Optimization of pig models for translation of subcutaneous pharmacokinetics of therapeutic proteins: Liraglutide, insulin aspart and insulin detemir

KAREN-MARGRETHE PEDERSEN, ANNA KATRINA JÓGVANSDÓTTIR GRADEL, TRINE PAGH LUDVIGSEN, BERIT ØSTERGAARD CHRISTOFFERSEN, CAROLINE AMALIE FUGLSANG-DAMGAARD, KRISTIAN MOSS BENDTSEN, SUZI HØGH MADSEN, VALENTINA MANFÉ, and HANNE HOFFMANN FRØLUND REFGAARD
COPENHAGEN AND MÅLØV, DENMARK

Prediction of human pharmacokinetics (PK) from data obtained in animal studies is essential in drug development. Here, we present a thorough examination of how to achieve good pharmacokinetic data from the pig model for translational purposes by using single-species allometric scaling for selected therapeutic proteins: liraglutide, insulin aspart and insulin detemir. The predictions were based on non-compartmental analysis of intravenous and subcutaneous PK data obtained from two injection regions (neck, thigh) in two pig breeds, domestic pig and Göttingen Minipig, that were compared with PK parameters reported in humans. The effects of pig breed, injection site and injection depth (insulin aspart only) on the PK of these proteins were also assessed. Results show that the prediction error for human PK was within two-fold for most PK parameters in both pig breeds. Furthermore, pig breed significantly influenced the plasma half-life and mean absorption time (MAT), both being longer in Göttingen Minipigs compared to domestic pigs (P < 0.01). In both breeds, thigh vs neck dosing was associated with a higher dose-normalized maximum plasma concentration and area under the curve as well as shorter MAT and plasma half-life (P < 0.01). Finally, more superficial injections resulted in faster absorption, higher Cmax/dose and bioavailability of insulin aspart (P < 0.05, 3.0 vs 5.0 mm injection depth). In conclusion, pig breed and injection region affected the PK of liraglutide, insulin aspart and insulin detemir and reliable predictions of human PK were demonstrated when applying single-species allometric scaling with the pig as a pre-clinical animal model. (Translational Research 2022; 239:71–84)

Abbreviations: ANOVA = Analysis of Variance; AUC = Area under the plasma concentration vs time curve from time zero to infinity; AUC/dose = Dose-normalized area under the plasma concentration vs time curve from time zero to infinity; AUMC = Area under the first moment curve; AUC% Extrapolated = Percentage of AUCinf due to extrapolation from time of last
measurable concentration to infinity; BW = Body weight; 
C_{\text{max}} = \text{Maximum plasma concentration}; 
\frac{C_{\text{max}}}{\text{dose}} = \text{Dose-normalized maximum plasma concentration}; 
\text{CV} = \text{Coefficient of variation}; 
\text{GLP-1} = \text{Glucagon-like peptide 1}; 
F = \text{Bioavailability fraction}; 
i.v. = \text{Intravenous(ly)}; 
\text{LOQ} = \text{Lower limit of quantification}; 
\text{LOCI} = \text{Luminescence oxygen channeling immunoassay}; 
\text{MAT} = \text{Mean Absorption Time}; 
\text{MRT} = \text{Mean Residence Time}; 
\text{NCA} = \text{Non-Compartmental Analysis}; 
\text{PK} = \text{Pharmacokinetic(s)}; 
s.c. = \text{Subcutaneous(ly)}; 
\frac{T_{1/2}}{\text{dose}} = \text{Half-life in plasma}; 
T_{\text{max}} = \text{Time to maximum plasma concentration}

**AT A Glance Commentary**

**Background**
Prediction of human pharmacokinetics is typically done by allometric scaling on data from several animal species. This study applied single-species allometric scaling on data from two pig breeds to assess the predictions for liraglutide, insulin aspart and insulin detemir.

**Translational Significance**
Reliable predictions were achieved with single-species allometric scaling, suggesting that fewer species and animals can be used to assess the translatability of therapeutic proteins. Furthermore, depending on the desired pharmacokinetic properties of the compound, pig breed and injection region should be taken into consideration in pre-clinical studies since these factors affected the pharmacokinetics of the selected proteins.

**INTRODUCTION**
In order to increase drug development success rates, pre-clinical animal models with a high predictive validity are a prerequisite for predicting the pharmacokinetic (PK) and pharmacodynamic properties of the compound of interest in humans.

The pig is a commonly used large animal model in the pharmaceutical development of new therapeutics for treatment of diabetes and obesity. For many of these compounds, the PK profile is important for drug optimization, e.g. for prandial insulin preparations, a rapid absorption profile is required for a fast onset of action and consequently a more effective reduction of postprandial hyperglycemia, whereas for basal insulin and glucagon-like peptide (GLP)-1 analogues, the daily peak-to-trough ratios may affect the incidence of side effects like hypoglycemia and nausea. Many of these compounds are delivered subcutaneously (s.c.), and the close similarity in the anatomical and physiological characteristics of s.c. tissue between pigs and humans suggests that the pig could be a suitable animal model to predict the PK of s.c. delivered therapeutics. However, the absorption rate of some therapeutic proteins may depend on the injection site and injection depth used for s.c. dosing in both humans and pigs. Consequently, information on the most translational injection site and depth for a given compound in a relevant animal model would be of great value since it would allow for a more accurate prediction of human pharmacokinetics.

