.8</td>
<td>52.9±5.9</td>
<td>0.806</td>
<td></td>
<td>54.7±17.8</td>
<td>53.3±7.6</td>
<td>0.824</td>
</tr>
<tr>
<td>GIR\text{max} (mg/kg/min)</td>
<td>8.9±2.0</td>
<td>9.5±2.3</td>
<td>0.296</td>
<td></td>
<td>9.5±2.6</td>
<td>10.5±2.6</td>
<td>0.151</td>
</tr>
<tr>
<td>t\text{max} (min)</td>
<td>144±23</td>
<td>156±29</td>
<td>0.228</td>
<td></td>
<td>146±18</td>
<td>108±15</td>
<td>0.006</td>
</tr>
<tr>
<td>t\text{early 50%} (min)</td>
<td>61±11</td>
<td>65±15</td>
<td>0.523</td>
<td></td>
<td>60±11</td>
<td>43±7</td>
<td>0.001</td>
</tr>
<tr>
<td>t\text{late 50%} (min)</td>
<td>345±68</td>
<td>387±68</td>
<td>0.088</td>
<td></td>
<td>360±75</td>
<td>271±35</td>
<td>0.008</td>
</tr>
<tr>
<td>AUC 0-90 min (mg/kg x 90 min)</td>
<td>273±106</td>
<td>286±133</td>
<td>0.767</td>
<td></td>
<td>299±143</td>
<td>485±182</td>
<td>0.005</td>
</tr>
<tr>
<td>AUC 0-120 min (mg/kg x 120 min)</td>
<td>515±170</td>
<td>533±216</td>
<td>0.798</td>
<td></td>
<td>556±225</td>
<td>789±251</td>
<td>0.008</td>
</tr>
<tr>
<td>AUC 0-180 min (mg/kg x 180 min)</td>
<td>1031±272</td>
<td>1074±352</td>
<td>0.674</td>
<td></td>
<td>1108±361</td>
<td>1347±357</td>
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</tr>
<tr>
<td>AUC 0-600 min (mg/kg x 600 min)</td>
<td>2663±512</td>
<td>3011±547</td>
<td>0.122</td>
<td></td>
<td>3002±1084</td>
<td>2724±558</td>
<td>0.395</td>
</tr>
</tbody>
</table>

* paired t-test for comparison of the results within one group of subjects
Table 6.3 (opposite)
Pharmacokinetic and pharmacodynamic summary measures registered after subcutaneous injection of short-acting insulin in a U-40 formulation (U-40) vs. a U-100 formulation (U-100) and vs. subcutaneous injection of insulin aspart in nine and eight subjects (after subtraction of baseline rates).
Inhalative insulin

Insulin is subject to a specific and, even more, an unspecific destruction by proteases. That is why insulin is degraded rapidly in the gastrointestinal tract after oral administration and, therefore, requires parenteral administration. Since the discovery of insulin, a series of different routes of administration have been investigated for their applicability: rectal, intrascrotal, ocular, intracutaneous, perlingual, per vaginam, intraperitoneal, peroral, percutaneous, and intratracheal. Compared with other routes of administration, the subcutaneous insulin injection has a (relatively) good reproducibility and dosage precision, and it is easy to handle. Therefore, the injection or infusion of insulin into the subcutaneous tissue is the only route of administration which has become generally accepted in insulin replacement therapy in clinical routine. However, the disadvantages of this route of administration include: non-physiologic site of administration, relatively slow absorption from the subcutaneous tissue and local degradation of insulin.

The possible routes of insulin administration which have been studied more in detail for their clinical applicability include dermal, oral, nasal and inhalative. Even in the first experiments with percutaneous insulin administration, there was evidence that a certain amount of insulin reached the blood stream successfully, i.e., the blood glucose concentration decreased. Attempts to increase the small percentage of insulin penetrating the skin—in order to achieve clinical relevance for this route of administration—by applying an electrical field above the skin and by using insulin analogues with unilateral electrical charge, have failed. Another approach towards increasing the transport of substances through the skin is through the use of Transfersomes®. In animal experiments, the incorporation of insulin into the vesicle of such fat particles (Transfersulin®) have resulted in an increased insulin transport through the skin into the blood stream.

Immediately after the discovery of insulin, attempts were made to prevent inactivation of the insulin protein in the gastrointestinal tract, and to achieve sufficient intestinal absorption by embedding the insulin into capsules or into layers of fat. None of the various approaches tackled in the following decades have led to a clinically relevant result so far, although new breakthroughs in that direction have continuously been reported.

The spraying of an insulin-containing fluid into the nose leads to a rapid insulin absorption via the nasal mucosa into the blood stream, resulting in a pharmacokinetic profile of the serum insulin concentrations characterised by a rapid increase and decrease of plasma insulin levels. A bioavailability of <10% has been registered for nasal insulin administration, which could, however, only be achieved with the help of absorption enhancers. Considerable intra- and interindividual differences in the bioavailability have also been observed.

Studies on the time-action profile of nasally administered insulin and on the decrease of the blood glucose concentration have shown that in healthy subjects, the onset of action
occurs within 10 min after administration, a dose-dependent peak of the glucose-lowering effect is achieved after 20-45 min, and that this action persists over a period of 90 to 120 min. This profile, which appears to be more favourable for prandial insulin substitution compared with the time-action profile observed with subcutaneous administration of short-acting insulin, makes the nasal administration of insulin an attractive route of administration.

As expected, under experimental conditions, the substitution of the prandial insulin requirement by nasal insulin administration in patients with type 1 diabetes elicits a stronger reduction of the prandial blood glucose level than the subcutaneous administration. In long-term studies on patients with type 1 diabetes, however, no consistent improvement of metabolic control has been achieved with nasal insulin administration. In particular, the nasal insulin administration should be a simple and easy-to-use route of administration for covering the prandial insulin requirement of patients with type 2 diabetes. However, no satisfying results have been attained in the relevant studies. These surprising negative results in the clinical studies have been put down to the short duration of action of insulin preparations in the case of nasal insulin administration.

Subsequently, studies with patients on type 2 diabetes were conducted, in which two or even three high doses (60 and 120 U) were administered nasally at one meal. Only with this unrealistic frequency of nasal insulin administration could a good postprandial metabolic control be achieved. Since clinical studies have failed to reach any improvement of the prandial metabolic control by nasal insulin administration compared with subcutaneous insulin administration, this route of administration does not represent a realistic alternative to subcutaneous insulin replacement therapy.

