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DMD #15669

Lymphatic absorption of subcutaneously administered proteins: Influence of different injection sites on the absorption of Darbepoetin alfa using a sheep model

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Running Title: Lymphatic absorption of Darbepoetin alfa

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List of Abbreviations:

DA – Darbepoetin alfa

rHuEPO – Recombinant human erythropoietin

F_{abs} – Total fraction of dose absorbed

F_{blood} – Fraction of dose absorbed into the blood

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F_{lymph} – Fraction of dose taken up by lymphatics

QC – Quality control

LLOQ – Lower limit of quantification

FITC – Fluorescein isothiocyanate

FSD – Fractional Standard Deviation

k_{loss} – Rate of loss of drug at the injection site

k_{blood} – Rate of absorption of the drug into the blood

k_{lymph} – Rate of absorption of the drug into the lymph

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Abstract

The relative contribution of the lymph and blood in the absorption of Darbepoetin alfa (DA) from different subcutaneous (SC) injection sites was determined using a central lymph-cannulated sheep model. DA was administered to parallel groups either as a bolus intravenous (IV) injection (0.5 µg/kg) into the jugular vein, or as a bolus SC injection (2 µg/kg) into the interdigital space, the abdomen or the shoulder. In the lymph-cannulated groups, the thoracic lymph duct was cannulated for continuous collection of central lymph and blood samples were periodically collected via the jugular vein in all groups. The concentration of DA in serum and lymph was determined by ELISA. The total fraction of the dose reaching the systemic circulation and the fractions absorbed via the lymph and the blood were determined. A pharmacokinetic model was constructed to simultaneously fit the data from all treatment groups. Absorption was essentially complete for all three injection sites in non-lymph-cannulated SC groups, but the rates of absorption differed significantly. Based on the modelling results for the lymph-cannulated groups, the lymphatics represented the predominant absorption route for both the interdigital (90 ± 1%) and the abdomen (67 ± 9%) injection sites. FITC dextran visualization studies revealed that the lymph draining the shoulder injection site entered the thoracic lymph duct distal to the point of cannulation, effectively precluding collection of thoracic lymph from this site. For that reason, the contribution of the lymphatics following injection in the shoulder could not be determined using these cannulation procedures.

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Darbepoetin alfa (DA, Aranesp®) is a hyperglycosylated analogue of recombinant human erythropoietin (rHuEPO) which stimulates red blood cell production (erythropoiesis) by the same mechanism as rHuEPO (Macdougall, 2000). DA is a 165 amino acid protein containing 5 N-linked oligosaccharide chains whereas rHuEPO has only three oligosaccharide chains (Egrie and Browne, 2001). The additional carbohydrate chains increase the molecular weight of the glycoprotein from approximately 30,400 to 37,000 Da which results in a considerable prolongation in half-life to 25 h after IV administration relative to 8.5 h after IV administration of rHuEPO (Macdougall et al., 1999). The longer half-life for DA allows for once a week SC administration compared to two to three times per week administration for rHuEPO (Egrie and Browne, 2001). DA is currently used in the treatment of anemia induced by cancer chemotherapy, chronic renal failure and other disorders.

After SC administration, proteins enter the systemic circulation either by direct absorption into subcutaneous blood capillaries or indirectly via absorption into the lymphatic capillaries present within the interstitial space. Previous reports in the literature have consistently suggested that proteins larger than approximately 16 kDa are absorbed primarily via the lymphatics (Supersaxo et al., 1988; Supersaxo et al., 1990; Charman et al., 2000; Charman et al., 2001; McLennan et al., 2003; McLennan et al., 2005). Much of the information to date has been generated following protein injection into either the lower region of the hind leg or the interdigital space of the hind leg in sheep. These injection sites were used for practical reasons in that they allowed cumulative and quantitative recovery of the peripheral lymph draining the injection site via cannulation of the popliteal lymph duct which collects all the lymph from the lower leg. With this experimental design (i.e. collection of peripheral lymph), the absorption process could be studied without consideration of the effect of

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transport through the larger lymphatics and lymph nodes (Porter and Charman, 2001). However, the question remains as to whether these studies provide a realistic estimation of the contribution of the lymphatics to the absorption process given that the injection sites are not representative of those typically used in humans.