In the current studies, we therefore investigated the impact of two commonly used injection sites in pigs on the PK profile of three therapeutic proteins including the GLP-1 analogue, liraglutide, as well as two insulin analogues, insulin aspart and insulin detemir. The translatability of animal PK to human parameters was investigated by allometric scaling which is commonly used in drug development to predict human PK from animal data and has been used for translation of small molecules as well as therapeutic proteins. In the present study, we applied single-species allometric scaling for prediction of s.c. PK, to investigate the translatable potential of domestic pigs and Göttingen Minipigs, both of which represent commonly used pig breeds in drug development.

**MATERIALS AND METHODS**

**Animals and Housing.** All animal experiments were performed according to permissions granted by the Animal Experiments Inspectorate, Danish Ministry of Environment and Food of Denmark. Forty female domestic pigs (Landrace x Yorkshire x Duroc from Gundsoegaard, Roskilde, Denmark) and 10 female Göttingen Minipigs (Ellegaard Göttingen Minipigs, Dalmose, Denmark) were included in the study. The domestic pigs and Göttingen Minipigs had a body weight of 60-65 and 18-25 kg, respectively, at study start. Domestic pigs were fed 2.4 kg chow once daily (Danish TOP SI 601, Danish Agro, Karise, Denmark) and 10 female Göttingen Minipigs (Ellegaard Göttingen Minipigs, Dalmose, Denmark) were included in the study. The domestic pigs and Göttingen Minipigs had a body weight of 60-65 and 18-25 kg, respectively, at study start. Domestic pigs were fed 2.4 kg chow once daily (Danish TOP SI 601, Danish Agro, Karise, Denmark) and Göttingen Minipigs were fed 360 g chow once daily (Mini-Pig, Special Diets Services, Essex, UK). All pigs were individually housed with possibility for snout-to-snout contact with neighboring pigs. The pigs had bedding and straw as enrichment and free access to water at all times. The room temperature was maintained at 22 +/-2°C, relative humidity was between 30-70% and light was on from 6:00AM to 6:00PM. Windows allowed natural light in addition to the mentioned light cycle.
Intravenous Catheterization. One week prior to the experiment, a catheter was implanted in the domestic pigs using Seldinger technique into the jugular vein via the right or left auricular vein under general anesthesia (Secalon™ Seldy, 16 G/42 cm shortened to 28 cm, polyurethane catheter, ref: 681011, Merit Medical, DK-Frederiksberg,). At least one week prior to the experiments, the Göttingen Minipigs had been instrumented with two central venous catheters (Cook TPN™ REDO, silicone catheters, ref: C-TPNS-6.5-90-REDO, William Cook Europe ApS, Bjaeverskov, Denmark) implanted in the caudal vena cava under general anesthesia according to the procedure described by Larsen et al.24 The catheter procedures were followed by ultrasound scans, as described below.

Ultrasound Scanning of the Injection Site. In order to define the optimal area for s.c. dosing in the neck and thigh and to ensure correct dosing, the animals were ultrasound scanned under general anesthesia and the injection area marked by a permanent tattoo. With the animal in lateral recumbency, the neck and the rear thigh were shaved (Fig. 1E and 1F). For the neck region, the injection site was located in the area approximately 4-5 cm caudal to the ear base and 14-15 cm below the dorsal midline in domestic pigs, with the same distances being 3-4 cm and 7-8 cm, respectively, in minipigs of the given size. For the thigh region, the injection site was located approximately 15 cm below the tail base and 9-10 cm cranial to the perineal midline, with the distances being approximately 12 and 7 cm, respectively, in the minipigs. These measures depend on the size of the pig and it was always ensured by ultrasound that the injection area fulfilled the criteria of having at least 5 mm subcutaneous tissue as described below. Using an ultrasound scanner with a high frequency linear transducer (18 MHz) and a scan depth of max. 2 cm, a site with at least 5 mm total thickness of skin and s.c. fat was identified and marked by a tattoo (approx. 2 × 2 cm for the neck and thigh in minipigs, and 2 × 2 cm for neck and 4 × 4 cm for the thigh in LYD pigs). The average thickness of the skin (dermis and epidermis) was 1.8 and 1.7 mm for neck and thigh, respectively, leaving at least 3 mm of subcutaneous fat for the injection. For the neck region, where both superficial and deep s.c. fat layers are present, the aim was to dose only in the superficial layer, but with the possibility of reaching the deep s.c. layer in case of thin superficial layer (Fig. 1C).

