Towards in vitro in vivo correlation for modified release subcutaneously administered insulins

Frederik Bocka, Eva Linab,c, Claus Larsena, Henrik Jensena, Kasper Huusa, Susan Weng Larsenas
Jesper Østergaardab,⁎

a Department of Pharmacy, Faculty of Health and Medical Sciences, University of Copenhagen, Universitetsparken 2, Copenhagen DK-2100, Denmark
b Global Research Technologies, Novo Nordisk A/S, Novo Nordisk Park, Måløv DK-2760, Denmark

Abstract

Therapeutic proteins and peptides are mainly administered by subcutaneous injection. In vitro release testing of subcutaneous injectables performed using methods that take the structure and environment of the subcutaneous tissue into account may improve predictability of the in vivo behavior and thereby facilitate establishment of in vitro in vivo correlations. The aim of the study was to develop a biopredictive flow-through in vitro release method with a gel-type matrix for subcutaneously administered formulations and to explore the possibility of establishing a level A in vitro in vivo correlation for selected insulin products. A novel gel-based flow-through method with the incorporation of an injection step was used to assess selected commercial insulin formulations with different duration of action (Actrapid®, Mixtard® 30, Insulatard®, Lantus®). The in vitro release method provided the correct rank ordering in relation to the in vivo performance. For the modified release insulins Insulatard® and Lantus®, an in vitro in vivo correlation using non-linear time scaling was established based on the in vitro release data and in vivo subcutaneous absorption data of the 125I-labeled insulins taken from literature. Predicted absorption profiles were constructed using the in vitro in vivo correlation and subsequently converted into simulated plasma profiles. The approach taken may be of wider utility in characterizing injectables for subcutaneous administration.

1. Introduction

Proteins and peptides are often administered parenterally due to the challenges associated with oral administration including poor permeability, degradation in the gastrointestinal (GI) tract and first-pass metabolism (Porter and Charman, 2000). Subcutaneous administration is often preferred over intravenous injection since it is more convenient from a patient perspective (Kinnunen et al., 2015). Upon subcutaneous injection, the formulation is exposed to an extracellular matrix mainly comprising collagen, hyaluronic acid and chondroitin sulfate (Kinnunen and Mrsny, 2014; Richter et al., 2012; Scott, 1995). The subcutaneous tissue resembles a gel-like matrix rather than bulk fluid and the local environment may significantly influence the performance of the injected formulation by affecting drug precipitation, transport and eventually absorption into the systemic circulation (Hernandez and Exner, 2017; Kinnunen and Mrsny, 2014; Leung et al., 2017). In vitro release models taking into account the structure and environment at the injection site may have a better chance of predicting the in vivo behavior of subcutaneously administered injectables. Hydrogels have been proposed as potential substitutes for bulk fluids since they share common features with the extracellular matrix (Chen et al., 2008; Gietz et al., 1998; Hernandez and Exner, 2017; Hernandez et al., 2016; Jensen et al., 2015; Klose et al., 2009; Leung et al., 2017; McCabe, 1972; Ye et al., 2012).

In order to assess and eventually take advantage of biopredictive in vitro release testing methods, establishment of in vitro in vivo correlations (IVIVCs) should be pursued. Prediction of in vivo behavior of new injectable delivery systems using data from in vitro release studies has the potential to reduce the time and resources spent on performing in vivo studies of these formulations (Hernandez and Exner, 2017; Shen and Burgess, 2015; Solorio and Exner, 2015). A number of in vitro release testing approaches, typically categorized as sample and separate methods, continuous flow methods and dialysis membrane-based techniques (Larsen et al., 2009; Shen and Burgess, 2015), has been explored for the subcutaneous route of administration. For insulin formulations, the sample and separate (Al-Tahami et al., 2011; Gietz et al., 1998; Jensen et al., 2016; Leung et al., 2017; Naha et al., 2009), flow-through (Graham and Pomeroy, 1984) and membrane-
based (Kinnunen et al., 2015) in vitro release testing methods have also found use. For instance, the in vitro release of insulin from commercially available products (Gietz et al., 1998; Graham and Pomeroy, 1984; Kinnunen et al., 2015; Leung et al., 2017) as well as experimental formulations such as implants, PLGA/PLA microparticles and in situ forming PLA implants (Al-Tahami et al., 2011; Jensen et al., 2016; Naha et al., 2009) has been characterized. Correlation to in vivo data (biological activity given by manufacturer), other than simple rank ordering has to the best of our knowledge only been established in a single study (Gietz et al., 1998), where a linear correlation was achieved between biological activity and the logarithmic total mean time of in vitro release for five different insulin formulations. The work of Gietz et al. (1998) indicates that it is possible to correlate in vivo and in vitro release data for insulin products. Albeit, the increasing interest in developing generic in vitro methods suitable for subcutaneous formulations and establishing IVIVCs for non-oral drugs (Shen and Exner, 2017), regulatory approved or endorsed in vitro release testing methods for subcutaneously administered drugs remain to be established (Hernandez and Exner, 2017). Furthermore, the literature on IVIVCs for non-oral drugs in man is sparse. For transdermal dosage forms, a few IVIVCs have successfully been established (Kondamudi et al., 2016; Mateus et al., 2014; Raber et al., 2014; Shin et al., 2018; Yang et al., 2015), whereas for intramuscular (Rawat et al., 2012) and subcutaneous injection (Vlugt-Wensink et al., 2007) the number of correlations are even more limited.

An IVIVC is a predictive mathematical model describing the relationship between in vitro release data and in vivo absorption data. There are 5 levels of IVIVCs, level A, multiple level C, level B, level C and level D as listed from the most to the least informative (Emami, 2006; FDA, 1997). A level A IVIVC is typically a linear point-to-point relationship between in vitro release data and in vivo absorption data (Emami, 2006; FDA, 1997). In case a linear correlation is obtained, the relationship between % absorbed in vivo and % released in vitro versus time profiles are directly superimposable; However, for non-linear correlations a time scale approach can be applied to render the profiles superimposable. The most common way to time scale is by constructing a Levy plot. A Levy plot is a linear relationship between the corresponding in vitro and in vivo times for 0 - 90%, absorbed and released, respectively (FDA, 1997; Cardot, 2016; Cardot and Beyssac, 2002). If linear time scaling (Levy plot) has been investigated without satisfactory results non-linear time scaling can be pursued in order to establish a correlation between in vitro and in vivo data (Cardot, 2016; Cardot and Davit, 2012; Ruiz Picazo et al., 2018).