We have recently reported that DA is predominantly taken up by the lymphatics with approximately 90% of the dose recovered in peripheral lymph following injection into the interdigital space of the hind leg using a sheep model (McLennan et al., 2006). In these studies, the rate of absorption following SC injection was considerably faster than that seen in humans with a time to the maximum concentration (T_{max}) of 8 h in comparison to 36-72 h in humans (Macdougall et al., 1999). It was proposed that the more rapid absorption of DA in sheep could have been due to the use of the interdigital injection site in comparison to injection in the arm, thigh, or abdomen in humans.

The influence of different SC injection sites on the rate and extent of protein absorption has been demonstrated for several different proteins in humans (Koivisto and Felig, 1980; Beshyah et al., 1991; Macdougall et al., 1991; Jensen et al., 1994; ter Braak et al., 1996; Owens et al., 2000). Though the extent of absorption is typically consistent for different injection sites, variability in the rate of absorption has been reported. These differences are likely due to differences in the subcutaneous blood and lymph flow in different anatomical regions, however the relative contributions of the blood and the lymph in the overall absorption profile from different subcutaneous injection sites has not been reported previously.

The current study was conducted to explore the role of injection site in dictating the absorption kinetics of DA, and to characterize the role of the lymphatics in the absorption process. DA was used as a model protein for these investigations

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since it is known to be largely absorbed via the lymphatics (McLennan et al., 2006) and it can be readily assayed by ELISA. In order to study the influence of different injection sites, a central lymph-cannulated sheep model (Porter et al., 2001) was used with the assumption that the majority of the lymph draining the different anatomical injection sites is collected in the thoracic lymph duct prior to its entry into the systemic circulation (Guyton and Hall, 1997).

METHODS

Materials

Formulated DA and blank placebo for dilution were generously provided by Amgen Inc. (Thousand Oaks, CA). Commercial Quantikine in-vitro diagnostic human erythropoietin enzyme linked immunosorbent assay kits were purchased from R&D Systems (Minneapolis, USA). Intravenous catheters (133 mm, Angiocath™, Beckton Dickinson, Australia) were used to catheterize the jugular vein for blood sampling. Sterilised medical grade polyvinyl cannulae with a 1.40 mm internal diameter and 1.90 mm external diameter (Paton Scientific, South Australia, Australia) were used for cannulating the thoracic lymph duct. The blood samples were collected into 5 mL glass tubes (Vacutainer®, Becton Dickinson) and central lymph was collected into 120 mL polypropylene tubes (Sarstedt Avst., Australia). A Heraeus Biofuge (Krendo Laboratory Products, Germany) was used to centrifuge the blood and lymph samples.

Study Design

The animal studies were conducted in accordance with the “Principles of laboratory animal care” (NIH Publication #85-23, revised 1985) and were approved by the University of Melbourne Animal Experimentation Ethics Sub-Committee. A

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parallel study design with IV control, SC control, and SC lymph-cannulated treatment groups was used in which a single dose of DA was administered to each animal. For the IV control group (n=4), 0.5 µg/kg was administered by IV bolus injection of approximately 1 mL into the jugular vein. For the SC treatment groups, three different injection sites were used for both the control and lymph-cannulated groups (n=4 for each group) and each sheep received 2 µg/kg by bolus SC injection of approximately 1 mL. During the experiment, sheep were housed in metabolic cages and had access to food and water *ad libitum*.

Surgical Procedures

Adult male merino wether sheep were used in these studies. The weight range for sheep in the IV group was 33-42 kg, whereas the weight ranges for the interdigital, abdomen and shoulder SC study groups were 38-57 kg, 40-61 kg and 42-67, respectively. Intravenous catheters were inserted into the jugular vein of all the sheep for collection of blood samples. For the central lymph-cannulated groups, the thoracic lymph duct was cannulated according to procedures described elsewhere (Porter et al., 2001). Antibiotics were administered by IM injection (600 mg procaine penicillin) to the sheep immediately after surgery to reduce the likelihood of post-operative infection. Following an initial recovery period, sheep were transferred to cages and allowed to recover for 14-16 hours before dose administration. The central lymph flow was monitored in the lymph-cannulated animals and animals with a lymph flow of less than 30 mL/h were excluded from the study (Porter et al., 2001).

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Dose Administration and Sample Collection

The formulation of DA was a sterile, clear and colourless solution containing 500 µg/mL DA. The dose for administration in the current study was prepared by dilution immediately prior to administration using a matching placebo formulation to obtain a 20 µg/mL solution for IV administration and an 80 µg/mL solution for SC administration.