Dosing and Blood Sampling. For a detailed overview of groups, formulations, dose volumes and needles used in the different experiments, the reader is referred to Supplementary Table S1. Briefly, on the days of dosing, the pigs were fasted from the afternoon before and until 5-8 h after dosing. Animals were dosed intravenously (i.v.) and s.c. with insulin aspart, insulin detemir or liraglutide (for s.c. dosing the clinical formulations were used, Novo Nordisk A/S, Bagsvaerd, Denmark). All doses were administered to unrestrained pigs that had been trained systematically for handling and in particular unstressed dosing. The calculated s.c. dose volume was rounded up to the nearest half unit and the dose was given approximately in the center of the tattooed area, with the needle perpendicular to the skin (without making a skinfold or causing depression of the skin). The needle was kept in the skin for approx. 10 seconds after dosing before it was withdrawn.

To assess the effect of injection site on compound PK, s.c. administrations were performed in the neck and thigh region (Fig. 1E and 1F). Furthermore, to investigate the effect of injection depth on insulin absorption, insulin aspart PK associated with the use of 3, 3.5, 4 and 5 mm needles was assessed in the thigh region of domestic pigs. In the domestic pigs, dosing in the neck was done opposite to the blood sampling site from the implanted jugular catheter. Blood samples of approx. 1 ml were collected from the central-venous catheter and transferred into EDTA-coated tubes (8 mM) just prior to administration of the test compounds and at regular intervals after dosing. The blood samples were kept on wet ice for maximum 30 minutes until centrifugation for 10 minutes at 4°C and minimum 1500G, and the resulting plasma samples were stored at −20°C until analysis as described below.

Exposure Analyses. The plasma samples were analyzed for levels of insulin aspart, insulin detemir and liraglutide using Luminescence Oxygen Channeling Immunoassay (LOCI)/Alpha-LISA, which is a homogeneous immunoassay method capable of quantitative determination of a wide range of analytes.25 Lower limit of quantification (LLOQ) was determined to be 200 pM for the liraglutide assay and 5 pM for the insulin detemir and insulin aspart assays. For further description of the analyses, please refer to the Supplementary material.

Pharmacokinetic calculations. The non-compartmental analysis (NCA) was performed applying a custom-made R shiny application (Rstudio.com, MA, US), validated against WinNonlin 5.1 (Certara, CA, US). Calculations were performed using individual plasma concentration-time profiles. The calculation of area under the plasma concentration-time curve extrapolated to infinity (AUC) was based on the “linear-up log-down” method and uniform weighting was used for estimation of the terminal rate constant. S.c. bioavailability was calculated as the dose-normalized AUC (AUC/dose) after s.c. administration divided by the AUC/dose after i.v. administration. Mean residence time (MRT) extrapolated to infinity was calculated from area under the first moment curve (AUMC).
Figure 1. Identification of injection site for subcutaneous administration. (A, B) anatomical localization of injection sites in the neck (A) and thigh (B), (A) 1-3: m. trapezius, 5: m. cleidocephalicus, 20: m. cutaneus colli (shaded area), injection site (white square). (B) 4: m. biceps femoris, 9: m. semitendinosus, injection site (white square). (C, D) Ultrasound scans of the neck (C) and thigh region (D) where important anatomical structures have been highlighted. D: dermis; ST: subcutaneous tissue; SSC: superficial subcutaneous tissue; DST: deep subcutaneous tissue; F: fascia separating the superficial and deep subcutaneous tissue; M: surrounding muscles; CT: connective tissue separating the biceps femoris (left) and semitendinosus muscle (right). (E, F) Tattooed areas show the localization of injection sites in the neck (E) and thigh region (F) that are used subcutaneous dosing in the pig. (G, H). Postmortem anatomical inspection of the dermal and subcutaneous tissue upon dissection in the thigh (G) and neck region (H) in domestic pig. The subcutaneous tissue in injection region in the neck is composed of a superficial and deep layer whereas the injection region in the thigh is not divided into layers.
extrapolated to infinity divided by AUC. Mean absorption time (MAT) was calculated as MRT from the individual s.c. profiles subtracted the average MRT from the i.v. profiles.

**Statistical Analysis.** The statistical analyses focused on the AUC/dose and the dose-normalized peak plasma concentration (Cmax/dose) as well as the plasma half-life (T1/2), MAT and MRT. Tmax (time to maximum plasma concentration) was not included in the statistical analyses as this parameter is highly influenced by the sampling regimen. Overall, several statistical analyses were carried out and some included repeated measurements in the same individual. F-test was used to assess the variability between groups and normality was tested using a Shapiro Wilks test. Log transformation of the data was performed when relevant to obtain a normal distribution and Welch's two sample t-tests was subsequently used to compare means between relevant groups (i.e. different injection sites, but only within same pig breed and compound). Please note that for the t-test, exclusion of some animals from the statistical analyses (see below) made a paired test infeasible which should be taken into consideration when interpreting the data since measurements from the same animal cannot be expected to be independent.

Linear mixed effect models were used for repeated measurements to assess the effect of injection site, breed and compound. Similar statistical analyses were performed to assess the effect of injection depth on PK for insulin aspart administered in the thigh in domestic pigs at different injection depths (5.0 mm vs 4.0, 3.5 and 3.0 mm respectively). For a more detailed description of the linear mixed effect models, please refer to the Supplementary material.