Insulin is used for the treatment of diabetes and several subcutaneously administered products with different duration of action are available. Available in vivo data provides an opportunity to explore possible IVIVCs. To this end, four commercial insulin formulations were assessed in vitro including a fast, an intermediate, a mixture of the fast and intermediate, and a long acting insulin, i.e. Actrapid®, Insulatard®, Mixtard® 30, and Lantus®, respectively (Table 1). The human soluble insulin (fast acting), isophane insulin (intermediate acting) and insulin glargine (long acting insulin analog) constitute three types of insulin with different onset and duration of action (DeWitt and Hirsch, 2003). Human soluble insulin (e.g., Actrapid®) has an onset after 0.5–1 h and a duration of action of 5–8 h (DeWitt and Hirsch, 2003). Isophane insulin (e.g., Insulatard®) has an onset after 2–4 h and a duration of action of 10–16 h (DeWitt and Hirsch, 2003). Different premixed ratios of human soluble insulin and isophane insulin are commercially available. Mixtard® 30, for example, consists of 30% human soluble insulin and 70% isophane insulin and has as an onset after 0.5–1 h and a duration of action of 10–16 h (DeWitt and Hirsch, 2003). Insulin glargine (Lantus®) is characterized by an onset after 2–4 h and a duration of action for 20–24 h (DeWitt and Hirsch, 2003).

The in vitro setup presented in the current work differs from already published in vitro release testing methods for injectables by incorporating an injection step and using a flow-through setup with a gel-type matrix, to assess the importance of the injection step and the matrix to restrain the dispersion of liquid formulations upon injection. The aim of study was to develop a biopredictive gel-based flow-through in vitro release testing method for subcutaneously administered formulations and to explore the possibility of establishing a level A in vitro in vivo correlation (IVIVC) for selected insulin products based on published subcutaneous absorption data.

### Table 1

<table>
<thead>
<tr>
<th>Product</th>
<th>API (100 U/ml)</th>
<th>Formulation</th>
<th>Composition</th>
<th>pI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actrapid®</td>
<td>Human soluble insulin</td>
<td>Solution (pH 7.0 – 7.8)</td>
<td>Glycerol, m-cresol, ZnCl2</td>
<td>5.4</td>
</tr>
<tr>
<td>Insulatard®</td>
<td>Isophane human insulin suspension</td>
<td>Suspension (pH 7.1 – 7.5)</td>
<td>Glycerol, m-cresol, phenol, ZnCl2, Na2HPO4 · 2H2O, protamine sulfate</td>
<td>5.4</td>
</tr>
<tr>
<td>Mixtard® 30</td>
<td>Human soluble insulin (30%) and isophane human insulin suspension (70%)</td>
<td>Suspension (pH 7.1 – 7.5)</td>
<td>Glycerol, m-cresol, phenol, ZnCl2, Na2HPO4 · 2H2O, protamine sulfate</td>
<td>5.4</td>
</tr>
<tr>
<td>Lantus®</td>
<td>Insulin glargine (insulin analog)</td>
<td>Solution (pH 4); in situ forming gel</td>
<td>Glycerol, m-cresol, ZnCl2 polysorbate</td>
<td>7.0</td>
</tr>
</tbody>
</table>

* EMA, 2009b.
* EMA, 2009d.
* EMA, 2009c.
* EMA, 2009a.
* Gillies et al., 2000; Wang et al., 2003.

2. Materials and methods

#### 2.1. Materials

Human insulin (2.3 Zn/hexamer; total peptide content of 89% (w/w)) and the insulin products Actrapid®, Mixtard® 30 and Insulatard® were obtained from Novo Nordisk (Bagsvaerd, Denmark), while Lantus® was obtained from Sanofi Aventis (Frankfurt am Main, Germany). Buffer salts, Na2HPO4 · H2O and NaH2PO4 · 2H2O, were obtained from Merck Emsure (Darmstadt, Germany). Tween 80 was obtained from Fluuka (Buchs, Switzerland). Sodium azide was obtained from Merck (Darmstadt, Germany). Sephadex G-25 (cross-linked dextran gel) fine was obtained from Pharmacia (Uppsala, Sweden). The chemicals used for preparation of buffers and mobile phases for the HPLC were of analytical grade. Purified water from a Milli-Q deionization unit (Millipore, Bedford, MA, USA) was used for the HPLC analysis and deionized water was used for the release studies.

The release medium consisted of 67 mM phosphate buffer, pH 7.4, containing 0.01% (w/v) Tween 80 to prevent adsorption to surfaces, 0.01% (w/v) sodium azide as preservative, and 0.42% (w/v) sodium chloride for isotonicity.
2.2. Methods

2.2.1. Packing of gel column/release column

The gel-based flow-through in vitro release model was based on a glass column with an inner diameter (i.d.) of 16 mm (C16/20) from GE Healthcare (Little Chalfont, England), which was modified to incorporate an injection port (0.5 cm i.d. and 2.5 cm in length). The injection port was sealed with a silicone stopper secured with an open screw cap allowing injection using syringe and needle (Fig. 1). The gel matrix consisted of Sephadex G-25 fine and was prepared by allowing approximately 4 g of dry powder to swell in the release medium for 3 h followed by degassing under vacuum for 15 min. The adaptor (AK 16 adaptor, GE Healthcare, Little Chalfont, England) with the outlet filter (pore size 25 µm, wetted with 20% (v/v) ethanol) was placed 6.5 cm beneath the injection port and the gel matrix was gently transferred to the column avoiding entrapment of air bubbles. After 1 h of settling, the adaptor with the inlet filter (pore size 25 µm, wetted with 20% (v/v) ethanol) and the tubing was attached to the column. Column packing was performed with the release medium applying a flow rate of 80 ml/h using a Pharmacia LKB pump P500 (Pharmacia, Uppsala, Sweden) for at least 1 h upon attainment of a constant bed height. After packing, the column was turned upside down (flow direction from bottom to top) and the release medium was circulated at a flow rate of 30 ml/h. The column length was 20 cm, giving 6.5 cm from the injection port to the outlet end.