Pulmonary administration of insulin has undergone fewer intensive studies in the past than, for instance, nasal administration. Pulmonary administration has some specific advantages, such as a great and well-perfused absorptive surface (in humans 50-140 m², 5 litres blood/min), the absence of some kinds of peptidases or proteases compared with the gastrointestinal tract, no direct metabolisation in the liver (‘first pass effect’), a thin alveolar-capillary barrier (in humans 2.2 µm) and small differences in the amount of mucus production. These conditions enable a fast absorption and a rapid onset of action of many drugs after inhalation. In recent years, the administration of proteins by inhalation has been followed with increasing interest, since a number of them are absorbed efficiently by the lung. The absorption mechanisms in the lung and the possibilities to influence them are currently being investigated intensively. Since the factors influencing absorption in the lung are not yet fully understood, improvements to this route of administration are still possible. The degree of absorption of, for instance, proteins varies between 0% and 95%.

In general, the preconditions for the generation of an aerosol and the transport of any drug into the blood are (Fig. 7.1):
The properties of the drug, in this case insulin, must be suitable for inhalation. If the drug is not available in liquid form, but only as a solid, it has to be micronisable.

The nebulising system must be able to generate particles of a suitable size in order to achieve an adequate deposition in the lung (see below). In most conventional inhalers used for nebulisation, the inspiratory airflow has an influence on the distribution of the particle sizes. To generate an aerosol with an adequate particle size, a liquid drug is usually first sprayed into a chamber, its content is then inhaled. This procedure causes a delay between the generation of particles and their inhalation. The humidity has an influence on particle size, too, so that a variability in the particle size distribution has to be reckoned on.

The way of inhalation is assumed to influence the amount of drug deposited in the lung. Depending on the inhalation pattern, the inhaled drug is either deposited in the larger bronchial branches or reaches the finer branches or the alveoli.

The degree of deposition of insulin in the lung depends on the anatomy and the functional status of the lung, i.e., pulmonary diseases or smoking influence the deposition.

The absorption properties of the drug have an impact on the transport via the pulmonary epithelium into the blood. The transport depends on the size of the molecules, their solubility in water and fat and their pK value. Smoking provokes
a massive enhancement of the transport via the alveolar-capillary barrier, as a consequence of an increased permeability of the membranes (see below).

- When the structure of the drug (in this case insulin) is not altered during this process, the biological effect should occur in an identical manner as with other routes of administration after absorption into the blood.

A major problem of the pulmonary administration of drugs is the generation of particles with an adequate particle size. In order to achieve an adequate deposition in the deep lung (alveoli), the particle size must be within a specific range. Only particles with a size <10 µm can be transported into the finer bronchial branches and alveoli with the airflow when inhaled (Fig. 7.2). Larger particles precipitate on the mucous membranes of the mouth and pharynx or in the larger bronchial branches. It is not known if and how much

![Diagram of lung and particle deposition](image)

**Figure 7.2**
*Deposition of inhaled particles in the lung as a function of their diameter.*
of this insulin is absorbed, becoming biologically active. Too small particles (<1 µm), on the other hand, are not deposited on the mucous membranes in the lung. They are exhaled. The measurement of the particle size and the frequency distribution of the particle sizes in an aerosol is difficult from a technical point of view, but possible. It is not the absolute diameter of the particles which is of decisive importance for their distribution in the air, but their aerodynamic diameter, and herein lies the problem. Particles with identical absolute diameters can have different aerodynamic diameters. The size depends on a number of properties of the particles, for instance, on whether the particles are liquid or solid. This problem disturbs the comparison of information on the particle sizes used in different studies (see overleaf) and reduces the meaningfulness of in vitro studies, since the in vivo conditions may differ from the in vitro conditions, especially in this respect. Only the use of lung simulation models, in which the aerodynamic conditions of the lung are partly imitated, provides information on the deposition properties in the lung with the particles sizes of a given insulin preparation. (For a summary of the findings obtained so far concerning the inhalation of insulin in in vitro studies and animal experiments see ref. 207.)

Due to the distribution of insulin to particles with various sizes and deposition properties, the inhalation of insulin cannot be expected to yield 100%, since only a certain part of the total dose of the administered insulin will precipitate. For other drugs administered by inhalation, 10 to 40% of the administered dose are assumed to be deposited in the lung. Since the volume of the particles increases to the third power in relation to the radius, a compromise must be chosen for the particle size between a great penetration in fine bronchial branches and alveoli (fast absorption, but small particles and a small insulin amount) or deposition in big bronchial branches (slow absorption, but large particles and higher insulin amount). Since the administration device determines the particle size (especially for liquid preparations), the amount of substance deposited in the lung depends directly on the inhaler (see below).

An aerosol with an appropriate particle size distribution can be obtained by nebulisation of an insulin solution (fog) or by pulverisation of solid insulin particles (smoke). With conventional inhalers, liquid insulin is often sprayed into a voluminous ‘spacing chamber’ by an atomiser (0.7 to 1.5 L) before the aerosol is inhaled in a complex procedure (see opposite). Up to 75% of the insulin appears to get lost by precipitation of the large liquid particles at the wall of the chamber or in the mouth piece. In another approach, the inhaler used was even larger and the subjects had to breathe in up to 60 times to inhale a sufficient amount of insulin. When using modern inhalers, the frequency distribution of the particle sizes seems to be such that a higher deposition within the deep lung is achieved. But, even with these systems, the subjects had to breathe in through the nebuliser over a period of 7 to 8 min in order to take in an adequate dose. The dimensions and the efforts required for inhalations considerably limit the practical usability of such systems.
When inhaling solid insulin by means of a powder inhaler, the insulin particles generated by micronisation must be deagglomerated upon inhalation to be transferred into an aerosol with a suitable distribution of particles. The energy source is represented by the inspiratory airflow, which vortexes the insulin particles in such a way that—with the flow velocities achieved—they break down into pre-formed microcrystalline particles with a relatively uniform particle size of <7 µm. This process leads to a good penetration of the particles into the lung and enables the deposition of considerable amounts of insulin in the deep lung. If the inhalation of solid insulin always results in a similar size distribution of the particles, this procedure should likewise be associated with a lower intra- and interindividual variability of the metabolic reaction than that of the inhalation of liquid insulin.

Whereas the principal effectiveness of inhalative insulin has been demonstrated several times, there haven’t been any studies showing the time-action profile obtainable with this route of administration as yet. In a study of our own, the time-action profile obtained after inhalation of a solid insulin preparation was investigated.