**Allometric Scaling.** Allometric scaling was applied for prediction of human PK parameters (s.c. injection into the abdominal region) from the animal PK data and individual animal weights (BW). The models employed were literature based and using half-life as an example the equation used was:

$$T_{1/2,\text{human}} = T_{1/2,\text{species}} \left(\frac{\text{BW}_{\text{human}}}{\text{BW}_{\text{species}}}\right)^{0.25}$$

The equations for scaling of clearance and volume of distribution are similar, but with the exponents 0.75 and 1, respectively. The estimates with two-fold error have been calculated.

Fold error for each compound and PK parameter was calculated as predicted/observed if the predicted value was bigger than the observed value or observed/predicted if the predicted value was lower than the observed value.

**Data Exclusions.** In the NCA and statistical analyses, 12 pigs were excluded due to remarks in relation to dosing (e.g. bent needle, backflow, incomplete sampling) and/or a profile that resulted in an area under the curve (AUC) that deviated more than 2 SDs from the mean AUC. This included three Göttingen Minipigs dosed with liraglutide (two neck and one thigh dosing), one Göttingen Minipig dosed with insulin aspart (three neck and one thigh dosing), two domestic pigs dosed with liraglutide and two dosed with insulin detemir (thigh dosing).

**RESULTS**

**Effect of Injection Site.** The PK profiles and parameters associated with s.c. dosing of liraglutide, insulin aspart and insulin detemir are shown in Fig. 2 and Table 1, respectively. An overview of the MAT and the MRT from s.c. and i.v. dosing of the three compounds is shown in Table 2.

For liraglutide, Cmax/dose, AUC/dose and bioavailability were higher (both breeds, but only significant in the Göttingen Minipigs) and apparent T1/2, MRT and MAT were shorter (both breeds, although MRT and MAT only significantly in domestic pigs) for thigh compared to neck dosing. Furthermore, in Göttingen Minipigs, the Tmax occurred earlier after neck vs thigh dosing. For insulin aspart, the apparent T1/2, MRT and MAT were significantly shorter for thigh compared to neck dosing in both breeds, whereas no significant differences were observed for any other PK parameters. For insulin detemir, Cmax/dose was significantly higher (both breeds) and apparent T1/2, MRT and MAT shorter (only significant in domestic pigs) for thigh versus neck dosing. In both pig breeds, Tmax occurred earlier after neck vs thigh dosing of insulin detemir.

Based on the three-way analysis of variance (ANOVA), pig breed significantly influenced the apparent T1/2, MAT and AUC/dose, although the effect to some degree depended on compound and injection site. None of the remaining PK parameters were affected by pig breed (Supplementary Table S3, S4, S5, S6, S7). Injection site across breeds significantly affected Cmax/dose, AUC/dose, apparent T1/2, MAT and MRT, with Cmax/dose and AUC/dose being higher and MAT, MRT and apparent T1/2 shorter for thigh vs neck (Supplementary Table S3, S4, S5, S6, S7).

S.c. dosing was associated with a longer plasma T1/2 compared to i.v. dosing for all compounds in both pig breeds (Table 1, Supplementary Table S2).

**Effect of Injection Depth.** To assess the effect of s.c. injection depth on insulin aspart PK, the PK profiles associated with 3, 3.5, 4 and 5 mm injection depths in the thigh region were assessed (Figure 3). More
Figure 2. Liraglutide, insulin aspart and insulin detemir plasma concentration versus time profiles. (A, B) Liraglutide levels in domestic pigs (A) and Göttingen Minipigs (B) upon subcutaneous administration of 2 nmol/kg liraglutide into the neck and thigh. (C, D) Insulin aspart levels in domestic pigs (C) and Göttingen Minipigs (D) upon subcutaneous administration of 0.8 nmol/kg (A) or 1 nmol/kg (B) insulin aspart into the neck and thigh. (E, F) Insulin detemir levels in domestic pigs (E) and Göttingen Minipigs (F) upon subcutaneous administration of 2 nmol/kg insulin detemir into the neck and thigh. T = 0 min represents time of dosing. Data are shown as mean ± SE. n = 10 (B, D, F), n = 2 × 8 (A, E) or n = 5–7 (B).
Table 1. Pharmacokinetic parameters in domestic pigs and Göttingen Minipigs upon subcutaneous administration of insulin aspart (0.8 and 1 nmol/kg, respectively) insulin detemir (2 nmol/kg) and liraglutide (2 nmol/kg).