2.2.2. Insulin in vitro release experiments

Release experiments were performed by injecting 0.25 ml of the insulin products (100 U/ml, Actrapid®, Mixtard® 30, Insulatard® or Lantus® into the gel-based column. The insulin products were slowly injected into the gel matrix through the injection port using a 1 ml syringe (Injekt – F, luer tip, B. Braun Melsungen AG, Melsungen, Germany) with a 22 gauge stainless steel needle for luer tip syringes from Rheodyne LLC (Rohnert Park, CA, USA) while the flow was arrested. Upon injection, the release medium was continuously pumped through the column at a flow rate of 0.25 ml/min. At the outlet, medium samples were collected using a LKB FRAC-2000 fraction collector (Pharmacia, Uppsala, Sweden). The duration of the release experiments was approximately 1, 3, 6, and 10 h for Actrapid®, Mixtard®, Insulatard® and Lantus®, respectively. Fractions were collected over 3 min, 3 min (10 min at t > 90 min), 15 min, and 30 min intervals for Actrapid®, Mixtard®, Insulatard® and Lantus®, respectively. The insulin content in each sample was determined by HPLC analysis. The release experiments were carried out in triplicate at 22–24 °C. After each release experiment, the column was conditioned by applying a flow of phosphate buffer (0.5 ml/min) for 1–2 h or until no precipitate was visible.

2.2.3. HPLC analysis

Reverse-phase HPLC was applied for quantification of insulin release using a Symmetry Shield RP C18 column (3.9 × 20 mm², 3.5 µm; Waters, Milford, MA, US). A LaChrom HPLC system (Merck-Hitachi, Tokyo, Japan), which consisted of a L-7100 pump, L-7200 autosampler, L-7200 Peltier Sample Cooler, D-7000 interface, and L-7400 UV detector was applied for the HPLC analysis using a linear gradient method. The following gradient method was used: 0 min 80% (v/v) A, 1.35 min 80% (v/v) A, 4.00 min 55% (v/v), 4.10 min 80% (v/v) A, and 6.00 min 80% (v/v) A. Mobile phase A consisted of 0.20 M sodium sulfate and 0.04 M sodium phosphate, pH 3.6, in 10% (v/v) acetonitrile and mobile phase B consisted of 70% (v/v) acetonitrile and 30% (v/v) water. The injection volume was 15 µl, the flow rate was set to 1 ml/min and the effluent was monitored at 215 nm at 30 °C. Quantification of insulin was done from peak area measurements relative to standard solutions of human insulin in the release medium analyzed under identical conditions. The insulin stock solution was prepared by weighing approximately 12 mg of human insulin and dissolving it in 10 ml of release medium. From this stock solution, serial dilutions were made covering the concentration range 4.1 × 10⁻⁶–3.3 × 10⁻⁸ M. The LOD and LOQ were determined as 9.3 × 10⁻⁸ M and 3.1 × 10⁻⁷ M, respectively.

2.2.4. Cumulated amount of drug released

The cumulated amount of insulin released (M(t)) was calculated as a function of time according to:

\[ M(t) = V_c C_a + \sum_{n=1}^{\infty} M_{b_{n-1}} \]

where \( V_c \) is the volume of the samples withdrawn at time \( t \), \( C_a \) is the insulin concentration in sample \( n \), and \( M_{b_{n-1}} \) represents the insulin amount in the previously collected samples.

2.2.6. Assessment of in vitro and in vivo correlation

The in vitro release profiles were corrected for column retention time such that time zero corresponded to the time where insulin was initially detected in the effluent. The in vitro disappearance profiles corrected for the column retention time were fitted to Equations (2) or (3):

\[ \% \text{insulin remaining} = b e^{-kt} \]

\[ \% \text{insulin remaining} = b + b_1 t + b_2 t^2 + b_3 t^3 \]

where \( b \) and \( t \) are the intercept with the y-axis and time, respectively, while \( b_1, b_2, b_3 \) are constants. Eq. (2) was used to calculate the in vitro times corresponding to 100, 90, 80, ..., 10% of insulin remaining in the column for Actrapid®, Insulatard® and Mixtard® 30. Eq. (3) was used to calculate the in vitro times corresponding to 100, 90, 80, ..., 15% of insulin remaining for Lantus®. In vivo insulin absorption studies corresponding to Actrapid®, Mixtard® 30, Insulatard® and Lantus® comprising 125I-labeled insulin were identified in the literature (Clauson and Linde, 1995; Henriksen et al., 1991; Hildebrandt et al., 1983, 1984, 1983; Kang et al., 1991; Lauritzen et al., 1982; Luzzio et al., 2003; Owens et al., 2000; Praamling et al., 1984; Sestoft et al., 1982). Insulin disappearance from the injection site was determined using 125I-labeled insulin and measuring the residual radioactivity at the injection site. The disappearance was used as a surrogate for absorption. The percentage of insulin remaining at the injection site was plotted against time, upon conversion of published graphs into numerical values using GetData Graph Digitizer (Version 2.26.0.20, Sergei Fedorov, Moscow, Russia). The studies of Luzzio et al. (2003) and Hildebrandt et al. (1983) were selected for investigating the possibility of establishing an IVIVC. Insulatard® and Lantus® in vivo data were taken from Luzzio et al. (2003). The disappearance of insulin from the injection site (subcutaneously in the abdomen) in 14 fasting type 2 diabetic patients was followed using 125I-labeled insulin over 48 h (Luzzio et al., 2003).
Actrapid® in vivo data were obtained from Hildebrandt et al. (1983), where the absorption of 125I-labeled insulin was followed upon subcutaneous injection (abdomen) in type 1 diabetic patients (n = 10) for 8 h. The mean in vivo absorption profiles (n = 10) for Actrapid® and the median in vivo absorption profiles (n = 14) for Insulatard® and Lantus®, respectively, were estimated from plots of residual radioactivity in percentage against time using GetData graph digitizer. GetData Graph digitizer was used for interpolation to obtain the in vivo times corresponding to 100, 90, 80…10% of insulin remaining at the injection site for Actrapid® and Insulatard® and to 100, 90, 80…15% remaining for Lantus®, these values were used for time scaling. The software was also used to interpolate the in vivo data in order to assess a potential IVIVC. In the exploration of a possible IVIVC, it was assumed that the disappearance of insulin from the injection site reflected absorption of the insulin. The percentage of insulin remaining in vitro and in vivo was made superimposable using non-linear time scaling (Section 3.3).