**Method**

Twelve subjects (age 25±2 years; body mass index 22.1±1.4 kg/m²; eleven non-smokers, one smoker; all of them negative for insulin antibodies) with normal pulmonary function (FEV₁ >80% of the reference value, maximum inspiratory flow 85±5 L/min, volume inspired 4.1±0.3 L, measured in the standing subject using a vitalograph MDI-compact; Vitalograph, Buckingham, United Kingdom).

Three study days, each of them with one of the following routes of administration: six inhalations in 3 min (= inhalation of 99 U insulin (1.4 U/kg body weight, mean body weight 72 kg)). The subjects lying in a bed were asked to expire deeply, then to raise the commercially available powder inhaler to their mouths and to inhale as deeply and strongly as possible (maximum inspiration flow 71±4 L/min, volume inspired 3.4±0.2 L, vitalograph arranged in series with the powder inhaler). After this procedure, they were asked to hold their breath in for 5 to 10 sec. This procedure resulted in an interval of 30 sec between the six inhalations (t=0 min after the last inhalation) and a total duration of the inhalation process of approx. 3 min. A subcutaneous injection of 10 U short-acting insulin (H-Insulin 100 Hoechst®; mean insulin dose 0.14 U/kg body weight) was administered at time t=0 min. Intravenous administration of 5 U short-acting insulin (H-Insulin 100 Hoechst®) was administered at time t=0 min. Baseline phase: 120 min; duration of the experiment: 360 min. This study was not designed as a bioequivalence study. Therefore, only one dose was studied with each route of administration.

Experiments in dogs have suggested a metabolic effect of the inhaled insulin of 10% as regards subcutaneously administered insulin (unpublished data, ASTRA, Uppsala, Sweden). Consequently, a dose, increased 10fold (compared with subcutaneous insulin administration) was used for inhalation. The mean differences between the individual
results obtained with subcutaneously administered insulin minus inhaled insulin were reported with a 95% confidence interval. The bioavailability was calculated as the ratio between the AUC under the serum insulin concentrations (corrected for the baseline serum insulin) after insulin inhalation, and the AUC after subcutaneous insulin administration (relative bioavailability) or after intravenous insulin administration (absolute bioavailability): i.e., (dose intravenous or subcutaneous/dose inhaled) x (AUC inhaled/AUC intravenous or subcutaneous) x 100. The intensity of insulin action was reflected by the glucose requirements during the glucose clamp experiments. For the calculation of the relative and absolute biopotency, the AUC under the insulin profiles was replaced by the AUC under the individual GIR profiles corrected for the baseline GIR.

All participants were declared non-smokers. However, measurement of the cotinine concentration (HPLC method, Bioscientia Laboratorien, Ingelheim, Germany) in urine samples, which were taken during the screening examination, revealed that one participant was a smoker, but he had already taken part in all three experiments. His results were excluded from the data analysis, since it is known that smoking leads to an increased absorption of inhaled insulin (see below).

Results and discussion
Starting from comparable basal concentrations, the serum insulin concentrations after the inhalation of insulin reached maximal concentrations faster than after subcutaneous administration (Fig. 7.3; Tab. 7.1). The comparable maximal concentrations (difference subcutaneous minus inhalation -52 (95% confidence interval -115 to 12) pmol/L) were achieved earlier with insulin inhalation (82 (51-113) min) than after subcutaneous insulin injection. With both routes of administration, the serum insulin concentrations decreased slowly from the maximal values to return to baseline values after 6 h (at the end of the experiment) (-11 (-23 to 2) pmol/L). Whereas the AUC was greater in the first 60 min after insulin inhalation than with subcutaneous injection (-3.3 (-6.0 to 0.7) pmol/L*60 min), the total AUC during the 360 min after administration was greater with subcutaneous insulin injection than with insulin inhalation (10.0 (1.1 to 19.1) pmol/L*360 min). $T_{\text{max}}$ was the only summary measure of insulin inhalation showing a significant correlation with the maximum inspiratory flow ($y=-1.0x+93.9; r=0.58; p<0.02$).

The intravenous administration of 5 U short-acting insulin resulted in pharmacological maximal serum insulin concentrations within one minute after administration in all subjects (Fig. 7.3; Tab. 7.1). The baseline values were reached again after 78±29 min. The absolute bioavailability of inhaled insulin, as regards the intravenous insulin administration, was 5.6±2.8%, and the relative bioavailability, as regards the subcutaneous insulin administration, 7.8±3.5%.

The continuous baseline intravenous insulin infusion, as well as the insulin administered at t=0 min resulted in serum C-peptide concentrations <0.5 nmol/L throughout the duration of the experiment.
The time-action profiles registered after inhalation of the insulin aerosol and after subcutaneous or intravenous insulin injection are represented in Fig. 7.3. The baseline glucose requirements were comparable on all three study days (Tab. 7.1). The onset of metabolic action was faster after insulin inhalation compared with subcutaneous administration, i.e., the $t_{\text{early}50\%}$ was achieved 23 (13 to 33) min earlier. Insulin inhalation led to a lower maximal metabolic activity ($GIR_{\text{max}}$ corrected for baseline values) than subcutaneous administration (2.9 (1.1 to 4.7) mg/kg/min), and this maximal activity was achieved earlier ($t_{\text{max}}$ 40 (8 to 70) min). After 6 h, the GIR was comparable for the two routes of administration (0.1 (-0.9 to 1.0) mg/kg/min) and still increased compared with the baseline GIR (both $p<0.001$). Within the first 60 min, the AUC under the GIR was greater after inhalation than after subcutaneous injection (-0.11 (-0.16 to -0.05) g/kg/60 min). During the total 6 h after insulin inhalation, on the other hand, the AUC was smaller than after subcutaneous insulin injection (0.46 (0.06 to 0.86) g/kg/360 min).

After intravenous insulin injection, the maximal metabolic activity was registered within 15 min, an additional glucose infusion (which was manually controlled) was needed to keep the blood glucose target value at an almost constant level, due to the rapid increase of the glucose requirements. The metabolic activity returned to baseline values within 245±47 min. The total AUC under the GIR profiles was lower after intravenous insulin administration than after insulin inhalation and after subcutaneous insulin injection. The calculation of the absolute biopotency (induced metabolic activity) of inhaled insulin in relation to intravenous administration turned out to be 9.5±4.1%. With reference to the subcutaneous insulin administration, the relative biopotency was 7.6±2.9%.