A bolus IV injection was administered to the IV control sheep via the non-catheterised jugular vein. SC doses were injected as a bolus into either the interdigital space of the hind leg, the abdomen ~2 cm ventral to the lateral spinus processes and ~2 cm cranial to the iliac fossa, or the shoulder ~2 cm caudal to the line of the mid humerous.

Blood samples were collected from all animals prior to dosing and the catheter for blood sampling was kept patent with heparinised saline (10 IU/mL). At the time of collection of each blood sample, an initial 1 mL volume of blood was withdrawn and discarded due to the presence of heparinised saline within the cannula, and a 5 mL blood sample was then collected. Following sample collection, the cannula was flushed with fresh heparinised saline. Blood samples for the IV and SC control groups were collected via the jugular vein catheter periodically over 168 h post-dose. For the central lymph-cannulated groups, blood samples were collected over 24-48 h post dose.

Central lymph was continuously collected from the cannulated animals into pre-weighed 120 mL polypropylene tubes and the lymph volume was determined gravimetrically. Lymph was collected for 30-60 minutes before dosing and post-dose lymph samples were collected at hourly intervals for 24 h in the interdigital and shoulder groups and for 48 h for the abdomen group. This limited blood and lymph

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sampling schedule in the lymph-cannulated animals relative to that used for blood sampling in the control groups was necessary to avoid dehydration of the animals with continuous lymph collection. The longer sampling schedule for the abdomen group was necessary given the significantly slower rate of absorption for this injection site.

Blood and lymph samples were allowed to clot for 60 min at room temperature and were then centrifuged at ~2600 *x g* for 10 min to separate the supernatant. The supernatant was collected and stored at -80°C until analysis.

Lymphatic Visualisation Studies

Evaluation of the lymph transport data obtained after injection of DA in the shoulder revealed a seemingly lower level of transport into the thoracic lymph than was expected. As such, the pattern of lymph drainage from the shoulder injection site was examined in more detail using a high molecular weight (21 kDa) fluorescent tracer (FITC dextran, Sigma-Aldrich, NSW, Australia). In these studies, 2 mL of a 4 mg/mL solution of FITC dextran was injected into the shoulder injection site in two anesthetised animals using the same injection technique that was employed for the shoulder SC treatment group. The surrounding lymphatics were carefully isolated to facilitate visualisation of the uptake and transport of the FITC dextran from the injection site to the thoracic duct. Visualisation of the fluorescent material was enhanced by viewing under an ultraviolet light.

Assay Method

The concentration of DA in serum and central lymph samples was determined using a commercial ELISA kit and the assay procedure was conducted as

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recommended by the manufacturer. Standards for DA were prepared in the corresponding blank matrix (i.e. serum or central lymph) using the same DA solution used for dosing. Standards for the calibration curve constituted eight concentrations over a range of 0.13-6.25 ng/mL.

Samples were diluted wherever necessary in the corresponding blank matrix before analysis to ensure that concentrations were within the range of the calibration curve. The pre-dose central lymph collected from each animal was used for sample dilution and standard preparation for the analysis of the central lymph samples. Blank sheep serum was collected from multiple sheep and pooled for the preparation of serum standards and for dilution of the serum samples.

Assay Validation

Quality control (QC) samples were prepared at three concentrations (0.25, 2 and 4 ng/mL) in each matrix (i.e. either blank sheep serum or lymph) and aliquots were stored along with the study samples. QC samples were included in each assay to monitor sample storage stability and to validate the assay performance. The lower limit of quantification (LLOQ) for the assay was defined as the lowest calibration standard which produced a signal at least twice the background absorbance, and which had an accuracy within ±15% and precision (% CV) of less than 15%.

The QCs in each assay were consistently within the ±15% of the nominal concentration demonstrating the accuracy of the method. The blank serum and lymph samples had no quantifiable concentrations of the endogenous sheep erythropoietin suggesting either low cross reactivity between human and sheep erythropoietin, or concentrations of endogenous erythropoietin in sheep which were not detectable.

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The dilution of the study samples was also validated by preparing high concentration samples which were within the expected range of the highest study sample concentrations. These high concentration QC samples were then diluted to fall within the assay calibration range and the concentration was estimated from the calibration curve. The measured concentrations were within ±15% of the nominal values confirming that the dilution approach was appropriate.