2.2.7. Validation of IVIVC

The in vitro profiles were converted to predicted in vivo absorption profiles using time scaling and the achieved IVIVC (Section 3.4). The predicted in vivo absorption profiles were subsequently compared with the absorption profiles from the literature. The time where 75% and 50% insulin remained to be absorbed (t75% and t50%) was used for comparison for Insulatard® and Lantus® (Luzio et al., 2003). The predicted t75% and t50% were calculated from the predicted in vivo absorption profiles by fitting them to either an exponential decay function (Insulatard®) or linear function (Lantus®). The error in percentage was calculated using the following equation:

$$\text{Error} (\%) = \frac{t_7 \text{ remaining pred.} - t_7 \text{ remaining lit.}}{t_7 \text{ remaining lit.}} \times 100\% \tag{4}$$

where $t_7$ remaining pred. is the predicted time and $t_7$ remaining lit. is the time found in the literature.

2.2.8. Simulation of insulin plasma profiles

The predicted in vivo absorption profiles were converted into simulated plasma or serum profiles using a quantitative and physiological-based model for the processes occurring upon subcutaneous injection of soluble and crystalline insulin (Soeborg et al., 2009). The model of Soeborg and co-workers was utilized due to the simplicity and documented ability to describe the pharmacokinetics of short and intermediate-acting insulins. Alternative models can be found, such as Tarin et al. (2005). Serum insulin and plasma concentrations were assumed to be similar. The predicted in vivo absorption profile was converted from% insulin remaining at the injection site to absolute amount of insulin released from the injection site, by converting the mean dose (26 U) (Luzio et al., 2003) to amount of substance (pmol). The achieved graphs were then fitted to either a one phase exponential growth function (Insulatard*) or a linear function (Lantus*). The generated expressions were differentiated and used to attain the input rate of insulin from the subcutaneous tissue to the plasma ($J_p$) in the model (Soeborg et al., 2009):

$$\frac{dC_{pl}}{dt} = \frac{1}{V_d} \cdot J_p - k_e \cdot C_{pl} \tag{5}$$

where $C_{pl}$ is the insulin concentration in plasma, $V_d$ is the volume of distribution of insulin in plasma (12 l) (Kraegen and Chisholm, 1984) and $k_e$ is the elimination rate constant of insulin in plasma (6.6 h⁻¹) (Normann Hansen, 2004). The integrals for Eq. (5) were solved for each formulation separately in order to achieve expressions describing the insulin plasma profile after a subcutaneous administration of 26 U of Insulatard® and Lantus*.

3. Results and discussion

Hydrogels, proposed to simulate the subcutaneous tissue in a drug release context (Chen et al., 2008; McCabe, 1972; Ye et al., 2012), have been used in vitro release studies for subcutaneous injectables involving insulin (Gietz et al., 1998; Jensen et al., 2016; Leung et al., 2017). The novel features of the presented in vitro release method lie in the addition of a continuous flow and incorporation of an injection step to the gel-like matrix (Fig. 1). A gel-like matrix may simulate more realistically the in vivo environment as compared to simply injecting the drug formulation into a solution. The Sephadex G-25 fine has been widely used in size exclusion chromatography as a gel filtration resin and, thus, selected for the ability to accommodate a porous matrix allowing a flow of dissolution medium through the column. The exclusion limit of Sephadex G-25 fine is ~5000 g/mol (GE-Healthcare, 2016), and strong retention on the column is therefore not anticipated (human insulin Mw 5800 g/mol). Here, a pragmatic approach was taken in form of a porous matrix and simple phosphate buffer, pH 7.4, with physiological ionic strength suited for a flow-through-based release testing system. The model was optimized with respect to the injection procedure and flow rate for repeatability and throughput (analysis time). Kinnunen and Mrsny (2014) have discussed the properties of the extracellular matrix and the interstitial fluid of the subcutaneous matrix in detail. Further refinement in terms matrix and fluid composition may well be possible, but was outside the scope of the present work. The column was operated at 22–24 °C rather than at 37 °C due to the column was found to occasionally crackle at the latter temperature.