One participant suffered from shortness of breath in the morning of the third day after insulin inhalation. It remained unclear whether this was a side effect of the inhaled insulin, or could be put down to a slight cold he had developed at that time. However, no changes of spirometric parameters were observed and the symptoms disappeared within one week.

Our study showed that the inhalation of solid insulin particles resulted in a time-action profile characterised by a rapid onset of action and a constant action over a period of 3 h. Afterwards, the action decreased slowly, but still was detectable after 6 h. Six to eight percent of the inhaled insulin was deposited in the lung and absorbed into the blood stream via the alveolar-capillary barrier (bioavailability). The inhalation of pure insulin without absorption enhancers resulted in a biopotency of 8-10%, in reference to the intravenous or subcutaneous insulin administration. The biological effect was thus at least as high as the effect achieved after nasal insulin administration in pharmacodynamic and clinical studies, even though this could only be achieved by using absorption enhancers.\textsuperscript{14,15,199,200,205}

The time-action profile of inhaled insulin observed in our study, the comparably low interindividual variability of the time-action profiles, as well as the (relatively) high biopotency, can be put down to the use of solid, microcrystalline insulin particles. The
Figure 7.3
Glucose infusion rates (top) and serum insulin concentrations (bottom) in 11 subjects after inhalation of 99 U microcrystalline insulin particles, subcutaneous injection of 10 U short-acting insulin and intravenous administration of 5 U short-acting insulin at the time t=0 min (arrow). 213
Table 7.1: Time-action profiles obtained with inhalative insulin.
frequency distribution of these insulin particles was within a narrower defined range than possible with an aerosol of liquid insulin particles. That the metabolic activity continued over a period of 6 h suggests that part of the inhaled insulin remained in the lung for a longer period. Further investigations are necessary to clarify why part of the insulin is absorbed rapidly whereas the action of another part is delayed. A possible explanation of this pharmacodynamic behaviour of inhaled insulin is that small insulin particles are dissolved and absorbed rapidly, whereas larger particles are dissolved more slowly. Even if the exact absorption mechanism of insulin through the lung epithelium is not known, it seems logical that dissolved insulin molecules are absorbed more rapidly as monomers. Given the high local insulin concentrations around the insulin particles, the dissolved insulin molecules may possibly remain in their association status (i.e., in the form of hexamers) for some time so that their absorption rate is reduced. Thus, the absorption properties of insulin might explain the delayed absorption after a rapid initial absorption. In *in vitro* studies with proteases from the lung, the degradation of insulin dimers was faster than that of hexamers, the investigation of the extent of degradation occurring in humans *in vivo* being difficult for methodological reasons. A rapid increase and a comparably slow decrease of the plasma drug concentrations was also observed for other drugs, such as budesonide or terbutaline (see above). Likewise, in these investigations the plasma drug concentrations were still higher than basal values after 6 h.

Earlier investigations on insulin inhalation used liquid insulin preparations which had been converted into an aerosol by different methods. The maximal metabolic activity (decrease of the blood glucose concentration) occurred within 15 to 60 min in both healthy subjects and in patients with diabetes. In patients with type 2 diabetes the blood glucose concentration fell (the basal values being high with >13.9 mmol/L) within some hours after inhalation. The reproducibility of the metabolic response is, however, considered to be poor. In a study on six non-smoking patients with type 2 diabetes, the inhalation of 1 U insulin/kg body weight led to a protracted action, as assessed by the slowly beginning and long-lasting decrease of the blood glucose concentration in the fasting patients. In a study on seven patients with type 2 diabetes, an acceptable suppression of postprandial glycaemia was observed after inhalation of 1.5 U insulin/kg body weight. In a study on six diabetic children, insulin inhalation could not provoke a relevant improvement of metabolic control. In this study considerable intra- and interindividual variations of blood glucose and serum insulin levels were observed. The inhalation of insulin led to only minor changes of the pulmonary function parameters examined. The inhalation of three different doses (40, 80 and 160 U) of liquid insulin, caused a dose-dependent decrease of blood glucose and a corresponding increase of serum insulin. The subjects had, however, to breathe longer via the nebuliser (7 to 8 min) than in our own investigations, and the maximum increase of serum insulin with a comparable dose (80 U) was just 8 µU/ml, whereas it was 40 µU/ml in our study. An insulin
aerosol can also be produced from insulin-zinc crystals, using a moistened gas, and
stored in that form for a longer period of time; however, no data from *in vivo*
investigations using this approach have been reported as yet.²²¹
In studies involving the inhalation of liquid insulin, the reported times to maximal
insulin concentrations and the concentration profiles for non-smokers have been
comparable to those of our own study.²⁰⁶,²¹⁰-²¹²,²¹⁹ Again, part of the liquid insulin seems
to remain in the lung for a longer period of time and to be absorbed slowly, resulting
in a concentration profile similar to that of subcutaneous administration, after the rapid
insulin absorption immediately after administration. It is not known if and how much of
the deposited insulin is degraded in the lung (e.g., by macrophages) (see above).
The permeability of the lung epithelium is known to be much higher in smokers, i.e., after
inhalation of drugs the plasma concentration in smokers is higher and is reached faster than
in non-smokers.²¹⁵,²²²,²²³ This is also true for insulin inhalation, where a stronger and faster
metabolic activity is observed in smoking patients with diabetes and in smoking subjects
without diabetes as compared with non-smokers.²⁰⁶,²¹⁴,²¹⁹ Accordingly, the smoker, who
erroneously participated in our study, had a higher metabolic activity after insulin inhalation
than the non-smokers, whereas after subcutaneous insulin administration, his glucose
consumption was comparably low. Whereas with other drugs, an earlier achievement of
maximal drug concentrations is observed in smokers, maximal insulin concentrations have
been reported to be reached at the same time in smokers and non-smokers.²¹⁴ This leads to
the question as to whether insulin is transported through the bronchial mucosa by selective
mechanisms or by simple diffusion. If membrane thickness or diffusion constants are different
in smokers and non-smokers, a membrane with a low permeability (as in non-smokers)
would be responsible for a later achievement of maximal activity. If, however, the number of
selective absorption mechanisms are different, the peak would be achieved at the same
point of time, but the maximal values would differ. For other substances (e.g., albumin,
horseradish peroxidase) an increased endocytosis (increased selective absorption) has been
found, after the bronchial mucosa had been damaged by ozone. The processes involved in
insulin absorption from the lung seem to be influenced by a series of properties of the
substance.²⁰⁶
Other studies with inhalative insulin have reported a comparable biological effect,
however, the investigational methods were not suited for a reliable quantitative
assessment.²⁰⁶,²¹⁸ The bioavailability calculated from the ratio between the AUC under
the insulin profiles obtained after inhalation and the insulin profiles achieved after
intravenous administration (i.e., not compared with subcutaneous insulin administration)
has been found to be 7 to 16% in patients with diabetes.²¹⁸ In a study on children, the
efficiency of insulin absorption was determined to be 20 to 25%, however, the calculations
computed to achieve this information cannot be traced back based on the data reported.²¹¹
With regard to subcutaneous insulin injection, an insulin absorption of 25% has been
determined in non-smokers after inhalation.²¹⁹ Note that, especially in older publications,
increased titres of insulin antibodies may have influenced the information on absorption kinetics.