<table>
<thead>
<tr>
<th>Pig strain</th>
<th>T&lt;sub&gt;max&lt;/sub&gt; (min)</th>
<th>C&lt;sub&gt;max&lt;/sub&gt;/dose (pM/nmol/kg)</th>
<th>AUC/dose (min kg/L)</th>
<th>T&lt;sub&gt;1/2&lt;/sub&gt; (h)</th>
<th>F (%)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Neck</td>
<td>Thigh</td>
<td>Neck</td>
<td>Thigh</td>
<td>Neck</td>
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<tr>
<td>Liraglutide</td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Domestic pigs</td>
<td>480</td>
<td>360</td>
<td>4443 ± 2335</td>
<td>6000 ± 2037</td>
<td>7123 ± 1374</td>
</tr>
<tr>
<td>(n=7 (N), n=5 (T))</td>
<td></td>
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<tr>
<td>Göttingen Minipigs</td>
<td>225</td>
<td>420</td>
<td>4011 ± 1315</td>
<td>7199 ± 3635</td>
<td>7137± 1820</td>
</tr>
<tr>
<td>(n=8 (N), n=9 (T))</td>
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<tr>
<td>Insulin aspart</td>
<td></td>
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<td></td>
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<tr>
<td>Domestic pigs</td>
<td>45</td>
<td>45</td>
<td>280 ± 48</td>
<td>297 ± 65</td>
<td>28 ± 3</td>
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<tr>
<td>(n=8)</td>
<td></td>
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<td></td>
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<tr>
<td>Göttingen Minipigs</td>
<td>25</td>
<td>25</td>
<td>185 ± 100</td>
<td>322 ± 188</td>
<td>17 ± 3</td>
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<tr>
<td>(n=7 (N), n=9 (T))</td>
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<tr>
<td>Insulin detemir</td>
<td></td>
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<td></td>
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<tr>
<td>Domestic pigs</td>
<td>38</td>
<td>60</td>
<td>613 ± 221</td>
<td>1009 ± 608</td>
<td>330 ± 88</td>
</tr>
<tr>
<td>(n=2 x 6 +2(N), n=2 x 5 +3(T))</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Göttingen Minipigs</td>
<td>35</td>
<td>50</td>
<td>544 ± 213</td>
<td>1211 ± 676</td>
<td>190**±32</td>
</tr>
<tr>
<td>(n=8 (N), n=7 (T))</td>
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</table>

Data are shown as mean ± standard deviation (SD); T<sub>max</sub> as median and T<sub>1/2</sub> as harmonic mean ± pseudo SD.

*P < 0.05

**P < 0.01 when log data was compared to thigh data by Welch’s t-test. ¹ Violation of normality (Shapiro Wilks test). ² AUC% Extrapolated was above 20%.

N: Neck dosing; T: Thigh dosing; T<sub>max</sub>: time to maximum plasma concentration (C<sub>max</sub>); C<sub>max</sub>/Dose: Dose-normalized maximum plasma concentration; AUC/Dose: Dose-normalized area under the curve from time zero to infinity; T<sub>1/2</sub>: half-life in plasma; F: Bioavailability. ³ Repeated dosing.
superficial injections resulted in earlier $T_{\text{max}}$, higher C$\text{max}$/dose, AUC/dose and bioavailability, and shorter MAT and MRT ($P < 0.05$ or less for comparison of 3 and 5 mm injection depths, Table 3). In the one-way ANOVA, injection depth significantly affected C$\text{max}$/dose and MAT (Supplementary Table S8).

**Prediction of Human Pharmacokinetics.** Single-species allometric scaling was used to assess the translatability of the results obtained from both Göttingen Minipigs and domestic pigs in terms of predicting the PK of liraglutide, insulin aspart and insulin detemir injected s.c. in the abdominal region in humans (Table 4). A direct comparison between human and pig PK data can be seen in Table 4 and Table 5. Although data presents with some degree of variation and divergences, a rough description is that prediction of animal PK data for all analogues is within two-fold of the human reported kinetic data. Of remark, slightly higher s.c. prediction error, between 2.1-2.6 fold, was observed for $T_{1/2}$ and distribution volume/bioavailability for liraglutide as well as $T_{1/2}$ and clearance/bioavailability for insulin aspart in Göttingen Minipigs. For the s.c. $T_{1/2}$ predictions, fold error tended to be lower for thigh dosing.

**DISCUSSION**

In order to evaluate the translatability of commonly used pig models for prediction of human s.c. PK, we have optimized and standardized dosing conditions by characterizing the dosing site and by handling/training the animals in order to reduce variation. We have characterized the Göttingen Minipig and domestic pig models for PK studies by assessing the PK of liraglutide, insulin aspart and insulin detemir after s.c. injection into the neck and thigh region. Furthermore, the effect of injection depth on insulin aspart PK was investigated. Several of the investigated parameters influenced the PK of the three proteins, but altogether the data also showed promising results in terms of single-species allometric scaling.

We found minor differences in the evaluated PK parameters between the two breeds whereas both injection site and injection depth significantly affected the PK. The s.c. injection site affected the PK parameters of all three tested proteins, but more pronounced for liraglutide and insulin detemir where administration in
the thigh vs neck region generally resulted in shorter MRT, MAT and apparent T 1/2 in addition to a higher Cmax/dose and AUC/dose. The data thus indicate a faster and more complete absorption with thigh injection, although the shorter MAT was not associated with an earlier T max. A slower absorption from the neck vs other injection sites (inguinal area, flank, interscapular area) has also been reported in minipigs dosed with the monoclonal antibody, Tocilizumab16, although it should be mentioned that Tocilizumab has a significantly higher molecular weight compared to the analogues used in our study and therefore is likely to enter the systemic circulation through the lymphatic route rather than through s.c. capillaries. 31