3.1. In vitro release setup

The selected insulin products were injected into the gel-based column via the dedicated port in absence of flow to allow time for the injected insulin formulations to settle inside the column matrix. The in vitro injection volume of 0.25 ml was selected based on typical in vivo injection volumes (0.2–0.3 ml) for the intermediate and long acting formulations, e.g., Luzio et al. (2003), Hubinger et al. (1992), Becker et al. (2015), and Zhang et al. (2017). The dose of short acting insulin is usually lower than that of intermediate and long acting insulins. Therefore, the injected volume of short acting insulin in vitro is higher than the commonly injected volume in vivo, which is around 0.05–0.15 ml (100 U/ml) (Clauson and Linde, 1995; Hubinger et al., 1992). Upon completion of the injection, a flow of 0.25 ml/min was applied to elute insulin. Insulin disappearance-time profiles were obtained upon quantification of insulin in the collected effluent and correction for the column retention time. The observed column retention times (the time of initial insulin detection in effluent) were 0.29 h, 0.35 h, 0.25 h and 0.58 h for Actrapid®, Mixtard®, Insulatard® and Lantus*, respectively. The variation in retention times is most likely reflecting the different physical properties of the formulations (Table 1). Similar retention times were found for Actrapid®, Mixtard® and Insulatard® whereas the increased retention time of Lantus® was attributed to extensive precipitation. Lantus* (insulin glargine) has a higher isoelectric point (pl 7.0) as compared to human insulin (pl 5.4) (Gillies et al., 2000; Wang et al., 2003), leading to higher solubility in acidic environments such as the formulation medium, and a comparatively low solubility at near neutral pH found at the injection site (Andharia et al., 2017). Thus, insulin glargine forms microprecipitates upon contact with the subcutaneous tissue, which reduces the systemic absorption rate (Gillies et al., 2000; Wang et al., 2003). The micro-precipitates have reported hydrodynamic radii ranging from 160 ± 30 nm to 1800 ± 200 nm at insulin concentrations ranging from 0.025 mg/ml to 1.8 mg/ml at pH 7 as determined by light scattering (Coppolino et al., 2006). For each insulin product, retention time corrected insulin disappearance-time profiles with good repeatability were obtained indicating that the injection step did not compromise repeatability (Fig. 2A). Distinct insulin disappearance-time profiles
Fig. 2. Insulin disappearance-time profiles for Actrapid® (×), Mixtard® 30 (■), Insulatard® (∆) and Lantus® (●). (A) Column retention time corrected in vitro data providing the fraction of insulin remaining in the column over time using the flow-through model (error bars indicate 1 SD, n = 3). The solid lines represent the obtained fits which are given in Equations (6)–(9). (B) Median and mean in vivo data in man redrawn from (Hildebrandt et al., 1983; Luzio et al., 2003) providing the residual radioactivity at the injection site (lower abdomen) over time upon injection of 125I-labeled insulin.

Fig. 3. Insulin in vivo absorption studies for Actrapid® (×), Insulatard® (∆) and Lantus® (●) utilizing 125I-labeled insulin in man. The amount of insulin remaining at the injection sites is depicted as residual radioactivity in percentage versus time. The data were replotted from the literature (Clauson and Linde, 1995; Henriksen et al., 1991; Hildebrandt et al., 1984, 1983; Kang et al., 1991; Lauritzen et al., 1982; Luzio et al., 2003; Owens et al., 2000; Pramming et al., 1984; Sestoft et al., 1982). The in vitro insulin disappearance-time profiles were fitted to Eq. (2) for Actrapid®, Insulatard® and Mixtard® 30 and Eq. (3) for Lantus® (Fig. 2A) with the following results:

Actrapid®: % remaining = 99.77 e^{−18.06t} \quad (6)

Mixtard® 30: % remaining = 99.65 e^{−3.44t} \quad (7)

Insulatard®: % remaining = 100.20 e^{−0.78t} \quad (8)

Lantus®: % remaining = 103.80 − 27.70t + 2.54t^2 − 0.08t^3 \quad (9)

where t is the time given in h. The confidence intervals for the fitted parameters can be seen in supplementary Table 2. The insulin disappearance-time profiles were depicted as percentage remaining as a function of time, t, to facilitate comparison to the in vivo absorption data from the literature. The exponential decay function Eq. (2) was selected for Actrapid®, Insulatard® and Mixtard® 30 as it provided good fits for the in vitro data with R^2 > 0.99. However, it did not describe the insulin disappearance from Lantus® well and therefore other mathematical functions were investigated. Eventually, a third order polynomial, Eq. (3), was found to describe the profile for Lantus® well (R^2 > 0.99). To this end, Eqs. (2) and (3) as well as the obtained Eqs. (6), (8) and (9) should be viewed merely as mathematical descriptions of the profiles that were used to construct a time scaling plot.

3.2. In vivo behavior of insulins

Insulin in vivo absorption studies with the formulations Actrapid® (Clauson and Linde, 1995; Hildebrandt et al., 1983; Kang et al., 1991; Pramming et al., 1984; Sestoft et al., 1982), Insulatard® (Henriksen et al., 1991; Hildebrandt et al., 1984; Lauritzen et al., 1982; Luzio et al., 2003; Owens et al., 2000; Pramming et al., 1984) and Lantus® (Luzio et al., 2003; Owens et al., 2000) comprising 125I-labeled insulin were identified from the literature and the results were replotted in Fig. 3. 125I-labeled insulin in vivo data were not available for Mixtard® 30.