Modifications of the inhalation procedure and of the insulin preparation may cause changes of the time-action profile of inhaled insulin, resulting in an even faster and stronger onset of action, as well as in a shorter duration of action. Moreover, an increase of the biological effect would be desirable, which might require the use of absorption enhancers. A number of substances have been investigated for their suitability in studies with nasal insulin (see pg. 106), even though the experiences made there were not necessarily applicable to pulmonary insulin administration. It is not only the immediate irritating effects of such absorption enhancers that are problematic, but the long-term effects which may occur after multiple daily administrations over several years. It must be considered an advantage of the particular insulin preparation under study, that a biopotency of 10% has been reached without absorption enhancers. In the end, such developments may lead to time-action profiles comparable to those registered with rapid-acting insulin analogues (see pg. 54).

An interesting alternative to the modification of the time-action profile of inhaled insulin would be the use of corresponding rapid-acting insulin analogues. So far, it has not yet been investigated whether these insulin analogues are absorbed more rapidly in the lung—such as after subcutaneous administration.

Another difficulty of pulmonary insulin administration (comparable with that of nasal insulin administration) is that the meaningfulness of animal experiments is limited, since there are dramatic differences in the surface of the lung (in proportion to body weight and height) among species. For instance, anaesthetized or intubated animals do not inhale insulin preparations arbitrarily as humans can do. The same is true with absorption enhancers. Our study used a powder inhaler. Such devices work best with an inhalation airflow of 60 L/min. When it is only 30 L/min, a decrease in the percentage of particles reaching the lungs as well as a deposition in the airways is observed. On the other hand, such administration systems enable the inhalation of higher doses per breath than is possible with systems with liquid insulin. The micronised insulin in the powder inhaler is moisture-sensitive, thus reducing its usability in environments with high humidity and requiring an adequate storage and handling of the inhaler. Breathing into the powder inhaler deprives the device of its functionality, at least for a couple of hours. An advantage for pulmonary administration of the solid insulin preparation used in our study, is that no admixtures are required, as they are commonly used for liquid insulin preparations to adjust the pH to achieve isotonicity or for antimicrobial purposes.

Still a number of factors should be clarified with inhaled insulin, such as: optimum inhalation procedure, precision of dosage, dependency of the administration on the inhalation airstream, reproducibility of the insulin action (intra- and interindividual variability), shape of the dose-response curve, influence of smoking habits, and significance of pulmonary diseases. According to some reports, the pulmonary function of patients with diabetes *per se*, is inferior to that of
healthy people in terms of the quality of metabolic control—being considered to contribute to the quality of pulmonary function. Under these conditions, the predictability of the metabolic response to the inhaled insulin would be distorted.224-229 Clinical studies with inhalative insulin should include investigations concerning the safety and possible side effects of long-term administration of inhalative insulin. There are alterations in the pulmonary function, irritations, allergic reactions or the development of insulin antibodies.207
Prandial insulin substitution with a rapid-acting insulin analogue

Compared with short-acting insulin, rapid-acting insulin analogues have advantages in their time-action profiles which should enable a better substitution of the prandial insulin requirements. One cannot start from the assumption that pharmacokineti c and pharmacodynamic properties of insulin preparations, as studied in non-diabetic subjects, are valid in the same form in patients with diabetes mellitus. Although the results of glucose clamp studies with healthy subjects are very helpful in describing the properties of insulin preparations, only studies in patients can show whether the advantages of rapid-acting insulin analogues really are of real benefit for the prandial insulin substitution in patients with diabetes.

It was studied whether the subcutaneous injection of the rapid-acting insulin analogue insulin lispro before a meal rich in rapidly absorbable carbohydrates, led to a smaller excursion of postprandial blood glucose than the injection of short-acting insulin. With respect to the importance of preprandial blood glucose concentrations for the level of prandial excursions, a comparable baseline metabolic situation was established on both study days. Therefore, a study protocol was used in which a comparable and constant blood insulin and blood glucose concentration was maintained over a period of >3 h before the meals. The meal was prepared identically and consumed in the same order and according to the same time schedule. The profile of the prandial blood glucose level within 4 h after the meal was compared on both study days.

Method

Double-blind randomised study; 10 C-peptide-negative patients with type 1 diabetes (glycosylated haemoglobin 7.0±0.5% (DIAMAT, Biorad, Munich; reference range 4.2 to 6.1%); age 29±3 years; duration of diabetes 11±3 years; body mass index 24.2±2.0 kg/m²); last injection of long-acting insulin in the evening of the previous day, during the night and in the morning of the study day only short-acting insulin to achieve metabolic control; all patients were on an intensive insulin therapy regimen; the five patients using a continuous subcutaneous insulin infusion for their insulin therapy switched off their pump at 6 a.m. On the two study days, the patients arrived in the function test room at 8.30 a.m. and were connected to a Biostator to establish a glucose clamp (Fig. 8.1); the blood glucose concentration was kept constant at 6.7 mmol/L during the following 180 min (baseline phase) with a constant intravenous infusion of short-acting insulin (0.2 mU/kg/min); hence the participants had comparable insulin concentrations on the two study days and a sufficient basal insulin supply during the time after the meal. After the baseline phase the patients received a pizza (94 g carbohydrates, 22.4 g protein, 15 g fat), tiramisu (Zott, Mertingen; 100 g, 5.4 g protein, 19.5 g fat and 22.2 g carbohydrates) and a classic coke (Coca-Cola AG, Hamburg; 330 ml, 23.8 g carbohydrates); total carbohydrate content of the meal 140 g (67% pizza, 17% coke, 16% tiramisu; total energy content 4254 kJ; 56% carbohydrates, 32% fat and 12% protein). Until the end of the experiment, the patients
did not receive any further food, but mineral water as required. Immediately before the meal, 15.4±3.5 U insulin (either insulin lispro (Humalog®, ELI LILLY) or short-acting insulin (Humulin R, U-100), 0.14-0.27 U/kg) were injected subcutaneously in the abdominal wall, the individual insulin dose being determined by the patients themselves (identical to the dose on the second study day). The profile of the blood glucose concentration was registered continuously for the subsequent 4 h.