Non-Compartmental Analysis

Non-compartmental analysis was conducted using WinNonlin (v. 4.0, Pharsight Corporation, Mountain View, CA, USA) for individual animals in the control groups. The area under the serum concentration-time profile from zero to infinity (AUC_{IV}), terminal half life ($t_{1/2}$), terminal rate constant (k_{el}), mean residence time (MRT), serum clearance (CL) and volume of distribution at steady state (V_{ss}) were determined following IV dosing. For the SC groups, the area under the serum concentration-time profile extrapolated to infinity (AUC_{SC}), $t_{1/2}$, $k_{el,}$ were calculated and the maximum serum concentration (C_{max}) and the time to reach the maximum concentration (T_{max}) were taken directly from the data. The fraction absorbed (F_{abs}) in the SC control groups was calculated from equation 1 where AUC_{sc} and AUC_{iv} are the areas under the concentration-time profiles extrapolated to infinity for individual SC control animals and the mean of the IV group, respectively. $Dose_{iv}$ and $Dose_{sc}$ are the administered doses in µg/kg for the IV and SC control groups, respectively.

$$F_{abs} = \frac{Dose_{iv} * AUC_{sc}}{Dose_{sc} * AUC_{iv}} \text{ x } 100 \qquad (1)$$

For the lymph-cannulated groups, the cumulative mass of DA collected in lymph was expressed as a fraction of the administered dose (F_{lymph}, Equation 2). The total fraction absorbed (F_{abs}) in the lymph-cannulated groups was calculated as a sum

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of the fraction of the dose recovered in lymph (F_{lymph}) and the fraction in blood (F_{blood}), the latter of which was obtained by comparing the serum data for individual animals in the lymph-cannulated groups to the mean IV data using equation 1.

$$F_{lymph} = \frac{\text{Amount recovered in lymph (µg)}}{\text{Dose administered (µg)}} \text{ x } 100 \qquad (2)$$

Compartmental Analysis and Modelling

Compartmental analysis of the data was performed using SAAM II (Version 1.2; SAAM Institute, University of Washington, Seattle). The mean data for each injection site were fit using the fractional standard deviation (FSD) for each datum based on the coefficient of variance for the corresponding mean data point. The model used for the IV control group was a standard two compartment model. The models for the SC control and the SC lymph-cannulated groups are shown in Figure 1. For the interdigital and abdomen injection sites, the data for the IV, SC control and SC lymph-cannulated groups were fit simultaneously while constraining the parameters k_{12}, k_{21}, k_{10}, and V_c to be the same for each group (Figure 1A and 1B). In the case of the shoulder injection site, only the data for the IV and SC control groups were fit to the model (Figure 1C, same parameters constrained). The absorption rate constants (k_{lymph} and k_{blood}) in the non-cannulated SC model were constrained to be equal to the corresponding rate constants in the cannulated SC model (interdigital and abdomen injection sites only). A rate constant for loss at the site of injection (k_{loss}) was introduced in the SC models and was constrained to be equal for the non-cannulated and cannulated groups. Goodness of fit for each of the models was assessed by the convergence of the least square regression, weighted residual sum of squares (WRSS) and the precision of parameter estimates.

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The equations used for the secondary parameter estimates in the lymph-cannulated model were:

$$F_{abs} = \frac{k_{lymph} + k_{blood}}{k_{lymph} + k_{blood} + k_{loss}} \quad (3)$$

$$F_{lymph} = \frac{k_{lymph}}{k_{lymph} + k_{blood} + k_{loss}} \quad (4)$$

$$F_{blood} = \frac{k_{blood}}{k_{lymph} + k_{blood} + k_{loss}} \quad (5)$$

Equation 3 describes the total fraction of the dose absorbed while equations 4 and 5 describe the individual fractions absorbed via the lymph and the blood, respectively.

Statistical analysis

Statistical analysis was performed using SPSS (Version 11.5.0) and the significance was tested at α=0.05.

RESULTS

Non-Compartmental Pharmacokinetics of DA

After IV administration, the serum concentrations of DA declined in a bi-exponential manner demonstrating an initial distribution phase and a subsequent elimination phase (Figure 2, Panel A). The terminal half life ($t_{1/2}$) and serum clearance (CL) were 26.3 ± 3.4 h and 2.24 ± 0.31 mL h^{-1} kg^{-1}, respectively. It is evident from the volume of distribution at steady state (V_{ss}, Table 1) that DA was not extensively distributed outside the serum. The values obtained are consistent with those previously described using the same animal model (McLennan et al., 2006).