In humans, the recommended injection sites for s.c. administration of insulin and GLP-1 receptor agonists include the abdomen, thigh, upper arm and buttock. 32-35 Whereas no dose adjustment is needed in humans with change of injection site when administrating liraglutide33, the absorption of human insulin and certain insulin analogues is affected by the injection site with a faster absorption from the abdomen vs thigh representing a consistent finding.7-13 However, this impact of injection site does not necessarily apply to all insulin preparations as no effect of injection site has been reported for insulin aspart36, insulin glargine 37 and NPH insulin. 38 Furthermore, although the injection site may affect the absorption of acylated analogues such as insulin detemir 14 and insulin degludec 39, it may not be clinically important after repeated dosing since the long half-lives of these analogues result in accumulation and steady state plasma concentrations that are less susceptible to changes in s. c. absorption rate.34,39 Nevertheless, our results show that as for insulin in humans and as reported for Tocilizumab in minipigs16, the injection site in pigs can affect the PK of s.c. administered proteins.

In domestic pigs, more superficial injections were shown to result in a more rapid absorption of insulin aspart which was reflected by an earlier T max, increased Cmax/dose, increased AUC/dose and a shorter MAT. These findings are in agreement with reports from clinical studies in humans where insulin aspart PK has been reported to differ with injection depths of 3 and 3.5 mm vs 4 mm.15 Another study has however shown in domestic pigs that as for insulin in humans, injection site in pigs can affect the PK of s.c. administered proteins. These findings indicate that insulin aspart which was administered in a more rapid absorption from the thigh represents a consistent finding.7-13 Although the injection site may affect the absorption of acylated analogues such as insulin detemir and NPH insulin, it may not be clinically important after repeated dosing since the long half-lives of these analogues result in accumulation and steady state plasma concentrations that are less susceptible to changes in s. c. absorption rate.34,39

In humans, the recommended injection sites for s.c. administration of insulin and GLP-1 receptor agonists include the abdomen and upper arm for insulin aspart and in humans with change of injection site when administrating insulin detemir 14 and insulin degludec 39, it may not be clinically important after repeated dosing since the long half-lives of these analogues result in accumulation and steady state plasma concentrations that are less susceptible to changes in s. c. absorption rate.34,39

Data are shown as mean ± standard deviation (SD). T max is shown as median and T 1/2 as harmonic mean ± pseudo SD.

* P < 0.05 and ** P < 0.01 when compared to values at 5 mm needle length by Welch's t-test on log transformed data.

T max: Time to maximum plasma concentration; Cmax/Dose: Dose-normalized maximum plasma concentration; AUC/Dose: Dose-normalized area under the curve from time zero to infinity; T 1/2: Half-life in plasma; F: Bioavailability; CV: Coefficient of variation; MRT: Mean Residence Time; MAT: Mean Absorption Time (calculated using the MRT from an IV study of aspart in domestic pigs i.e. MRT IV = 7.7 min). AUC% Extrapolated: was below 20 % for insulin aspart for all injection depths. §Repeated dosing.

Table 3. Pharmacokinetic parameters in domestic pigs upon subcutaneous administration of 0.8 nmol/kg insulin aspart in the thigh using injection depths of 3.0, 3.5, 4.0 and 5.0 mm

<table>
<thead>
<tr>
<th>Injection depth (mm)</th>
<th>T max (min)</th>
<th>Cmax/Dose (pM/nmol/kg)</th>
<th>AUC/Dose (min kg/L)</th>
<th>T 1/2 (min)</th>
<th>MRTCSC (min)</th>
<th>MAT (min)</th>
<th>F (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.0 (n=2 × 7+1)</td>
<td>20</td>
<td>424** ± 181</td>
<td>23 ± 2</td>
<td>38 ± 8</td>
<td>71** ± 16</td>
<td>65 ± 16</td>
<td>45.6 ± 8.5 (CV%)</td>
</tr>
<tr>
<td>3.5 (n=2 × 6+2)</td>
<td>30</td>
<td>285 ± 77</td>
<td>22 ± 3</td>
<td>36 ± 6</td>
<td>76 ± 11</td>
<td>68 ± 11</td>
<td>43.6 ± 12.2 (CV%)</td>
</tr>
<tr>
<td>4.0 (n=2 × 8+)</td>
<td>30</td>
<td>275 ± 72</td>
<td>22 ± 2</td>
<td>33 ± 6</td>
<td>78 ± 13</td>
<td>70 ± 13</td>
<td>43.9 ± 7.7 (CV%)</td>
</tr>
<tr>
<td>5.0 (n=2 × 8+)</td>
<td>45</td>
<td>246 ± 60</td>
<td>22 ± 2</td>
<td>35 ± 6</td>
<td>81 ± 12</td>
<td>74 ± 12</td>
<td>42.4 ± 10.7 (CV%)</td>
</tr>
</tbody>
</table>

Data are shown as mean ± standard deviation (SD). T max is shown as median and T 1/2 as harmonic mean ± pseudo SD.

* P < 0.05 and ** P < 0.01 when compared to values at 5 mm needle length by Welch's t-test on log transformed data.