**Results and discussion**

The blood glucose concentrations during the baseline phase were identical on the two study days (insulin lispro vs. short-acting insulin: 6.7±0.3 vs. 6.7±0.3 mmol/L; CV 3.9 vs. 4.0%), the glucose infusion rates being comparable (2.2±1.2 vs. 1.6±1.2 mg/kg/min; N.S.). After the meal the blood glucose concentration reached maximal values of 10.0±1.5 mmol/L with insulin lispro and 11.8±2.8 mmol/L with short-acting insulin within the first 3 h (Fig. 8.2; p<0.05). The maximal blood glucose concentrations were achieved within 41±7 min with insulin lispro and within 66±37 min with short-acting insulin (p<0.05). The maximal differences in the blood glucose concentrations (short-acting insulin minus insulin lispro) after the meal amounted to 4.8±2.2 mmol/L (p<0.0001 towards zero) and were achieved after 110±37 min (Fig. 8.3). The AUC under the
blood glucose curve was only 62% when using insulin lispro as compared with that of short-acting insulin (0.71±0.25 vs. 1.14±0.48 mol/L*180 min; p<0.01; values >3.3 mmol/L; 180 min after insulin injection).

The mean free plasma insulin concentrations during the baseline phase were comparable (93±24 vs. 80±33 pmol/L; N.S.). After the subcutaneous injection of insulin lispro the plasma insulin concentrations reached maximal values of 372±72 pmol/L within 68±18 min. Contrary to the fast increase and fall of the plasma insulin after subcutaneous injection of insulin lispro, the injection of short-acting insulin resulted in a comparably flat insulin profile with maximal values of 264±60 pmol/L after 117±25 min (Fig. 8.4).

This study showed that after a meal rich in rapidly absorbable carbohydrates, the subcutaneous injection of the rapid-acting insulin analogue insulin lispro leads to lower postprandial glucose excursions than the injection of short-acting insulin. A rapid increase in the metabolic activity immediately after injection, as observed in the investigations on the time-action profile of insulin lispro (and insulin aspart), appears to be important in order to limit postprandial hyperglycaemia.

When the time-action profiles of subcutaneously injected insulin preparations are registered during euglycaemic glucose clamps, the measured glucose consumption reflects especially the insulin sensitivity of the peripheral tissues (i.e., predominantly glucose uptake of muscle tissue). The hepatic glucose production is largely suppressed, and the liver hardly takes up any glucose. After a meal, however, the liver plays an important role in limiting postprandial glycaemic excursions. It takes up a large part of the glucose absorbed from the small intestine into the blood, and stores it in the form of glycogen. The hepatic insulin sensitivity is an important factor in the prandial insulin requirement. Our clinical-experimental study showed that the beneficial properties of rapid-acting insulin analogues, as observed in pharmacodynamic studies in healthy subjects (i.e., a higher metabolic activity during the period immediately after subcutaneous injection with insulin lispro as compared to short-acting insulin), lead to a considerable improvement of prandial metabolic control in patients with type 1 diabetes mellitus. In spite of the differences in the metabolic situations of healthy subjects and patients with diabetes, the determination of the time-action profiles in healthy subjects allows the drawing of conclusions concerning the effects of the insulin preparations under everyday conditions in patients with diabetes. The differences in postprandial hyperglycaemia would have been smaller if the short-acting insulin had been injected 30 min before the meal. However, this procedure would not have been in compliance with the recommendations the patients with diabetes mellitus get from experienced diabetologists and would also not have reflected the real patient behaviour.

If future studies were to investigate the effect of the interval between injection and meals on the postprandial blood glucose level, this would require two subcutaneous injections at a meal in order to keep the double-blind study approach. This being the case, the investigator would not know which injection contains the active treatment.
Figure 8.2
Postprandial blood glucose profile before and after the carbohydrate-rich meal, with injection of insulin lispro (solid line) or short-acting human insulin (dashed line) immediately before the beginning of the meals in a dose chosen by the patients on both study days.

Figure 8.3
Difference (solid line, dotted lines ± 1 standard error) in blood glucose on the two study days with insulin lispro and short-acting insulin. The values obtained on the day with insulin lispro have been subtracted from the values obtained on the day with short-acting insulin.
Figure 8.4
Free plasma insulin profile within 4 h after the carbohydrate-rich meal with subcutaneous injection of insulin lispro (solid line) or short-acting human insulin (broken line) at t=0 min.

It has been proven repeatedly that the degree of postprandial blood glucose excursions depends critically on the preprandial glycaemia.\textsuperscript{233,234} Therefore, comparative studies on the blood glucose-raising effect of various foods only make sense when identical baseline conditions are established. This problem has not been taken adequately into account in other studies on the influence of rapid-acting insulin analogues on meal-induced blood glucose excursions.\textsuperscript{235-237} For instance, in a study with various rapid-acting insulin analogues, the blood glucose excursions observed in six insulin-dependent patients with diabetes mellitus as a response to a meal were lower after injection of B9AspB27Glu (not significant with B10Asp) than after injection of short-acting insulin.\textsuperscript{238} However, the authors put down the attenuation of the postprandial blood glucose profiles (maximal blood glucose 13.3 vs. 20 mmol/L) after injection of the insulin analogue solely to the more rapidly rising blood insulin, without taking into account that the preprandial blood glucose level was on average 21\% higher in the experiments with short-acting insulin than in those with the insulin analogue (9.4±1.6 vs. 7.8±1.4 mmol/L). Since the authors had one clearly overweight patient (with a body mass index of 29.6 kg/m\textsuperscript{2}) and also patients with poor metabolic control (HbA\textsubscript{1c} 11.2\%) among the six patients investigated, the study was performed in patients with different insulin sensitivities. In our study, only C-peptide
negative patients with type 1 diabetes with a good metabolic control were studied in order to avoid, as far as possible, interindividual differences in insulin sensitivity. In a study on 10 patients with type 1 diabetes with a study design similar to ours, the postprandial glycaemic excursions were lower after subcutaneous injection of insulin lispro 5 min before a meal than after injection of short-acting insulin 30 min before the meal.\textsuperscript{116} The subcutaneous injection of insulin lispro was reported to cause such a high increase of the blood insulin in the portal blood that (unlike the situation with short-acting insulin) the hepatic glucose production was almost completely suppressed.\textsuperscript{239,240} The lower course of the postprandial blood glucose concentration in the case of subcutaneous injection of insulin lispro can thus be considered a synergistic effect of the peripheral glucose uptake, which is increased rapidly after the meal, and of the reduced glucose production in the liver. In conclusion, our study showed that a reduction of the postprandial hyperglycaemic excursion can be achieved by subcutaneous injection of the rapid-acting insulin analogue insulin lispro vs. short-acting insulin prior to a carbohydrate rich meal. However, an excessive postprandial hyperglycaemia can also be prevented when using an appropriate dose of short-acting insulin.
9 Clinical implications of the results