Fig. 3 shows that the absorption of Actrapid®, Insulatard® and Lantus® is easily differentiated in vivo. Fig. 3 also reveals significant variability between studies with the same formulation. Subcutaneous absorption of insulin is known to be characterized by relatively large variability both inter-individually and intra-individually (Gradel et al., 2018; Heinemann, 2002). The multiple factors causing the variability may fall in three groups, the dose and concentration of the insulin product, difference between injection sites and changes of the injection site. The influence of dose and concentration on the variability of in vivo absorption data has been shown in various studies for different insulin formulations (Becker et al., 2015; Heinemann, 2002; Hildebrandt et al., 1984, 1983; Lauritzen et al., 1982; Owens et al., 2000). These studies found that a higher dose or a higher insulin concentration in the formulation were associated with a slower insulin absorption as compared to a lower dose or a lower insulin concentration (Becker et al., 2015; Heinemann, 2002; Hildebrandt et al., 1984, 1983; Lauritzen et al., 1982; Owens et al., 2000). Differences between injection sites covers factors such as anatomical region of injection, injection depth and obesity (Heinemann, 2002). The influence of these factors has been shown in these studies (Clauson and Linde, 1995; Hildebrandt et al., 1983; Owens et al., 2000). Changes related to the injection site include factors such as temperature, physical activity, and substances known to
increase blood flow (Heinemann, 2002). The use of $^{125}$I-labeled insulin constitutes a common way of determining insulin absorption. Sohoel et al. (2007) compared the absorption rate determined by the disappearance of radioactivity at the injection site to the absorption rate of non-labeled insulin Aspart® obtained by deconvolution in pigs. The absorption of non-iodinated insulin Aspart® was $\sim 20\%$ faster than iodinated insulin, suggesting that absorption rates determined by $^{125}$I-labeling might underestimate the true absorption rate (Sohoel et al., 2007). However, other studies have found similar absorption rates for iodinated and non-iodinated insulin for isophane insulin (Deckert et al., 1982 and four other formulations (Binder, 1969). Thus, labeling of the protein is associated with a risk of affecting the in vivo behavior. The magnitude of this error may be difficult to gauge. In the pursuit of an IVIVC, studies with as many as possible factors in common were selected in order to minimize the variability. In the present study, in vivo data obtained by Luzio et al. (2003) and Hildebrandt et al. (1983) were selected for exploration of an IVIVC as these studies used the lower abdomen as the injection site and the insulin disappearance was followed for a relatively long time period compared to most other in vivo studies. However, the two studies differ with respect to the type of diabetic patients where Luzio et al. (2003) used type 2 diabetics and Hildebrandt et al. (1983) used type 1 diabetics. A further advantage of including the study of Luzio et al. (2003) was that it provides in vivo data for both Insulatard® and Lantus® performed using identical injection site (abdomen), administration method, fasting schedule and dose (single administration of mean dose 26 U (0.26 ml 100 U/ml insulin (0.3 U/kg body weight)).

### 3.3. IVIVC

The in vivo absorption (disappearance) profiles for Actrapid®, Insulatard® and Lantus® (Luzio et al., 2003; Hildebrandt et al., 1983) are shown in Fig. 2B. Eqs. (6), (8) and (9) were used to calculate the in vitro times corresponding to 100, 90, 80,...10% (15% instead of 10% for Lantus®) of insulin remaining in the column in order to make a Levy plot with Actrapid®, Insulatard® and Lantus®. The in vitro and in vivo times for 100–10% remaining were plotted on the x-axis and y-axis, respectively, for Actrapid®, Insulatard® and Lantus®. A Levy plot (linear fit) was drawn for all three formulations (supplementary Fig. 1) and for Insulatard® and Lantus® only (supplementary Fig. 2). Both of these graphs resulted in deviation from linearity and a $R^2 < 0.95$. Consequently, a non-linear time scaling approach was investigated, a second-degree polynomial fit resulted in a $R^2 = 0.95$, excluding Actrapid® (Fig. 4A). In case Actrapid® was included, the obtained IVIVC was not satisfactory, $R^2 < 0.95$ (see supplementary Fig. 3 for non-linear time scaling plot and attempted IVIVC including the three formulations). In contrast to Insulatard® and Lantus®, the absorption of insulin upon subcutaneous injection of Actrapid® is not controlled by a rate-limiting dissolution step of insulin crystals because the product comprises soluble insulin. For establishment of an IVIVC, it is considered important that the rate-limiting step is the same in vitro and in vivo in order for the in vitro drug release mechanism to be identical to that present in vivo (Burgess et al., 2004; Dadhaniya et al., 2015; Kinnunen et al., 2015; Larsen et al., 2009, 2013; Shen and Burgess, 2012). Apparently, the presented in vitro release method is lacking a diffusion barrier present in vivo controlling the absorption of insulin from the Actrapid® solution. This might explain why it was not possible to incorporate Actrapid® into the IVIVC. Eq. (10) was obtained from the non-linear time scaling plot for Insulatard® and Lantus® in Fig. 4A and was used to scale the in vitro times from the disappearance profiles (see supplementary Table 2 for confidence intervals on fitted parameters):

\[ I_{\text{in vivo}} = -0.42 + 16.16 I_{\text{in vitro}} - 1.36 I^2_{\text{in vitro}} \]  

(10)

where the times are given in h. The correlation depicted in Fig. 4B is % released/absorbed (converted from % insulin remaining) in accordance with standard practice for IVIVCs. The % of insulin absorbed in vivo corresponding to the same time as the scaled in vitro time points was plotted against the % insulin released in vitro for the scaled in vitro time points (Fig. 4B). This resulted in a linear correlation (see supplementary Table 2 for confidence intervals on fitted parameters) between the insulin release in vitro and the absorption in vivo for Insulatard® and Lantus®, $R^2 = 0.96$ (Fig. 4B):

\[ \% \text{absorbed}_{\text{in vivo}} = 0.90 \times \% \text{released}_{\text{in vitro}} + 5.16 \]  

(11)

### 3.4. Validation of IVIVC

Seeking to validate the acquired IVIVC, the internal predictability (FDA, 1997) was investigated. Since the in vitro disappearance-time profiles from Insulatard® and Lantus® were used to establish the IVIVC, the simulations utilizing these formulations represent internal validation. Actrapid® and Mixtard® 30 were not included in the validation or simulation of plasma profiles, since both of these formulations contain soluble insulin and were not adequately described by the IVIVC (Section 3.3). The in vitro insulin disappearance-time profiles for Insulatard® and Lantus® from Fig. 2A were converted to predicted in vivo insulin absorption profiles using Eqs. (10) and (11) for Fig. 4A and B, respectively. It was assumed that the disappearance of insulin from the injection site reflected absorption of the insulin. The predicted in vivo insulin absorption profiles (Fig. 5) followed the rank ordering observed in man (DeWitt and Hirsch, 2003); where Lantus® had a lower rate of absorption as compared to Insulatard®. The predicted in vivo insulin absorption profiles were compared to the insulin absorption profiles from the literature (Luzio et al., 2003). From Fig. 5, it can be seen that the predicted absorption profiles exhibit a reasonable degree of similarity to the absorption profiles from the literature. Internal predictability is normally considered acceptable when the average percent prediction error is below 10% for each $C_{\text{max}}$ and AUC, and none of the formulations have a prediction error greater than 15% defined by FDA for extended release oral drug products (FDA, 1997). For the insulin absorption profiles,
however, these parameters were not available, instead the predicted $t_{75\%}$ (time where 75% was remaining) and $t_{50\%}$ was compared with the $t_{75\%}$ and $t_{50\%}$ from the literature for Insulatard® and Lantus®, respectively (Table 2, supplementary Table 3 for confidence intervals). Table 2 shows that the internal predictability for $t_{75\%}$ is not within the limits of 15% error (albeit close) for both of the formulations, however, the average error is <10% (FDA, 1997). The internal predictability for $t_{50\%}$ is within the limits of 15% error for each formulations, and the average is <10% and consequently complies with the prediction error set by FDA for extended release oral products. Internal predictability showed that the achieved correlation cannot be designated a level A IVIVC, since it does not fulfill the requirements for $t_{75\%}$. We consider that the obtained IVIVC may still prove useful in estimating the absorption profile for intermediate and long-acting insulin formulations and may be suited in relation to the design of insulin formulations where dissolution is the rate controlling release mechanism. It should be noted that absorption profiles shown in Fig. 5 are for two different molecules. Insulatard® consists of NPH crystals whereas Lantus® is an insulin analog.