T max: Time to maximum plasma concentration; Cmax/Dose: Dose-normalized maximum plasma concentration; AUC/Dose: Dose-normalized area under the curve from time zero to infinity; T 1/2: Half-life in plasma; F: Bioavailability; CV: Coefficient of variation; MRT: Mean Residence Time; MAT: Mean Absorption Time (calculated using the MRT from an IV study of aspart in domestic pigs i.e. MRT IV = 7.7 min). AUC% Extrapolated: was below 20 % for insulin aspart for all injection depths. §Repeated dosing.
Table 4. Prediction of human pharmacokinetic parameters for a 75 kg person based on allometric scaling. Clinical data in humans (subcutaneous injection into the abdominal region) have been calculated from the references included in the parentheses.

<table>
<thead>
<tr>
<th>Subcutaneous</th>
<th>Intravenous</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>T1/2 (h)</strong></td>
<td><strong>Cl (L/h)</strong></td>
</tr>
<tr>
<td><strong>Cl/f (L/h)</strong></td>
<td><strong>Vz/f (L)</strong></td>
</tr>
<tr>
<td><strong>Liraglutide</strong></td>
<td>Domestic pigs (n=7 (N), n=5 (T))</td>
</tr>
<tr>
<td></td>
<td>Neck</td>
</tr>
<tr>
<td></td>
<td>Neck</td>
</tr>
<tr>
<td></td>
<td>Neck</td>
</tr>
<tr>
<td><strong>Insulin aspart</strong></td>
<td>Domestic pigs (n=2 × 8)</td>
</tr>
<tr>
<td></td>
<td>Göttingen Minipigs (n=7 (N), n=9 (T))</td>
</tr>
<tr>
<td></td>
<td>Humans</td>
</tr>
<tr>
<td><strong>Insulin detemir</strong></td>
<td>Domestic pigs (n=2 × 6)</td>
</tr>
<tr>
<td></td>
<td>Göttingen Minipigs (n=8 (N), n=7 (T))</td>
</tr>
<tr>
<td></td>
<td>Humans</td>
</tr>
</tbody>
</table>

Predictions based on domestic pigs and Göttingen Minipigs are shown as estimates with two-fold error (lower and upper limit). M: males, F: females. #Repeated dosing. T1/2: Half-life in plasma; Cl: Clearance; Cl/f: Cl divided by the bioavailability; F: Vz: Volume of distribution; Vz/f: Vz divided by the bioavailability. *Calculations based on area under the curve (AUC) and dose. NV: No literature value found.
hexamer or dodecamer, and the following dissociation to monomers/dimers (insulin aspart) or further self-association into multihexamers leading to a slow release of monomers/dimers (insulin degludec). Variations in the composition of s.c. connective tissue with pig breed, injection site and/or injection depth could therefore be speculated to affect the oligomeric state of insulin due to differences in depot structure/dilution. The absorption rate of insulin is also influenced by the s.c. blood flow, with increased capillary density leading to a shorter diffusion distance thereby accelerating absorption. Thus, the effects of needle length and injection site on insulin absorption in pigs and humans could be explained by differences in vascular supply, as will also be discussed further below. Furthermore, for acylated analogues, fatty acid composition of the analogue and the degree of albumin binding at the injection site and in the circulation could also be speculated to give rise to differences in PK.

Allometric scaling was used to assess human prediction based on each injection site in Göttingen Minipigs and domestic pigs. Results showed that the human parameters in general were within two-fold of the predicted parameters, irrespective of injection site or pig breed (Table 4 and Table 5). Comparable prediction results were also shown for the i.v. parameters (data on fold change in prediction not shown). Furthermore, just like in humans, all analogues displayed so-called flip-flop kinetics, reflected by a shorter plasma half-life associated with i.v. vs s.c. dosing in agreement with the elimination occurring more rapidly than the absorption.

Traditionally, allometric scaling is performed on data from three or more species. However, allometric scaling based on data from one or two species has more recently shown a reliable prediction of human PK, as reported for a number of intravenously administered compounds with various physicochemical properties in minipigs. Accordingly, the present study demonstrated reliable predictions when we applied single-species allometric scaling, using fixed exponents for each PK parameter. For all three proteins, the predictions at both injection sites were within two-fold error for most of the parameters which is better than the prediction accuracy of 2.5-3.5 average fold error reported for e.g. small molecules using multi-species allometric scaling or physiologically based pharmacokinetic methodologies. The fact that single-species allometric scaling results in reliable predictions of human PK represents a key finding, as such information would result in the use of fewer research animals compared to typical three-species allometric scaling which is highly relevant from a 3R perspective. Inclusion of e.g. fraction of unbound protein in the scaling could potentially even further improve the predictions.

Simple allometric scaling may, however, be less applicable for some drugs depending on their PK properties. These include drugs that e.g. are highly protein-bound in the circulation, drugs that are extensively metabolized or show a combination of hepatic metabolism and renal excretion. Some of these properties may challenge the PK translatability of liraglutide, insulin aspart and insulin detemir, e.g. due to high

### Table 5. Fold error associated with prediction of subcutaneous pharmacokinetic parameters in humans from Göttingen Minipig data or domestic pig data.