The first replacement therapy for hormone deficiency, introduced in 1896, involved treatment with thyroid extracts in patients with hypothyroidism. When insulin became available for the treatment of patients with diabetes mellitus in 1922, the replacement therapy for thyroxin was known as a simple, effective treatment, free of short- or long-term complications. For a number of reasons, the hope that the insulin replacement therapy would be just as simple, was not fulfilled. Insulin is not bound to plasma proteins, and the insulin secretion in healthy subjects changes dramatically, depending on the food intake or on physical activity.

The necessity to apply insulin by parenteral administration reverses the relevant physiological conditions, namely, higher insulin concentrations in the peripheral blood than in the hepatic blood. Ideally, insulin replacement therapy would consist of an insulin infusion into the portal vein and the infusion rate would be adapted automatically and immediately, based on the changes of the blood glucose concentration. Since with the technical possibilities available until now, such an invasive insulin therapy in a closed loop, is only possible in very few patients and for a relatively short period of time, this approach does not help the vast majority of patients with diabetes. As long as no alternative routes of insulin administration which provide more favourable time-action profiles are available, subcutaneous insulin treatment will remain the only common route of administration.

A series of pharmaceutical, pharmacokinetic and pharmacodynamic factors have an influence on the insulin absorption, bioavailability and action of subcutaneously injected insulin. An optimum substitution of insulin requirement can be achieved by taking into account important factors such as preprandial glycaemia, insulin sensitivity as a function of the time of the day, the amount of carbohydrates taken in with the meal, the type and absorbability of these carbohydrates, the degree of physical activity before or after the meal and situation-dependent conditions such as a short lunch-break at the canteen or restaurant or long meals at a celebration. With respect to the complex requirements of subcutaneous insulin substitution, a patient with diabetes has comparatively few possibilities to react at his disposal: he is just flexible in varying the insulin dose and the time of injection, given that the type, amount and composition of the meal are fixed.

For subcutaneous insulin therapy, it is crucial to have information about the pharmacodynamic properties of the insulin preparations used, because, as their time-action profiles are not optimal, only with an appropriate combination of short-acting insulin and long-acting insulin is it possible to adequately substitute the considerably varying insulin requirements and thus to continuously establish a good metabolic control. This has been proved to be possible with the currently available insulin preparations. However, more suitable time-action profiles would allow more patients with diabetes to achieve that goal. Compared with the common short-acting insulin preparations, rapid-acting insulin analogues have more suitable pharmacodynamic properties, providing a
more physiological insulin substitution. They should, therefore, facilitate an optimisation of the prandial insulin substitution.

When comparing the properties the ideal rapid-acting insulin analogue should have (see Tab. 5.1) to the properties of those which are currently available, only some of the requirements have been met. However, it is questionable, whether this goal can be achieved by subcutaneous insulin administration at all (see below).

As shown in our own study with a carbohydrate-rich meal and a rapid-acting insulin analogue, an improvement of prandial metabolic control can be achieved by the higher insulin action in the period immediately after the subcutaneous injection.

For the substitution of the basal insulin requirement between meals and during the night, insulin preparations with a more appropriate time-action profile are desirable. NPH insulin, which is frequently used due to its good miscibility with short-acting insulin, has a pronounced maximal activity some hours after injection, therefore its action is not as constant and long-lasting as it should ideally be. The long-acting insulin analogues developed so far do not exhibit such a marked peak in their time-action profile as NPH insulin. The time-action profiles recorded for long-acting insulin analogues are similar to that of Ultratard, i.e., the increase in activity is slower, and the maximal activity is lower and achieved later than with NPH insulin.

The success of an insulin replacement therapy in terms of an improved metabolic control depends on how successful the body’s insulin requirement is covered by an adequate insulin substitution, so that the rise in blood glucose after meals is as low as possible and that the hepatic glucose production is adequately suppressed between meals. The optimisation of the time-action profiles of the insulin preparations used is not a prerequisite to achieving this goal, but certainly supports such efforts.
10 Summary
This book describes the results of systematic clinical experimental investigations on the time-action profiles of insulin preparations. The use of a modified euglycaemic glucose clamp technique allows an assessment of the pharmacodynamic properties of insulin preparations under defined and reproducible conditions in healthy subjects, which has not been possible with former approaches.

The studies were conducted by means of a Biostator which—based on the continuously measured blood glucose concentration—uses a direct negative feedback algorithm to calculate the amount of glucose to be infused intravenously, and the amount required to maintain blood glucose concentrations at a defined level before and after a subcutaneous insulin injection. The amount of glucose required to keep the blood glucose concentration constant constitutes a measure of the biological effect of the subcutaneously injected insulin. The representation of the registered glucose infusion rates over time, after subcutaneous insulin injection, describes the time-action profile of an insulin preparation.

In order to determine the parameters, enabling a more adequate description of the time-action profiles and for their statistical analysis, a function was fitted to the registered time-action profiles. The general use of this glucose clamp method served to enable a comparison of the results of different studies on the time-action profile of insulin preparations, which had only been possible to a limited extent in the past.

A series of questions were investigated using this method. The purpose of the studies addressing these questions were: to describe the time-action profiles of various insulin preparations and insulin analogue preparations; to obtain information about factors with influence on the insulin action; to determine the metabolic activity of insulin with another route of administration; and to study the degree of postprandial hyperglycaemia in patients with type 1 diabetes mellitus under controlled conditions. The results of these studies are of considerable importance for practical insulin therapy.