### 3.5. Simulation of insulin plasma profiles

The process of achieving the present IVIVC was atypical since deconvolution of plasma profiles was not performed to obtain in vivo absorption profiles. Plasma profiles may be of particular interest in relation to estimating dosing, etc. Consequently, it was found of interest to investigate to what extent the achieved IVIVC was suited for simulating plasma profiles. The absolute amount of insulin absorbed from the injection site over time were obtained for Insulatard® and Lantus® (supplementary Fig. 4) by converting % remaining to absolute amount (supplementary Fig. 4) by converting % remaining to absolute amount. The absolute amount of insulin absorbed from the injection site over time were obtained for Insulatard® and Lantus® (supplementary Table 3 for confidence intervals). The corresponding fits are given by Eqs. (12) and (13) (see supplementary Table 4 for confidence intervals for the fitted parameters):

**Insulatard®:**

$$\text{Insulin absorbed (pmol)} = 152405\left(1 - e^{-0.066_{\text{in vivo}}}t\right)$$  \hspace{1cm} (12)

**Lantus®:**

$$\text{Insulin absorbed (pmol)} = 2603_{\text{in vivo}} - 474$$ \hspace{1cm} (13)

where $t_{\text{in vivo}}$ is given in hours. Differentiation of Eqs. (12) and (13) provided the systemic input rate of insulin from the subcutaneous tissue to plasma ($J(t)$) for the two formulations:

**Insulatard®**

$$J(t) = \frac{9144 - e^{-0.066_{\text{in vivo}}t}}{h}$$ \hspace{1cm} (14)

**Lantus®**

$$J(t) = 2603$$ \hspace{1cm} (15)

Eqs. (14) and (15) were introduced into one-compartment model for plasma insulin as described in Eq. (5). The respective integrals were solved and the resulting expressions simulate insulin plasma profiles after subcutaneous administration of 26 U for Insulatard® and Lantus®:

**Insulatard®**

$$c_{\text{in vivo}}(pM) = 117e^{-0.066_{\text{in vivo}}t} - 117e^{-6.6_{\text{in vivo}}t}$$ \hspace{1cm} (16)

**Lantus®**

$$c_{\text{in vivo}}(pM) = 33 - 33e^{-6.6_{\text{in vivo}}t}$$ \hspace{1cm} (17)

Expressions simulating the insulin plasma profiles assuming an insulin bioavailability of 50% were also calculated using a simplified approach where 50% of the insulin was bioavailable at time 0. This resulted in expressions similar to Eqs. (16) and (17), but where 50% of 117 and 33 were used instead, respectively. The simulated profiles were compared to plasma profiles (serum insulin) from the literature (Fig. 6). The plasma profiles for Insulatard® (Hubinger et al., 1992) and Lantus® (Zhang et al., 2017) from healthy subjects were corrected for endogenous insulin using the C-peptide concentration whereas no correction for endogenous insulin was required for the plasma profile for Lantus® obtained upon injection in to type 1 diabetic patients (Becker et al., 2015). The insulin concentrations were measured on day 8 upon administering insulin glargine (Lantus®) for 8 consecutive days and achievement of steady state conditions (Becker et al., 2015).

In the present work, the plasma profiles after subcutaneous administration of the insulin formulations were simulated using Eq. (5) (Soeborg et al., 2009) ($V_E = 12 \text{l}$ (Kraegen and Chisholm, 1984) and $k_e = 6.6 \text{ h}^{-1}$ (Normann Hansen, 2004)). The simulated plasma profiles for Insulatard® have a lower $t_{\text{max}}$ as compared to the plasma profile from the literature (Fig. 6A). This may be due to correction for column retention time of the in vitro release data that were used to obtain the simulated plasma profiles. The considerable higher plasma concentrations of the simulated plasma profile ($F = 1$) for Insulatard® (Fig. 6A) was due to the assumption that the bioavailability upon subcutaneous administration was 100%. It has been found that the bioavailability for suspensions such as Insulatard® due to degradation of insulin in the subcutaneous tissue by macrophages (Markussen et al., 1996; Soeborg et al., 2012) is lower than that of soluble insulin, which is 50–80% (Soeborg et al., 2012). Macrophages invade and degrade the injected insulin crystals thereby making some material unavailable for absorption (Markussen et al., 1996; Soeborg et al., 2012). Adjustment for bioavailability ($F = 0.5$), led to a closer degree of resemblance between the simulated and observed plasma profiles for Insulatard® (Fig. 6A). Invading macrophages may also explain why the in vivo absorption profiles, used to make the IVIVC shown in Fig. 4B, exhibit a considerable longer absorption time as compared to the observed duration of action in man (48 h versus 10–16 h for Insulatard® and 20–24 h for Lantus®) (DeWitt and Hirsch, 2003; Luzio et al., 2003). A fraction of the insulin injected may not be absorbed, but due to the labeling still contribute to the residual radioactivity measured. The invasion of macrophages has only been reported for Insulatard® (Markussen et al., 1996; Soeborg et al., 2012); however, it may also

---

Table 2: Internal validation of the IVIVC.