<table>
<thead>
<tr>
<th>Protein</th>
<th>T1/2</th>
<th>Cl/t</th>
<th>Vz/t</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Neck</td>
<td>Thigh</td>
<td>Neck</td>
</tr>
<tr>
<td>Liraglutide</td>
<td>1.5</td>
<td>1.0</td>
<td>1.1</td>
</tr>
<tr>
<td>Domestic Pigs</td>
<td>2.5</td>
<td>2.0</td>
<td>1.3</td>
</tr>
<tr>
<td>Göttingen Minipigs</td>
<td>13 h</td>
<td>0.6 L/h</td>
<td>11 L</td>
</tr>
<tr>
<td>Insulin aspart</td>
<td>1.4</td>
<td>1.8</td>
<td>1.4</td>
</tr>
<tr>
<td>Domestic Pigs</td>
<td>2.1</td>
<td>1.4</td>
<td>2.6</td>
</tr>
<tr>
<td>Göttingen Minipigs</td>
<td>1.0 h</td>
<td>120 L/h</td>
<td>NV</td>
</tr>
<tr>
<td>Insulin detemir</td>
<td>1.2</td>
<td>1.1</td>
<td>1.2</td>
</tr>
<tr>
<td>Domestic Pigs</td>
<td>1.8</td>
<td>1.6</td>
<td>1.1</td>
</tr>
<tr>
<td>Göttingen Minipigs</td>
<td>5.4 h</td>
<td>16.8 L/h</td>
<td>NV</td>
</tr>
</tbody>
</table>

Fold error calculated as the ratio between predicted and observed values in Table 4. Average human observed values are also shown. Please refer to Table 4 for references on human data.

T1/2: Half-life in plasma; Cl: Clearance; Cl/t: Cl divided by the bioavailability, F; Vz: Volume of distribution; Vz/t: Vz divided by the bioavailability. NV: No literature value found.

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albumin binding for liraglutide and insulin detemir. Furthermore, since the compounds are all administered by the s.c. route, differences in physiology and morphology of the s.c. tissue may also contribute to inter-species variation in s.c. PK. However, compared to e.g. rodents that represent the most commonly used pre-clinical animal model to study metabolic disorders, the skin and s.c. tissue in humans and pigs show several anatomical similarities. This is for example reflected by a firm attachment between the skin, the s.c. tissue and the underlying muscle in both species, a comparable vascular supply, the presence of both a superficial and deep s.c. fat layer in certain regions (abdomen/paralumbar/gluteal/rear thigh in humans and side of the neck in pigs), whereas in other regions, the s.c. tissue only consist of one layer corresponding to the superficial layer (front thigh in humans and rear thigh in pigs). However, to our knowledge, use of other species for single-species allometric scaling for prediction of s.c. PK of therapeutic proteins remains to be described.

 Altogether, the information obtained in the current study could be useful in the design of pre-clinical studies in pigs. Based on our results and experience, the neck-region as dosing site has at least equal translational value compared to the thigh region, is better tolerated by the animals and requires less training for an unstressed and accurate injection. Therefore, we recommend using the neck injection site for translational purposes, although the effect of injection site may vary depending on the specific protein/preparation of interest. This recommendation is also supported by the superior translation to humans of tocilizumab PK after SC administration “behind the ear” as compared to three other subcutaneous sites in Göttingen Minipigs — an injection site comparable to the neck region used in our study.

 A limitation in relation to comparing the two breeds is that they differ in age and also sexual maturity which could influence clearance of the drugs. The position of the sampling catheter in relation to the injection site could also have influenced the measured drug concentration as sampling close to the injection site could potentially result in falsely elevated drug concentrations if the absorbed drug has not yet been systemically distributed. In our case, the position of catheters in the two breeds differed, potentially contributing to some of the observed differences between the breeds. Also of importance, the assay used for measuring exposure in the clinical studies was not the same as in the pre-clinical studies. Hence, the absolute values (e.g. for volume of distribution and clearance) may not be directly comparable when based on different assays whereas derived values (e.g. T1/2) should be comparable. Finally, we prioritized to dose unrestrained animals and did not confirm by ultrasound that the drug was dosed in the s.c. tissue. However, as described in the methods section, an extensive effort was made to select the optimal injection site and ensure accurate delivery of the drug in the s.c. tissue prior to dosing.

 In conclusion, this study assessed the translatability of subcutaneous PK of therapeutic proteins in pigs under optimized and standardized conditions using ultrasound characterization of the dosing site and training of the animals for unrestrained dosing. Both pig breeds and injection sites showed good translation with respect to human PK of the investigated proteins, with predictions overall being within two-fold from human data. In both breeds, injection site influenced some of the PK parameters with neck dosing generally giving rise to longer MAT and apparent half-life as well as lower dose-normalized Cmax and AUC. Injection depth significantly influenced absorption kinetics for insulin aspart, and needle length should therefore be taken into consideration in the design of pre-clinical studies. Based on our data for the selected protein therapeutics, both pig breeds and injection sites can be used in pre-clinical PK studies for prediction of human PK, although the neck region has some advantages over the thigh region and therefore is our recommended injection site. Whether our conclusions can be extended more broadly to encompass other peptide and protein therapeutics remains to be investigated.

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SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.trsl.2021.08.005.

REFERENCES