The time-action profiles of three commonly used preparations (short-acting (regular) insulin, NPH insulin, 25/75 premixed insulin) were investigated in a comparative study. The subcutaneous injection of 0.3 U short-acting insulin per kg body weight resulted in a time-action profile characterised by an onset of action 15-30 min after injection, a maximal activity after 2 to 3 h and a duration of action of up to 10 h. The time-action profile of short-acting insulin is highly dependent on the selected dose. The injection of the same dose of NPH insulin induced a maximal insulin action as early as after 4 h. Afterwards, the action declined slowly and steadily to return to baseline values after 18-20 h. The studied mixture of short-acting insulin and NPH insulin induced a time-action profile located between those of the two pure preparations, reflecting the proportions of the mixture. During the first hours after injection a higher activity was registered, with a maximal activity after 3 h. After 7 h, the course of the time-action profile corresponded to that of NPH insulin. The time-
action profile of premixed insulin did not exhibit, as could have been expected, a biphasic pattern (i.e., two peaks in the metabolic action, the first induced by the proportion of short-acting insulin and the second by the proportion of NPH insulin), but a unimodal shape.

The properties of the currently available insulin preparations are not ideal for insulin replacement therapy. Insulin preparations, which are absorbed more rapidly or more slowly from the subcutaneous insulin depot (thus having a shorter or longer duration of action), can be generated by modifying the primary structure of insulin, i.e., by changing the amino acids at certain sites. Such changes in the pharmacokinetic and pharmacodynamic properties result from the fact that these insulin analogues associate less firmly or more firmly into slowly absorbable hexamers, as the insulin molecules in insulin preparations will do during the process of self-association. Even in insulin analogues with a rapid onset of action, the insulin molecules associate into hexamers, but the cohesional forces are minor compared with human insulin.

The subcutaneous injection of rapid-acting insulin analogues (insulin lispro and insulin aspart) induced a stronger metabolic activity in the first 1-2 h after injection compared with short-acting insulin. Not only the maximal activity was achieved more rapidly, the subsequent return-to-baseline values were also more rapid than with the short-acting insulin.

Premixed insulin preparations using a rapid-acting insulin analogue instead of short-acting insulin had time-action profiles in which the properties of the analogue were maintained, i.e., immediately after injection the action was more pronounced than with the corresponding combinations with human insulin preparations. It must be noted that the long-acting insulin used in such mixtures has to be formulated with the rapid-acting insulin analogue as well, in order to avoid interference of the human insulin in the NPH insulin with the dissolved rapid-acting insulin analogue after prolonged storage.

To cover the basal insulin requirement, the available long-acting insulin preparations are not ideal, due to their time-action profile. The investigation of the time-action profile of an intermediate product, resulting from the bioconversion from proinsulin to insulin (des(64,65)-proinsulin), did not show the flat time-action profile desired for a long-acting insulin. Since the injected insulin is degraded in the subcutis, a new approach was developed to delay the insulin effect, which is no longer based on the protraction of absorption, but on the coupling of a fatty acid to the insulin molecule in order to ensure the binding to the corresponding binding sites of albumin. As a consequence, the insulin molecules are slowly released from the bond, which results in a delayed metabolic activity.

Subcutaneous injection of three different dosages of this long-acting insulin analogue led to a linear, proportional and dose-dependent increase in albumin loading, but there were no corresponding dose-dependent differences in the metabolic effect. Compared to NPH insulin, the time-action profile of this insulin analogue did not show a pronounced maximal activity some hours after injection, but a more even pattern.
The shape of the time-action profiles is influenced by a series of factors. It could be shown that—in addition to the known factors—the skin temperature *per se* (i.e., without specific local modifications) influences the insulin absorption rate from the subcutis. The concentration of subcutaneously injected insulin is another factor influencing the velocity of insulin absorption and insulin action. On the other hand, the administration of commercially available short-acting insulin (U-40 or U-100) did not result in significant pharmacodynamic differences. The subcutaneous administration of short-acting U-40 insulin did not result in a time-action profile that was more comparable to that of a rapid-acting insulin analogue (insulin aspart) than to that of short-acting U-100 insulin.

All factors having an influence on insulin absorption and insulin action led to an intraindividual variability of 15 to 35% and to an interindividual variability of 25 to 45%, depending on the parameter observed, after repeated subcutaneous injection of short-acting insulin or insulin aspart—even under controlled experimental conditions.

Attempts to obtain more adequate time-action profiles by using other routes of insulin administration have not yet been successful. Various routes of administration, such as dermal, oral and nasal administration, have been studied more in detail, but not pulmonary insulin administration. Our study showed that the inhalation of solid insulin particles (99 U) via a powder inhaler resulted in a faster onset of action compared with the subcutaneous administration of short-acting insulin (10 U), whereas the duration of action was similar. With reference to the subcutaneous insulin administration, the inhaled pure insulin had a relative biopotency of 7.6±2.9%. It remains to be investigated whether modifications of the inhalation procedure and/or the insulin preparation will result in a time-action profile which is even more suitable for the substitution of the prandial insulin requirements.

As shown by an investigation on patients with type 1 diabetes mellitus, the subcutaneous administration of the rapid-acting insulin analogue, insulin lispro, caused a more adequate coverage of the prandial insulin requirement than short-acting insulin at a meal which was rich in rapidly absorbable carbohydrates. Due to the properties of insulin lispro, the stronger metabolic activity immediately after subcutaneous injection provoked a smaller increase of postprandial blood glucose concentrations and a faster return to baseline values. The study design used in this investigation, enabled a comparative evaluation of insulin preparations in patients with diabetes mellitus under standardised conditions.

The findings concerning the time-action profiles of different insulin preparations and a series of factors provide the fundamental data for an optimisation of insulin replacement therapy. The consideration of the described results may allow a better substitution of the prandial and basal insulin requirements in patients with diabetes mellitus.
11 References


63. Diamond MP, Simonson DC, DeFronzo RA: Menstrual cyclicity has a profound effect on glucose homeostasis. *Fertility and Sterility* 52:204-208, 1989


91. Heinemann L, Richter B: Clinical pharmacology of human insulin. Diabetes Care 16 (Suppl. 3):90-100, 1993


140. Rhodes CJ, Alarcon C: What beta-cell defect could lead to hyperproinsulinemia in NIDDM? Some clues from recent advances made in understanding the proinsulin-processing mechanism. *Diabetes* 43:511-517, 1994


11 References


216. Thorsson L, Edsbäcker S, Conradson TB: Lung deposition of budesonide from Turbuhaler is twice that from a pressurized metered-dose inhaler P-MDI. *Eur Respir J* 7:1839-1844, 1994