<table>
<thead>
<tr>
<th>Internal validation</th>
<th>$t_{50%}$ from the literature*</th>
<th>Predicted $t_{50%}$ (error)</th>
<th>$t_{50%}$ from the literature*</th>
<th>Predicted $t_{50%}$ (error)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulatard®</td>
<td>6.5 h</td>
<td>5.4 h (−15.6%)</td>
<td>13.4 h</td>
<td>13.5 h (0.7%)</td>
</tr>
<tr>
<td>Lantus®</td>
<td>15.0 h</td>
<td>15.1 h (0.7%)</td>
<td>26.3 h</td>
<td>30.0 h (14.1%)</td>
</tr>
<tr>
<td>Average error</td>
<td>8.2%</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Luzio et al., 2003.
occurred for Lantus®. The model depicted in Eq. (5) was developed for soluble and crystalline insulins (Soeborg et al., 2009). Insulin glargine (Lantus®) is metabolized into the metabolites M1 (21^Gly-human insulin) and M2 (21^Gly-des-29^Thr-human insulin) (Boll et al., 2012; Kuerzel et al., 2003). M1 has been found to account for >90% of the plasma insulin after subcutaneous injection of insulin glargine (Lucidi et al., 2012). M1 has been found to possess similar metabolic properties as endogenous insulin (Lucidi et al., 2012) and insulin glargine to have similar elimination half-life as human insulin (EMA, 2009). Consequently, it was considered of interest to apply the model to insulin glargine. The simulated profiles for Lantus® describe a constant plasma concentration over a prolonged period of time (Fig. 6B) as a result of a linear disappearance profile of Lantus® (constant $K_j$). Lantus® (Gillies et al., 2000; Lepore et al., 2000) is characterized by a flatter time-action profile compared to Insulatard®, which was captured by the simulation (Fig. 6). The simulated plasma profiles for Lantus® will decrease at $t = 60$ h where the predicted% insulin remaining at the injection site was zero using a dose of 26 U. Adjustment for bioavailability (50%) did not improve the simulated plasma profile for Lantus® (Fig. 6B). The lack of agreement between the simulated plasma profiles and those obtained in the literature for Lantus® suggests that the model needs further refinement in order to properly simulate plasma profiles for Lantus®. Combining the simulated insulin absorption profiles with the pharmacokinetic model provides simulated plasma concentration-time profiles, which we believe capture the most distinct and characteristic features of the formulations. In order to achieve a quantitative agreement, refinement in terms of incorporating a diffusion component in the release model is required for the IVIVC to include soluble insulin. At the current level of development, the in vitro setup along with the IVIVC may be useful in ranking the in vivo action of other intermediate and long acting insulins, where a dissolution step is present.

4. Conclusions

The novel flow-through based in vitro release method sought to mimic the in vivo environment at the subcutaneous injection site and to incorporate features of the injection step, which renders the approach of particular interest for liquid formulations and in situ forming formulations administered subcutaneously. The flow-through based in vitro release method was found to provide the correct rank ordering in relation to the in vivo absorption of the 4 insulin products, Actrapid®, Mixtard® 30, Insulatard® and Lantus®. Using literature in vivo data and in vitro release data generated in the novel gel column, the potential of establishing an IVIVC was explored. A correlation between in vitro release and in vivo absorption (IVIVC) was established using non-linear time scaling for the suspension-based and in situ precipitating formulations Insulatard® and Lantus®. Actrapid® did not fit into the correlation, most likely because it consists of soluble insulin hence not endowed with a rate controlling dissolution step. The in vitro method is lacking the diffusion barrier, which is present in vivo and therefore not suited for injectable designs as a simple solution. The IVIVC obtained for Insulatard® and Lantus® was used to construct predicted in vivo absorption profiles and these were compared to the absorption profiles found in the literature by comparing the time where 75% and 50% insulin was remaining ($t_{75\%}$ and $t_{50\%}$) at the injection site. The average error of both formulations was less than 10% for $t_{75\%}$ and $t_{50\%}$, however the predicted $t_{75\%}$ for Insulatard® deviated >15% (15.6%) from the $t_{75\%}$ found in literature. Therefore, the IVIVC did not fulfill the requirements set for predictability of a level A IVIVC by FDA for extended release oral drug products. Simulated plasma profiles based on the predicted in vivo absorption profiles were compared to plasma profiles found in the literature. The simulated profiles for Insulatard® exhibited a shape resembling the plasma profile obtained in man and adjustment for bioavailability resulted in a close degree of resemblance between the simulated and observed plasma profiles for Insulatard®. The simulated plasma profile for Lantus® captures some of the characteristics (flatter plasma profile compared to Insulatard®) of the in vivo profiles from the literature. However, the simulated profiles for Lantus® differed from those obtained in literature even after adjustment of bioavailability. This suggests that the model needs further refinement in order to simulate plasma profiles after subcutaneous injections of Lantus®. The IVIVC for insulin found in this study provides a tool for ranking the absorption behavior of intermediate and long-acting insulins as well as to simulate plasma concentration-time profiles. This work demonstrates that it is possible to design an in vitro release model incorporating an injection step in a repeatable manner. The correlation obtained for modified insulin release injectables suggests that the developed type of flow-through gel column in vitro release testing setup is suited for depot injectables. However, ideally, a diffusion step should be incorporated in particular for solution-based drug formulations. The in vitro setup may be of wider interest in characterizing depot injectables for subcutaneous administration.

Funding

This work was supported by Novo Nordisk A/S, Måløv, Denmark.

Supplementary materials


References

Al-Tahami, K., Oak, M., Singh, J., 2011. Controlled delivery of basal insulin from phase-