In the non-cannulated SC control groups (Figure 2, Panel B), the absorption from the interdigital and the shoulder injection sites was relatively fast with a T_{max} of

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approximately 8 h for each compared to approximately 18-72 h for the abdomen group. The overall bioavailability was essentially complete for each of the three injection sites (Table 1). The terminal elimination half life for each of the non-cannulated SC control groups was not statistically different to that of the IV control group (Table 1).

In the central lymph-cannulated group in which DA was administered by SC injection into the interdigital space (Figure 3), DA entered the systemic circulation predominantly via the lymphatics with 92 ± 15% of the administered dose recovered in thoracic lymph over 24 h. The total fraction absorbed, obtained by summing the fraction of the dose absorbed via the lymph and the blood, was not statistically different to that for the non-cannulated control group using the same injection site (Table 1).

The lymphatic recovery of DA following injection into the abdomen in the lymph-cannulated group could not be completely quantified over the 48 h post-dosing period which represents the maximum cannulation period which can be utilised with this animal model to avoid dehydration (Porter et al., 2001). The serum and lymph profiles for the abdomen group (Figure 4) suggest that absorption was still occurring at the conclusion of the 48 h sampling period. The estimated lymphatic recovery of DA following injection into the abdomen during the 48 h lymph collection period was 28 ± 4% and the dose recovered in the systemic circulation over the same time period was 8.2 ± 3.4%, the sum of which (37 ± 7%) was considerably lower than that for the non-cannulated control group where the calculated F_{abs} was 85 ± 20%. Calculation of the fractional AUC in the non-cannulated abdomen group up to 48 h gave a value of only 33 ± 5% which is close to the total percent recovered in blood and lymph in the lymph-cannulated abdomen group over the 48 h collection period.

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The initial data analysis for the absorption of DA following SC injection into the shoulder indicated that absorption occurred predominantly via the blood with 75 ± 10% of the administered dose recovered in the blood while only a very small fraction was recovered in the lymph over 24 h. This surprisingly low recovery of DA in lymph following injection into the shoulder prompted an additional visualization study in which a 21 kDa FITC dextran was injected into the shoulder region in two sheep in an attempt to follow the transfer pathway from this injection site into the systemic circulation. Previous studies conducted with proteins of a size similar to that of the FITC dextran have indicated that a large proportion of the dose would be absorbed via the lymphatics (Charman et al., 2000). In both animals, visual assessment using an ultraviolet light indicated that the FITC dextran was taken up by the surrounding lymphatics and entered the thoracic lymph duct at the junction with the internal jugular vein (see Figure 5). This site of entry was approximately 2 cm distal to the site of cannulation employed in the lymph-cannulated animals such that DA absorbed from the shoulder injection site into the local lymphatics would have escaped collection via the thoracic lymph duct cannula and instead, would have been returned to the systemic circulation via the jugular vein (i.e. “downstream” of the thoracic cannulation site). This is consistent with the high recovery in blood and the low recovery in lymph following injection into the shoulder region in lymph-cannulated sheep. Unfortunately, given that the lymphatic entry point was close to the junction of the thoracic duct and the jugular vein, it was not possible to change the lymph cannulation site in a manner which would have ensured collection of the lymph from this injection site. Attempts to obtain photographs which showed the absorption pathway of the FITC dextran injected into the shoulder region were unsuccessful and it was not possible to visualise the fluorescence without the aid of a UV light.

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Compartmental Modelling

A two compartment model with first order elimination from the central compartment best described the disposition of DA after IV administration. The model predicted data are shown in Figure 2A and the parameter estimates are shown in Table 2.

For the SC groups, a two compartment model was constructed to simultaneously fit the serum data for the interdigital and abdomen groups and the cumulative amount of DA in lymph (Figure 1A and 1B). Despite the monoexponential decline in the concentration of DA in serum following SC administration, a two compartment model was adopted based on the biexponential decline following IV administration. The mean volume of the central compartment (V_c), elimination rate constant (k_{10}) and inter-compartmental distribution constants (k_{12} and k_{21}) were constrained to be equal to the corresponding rate constants in the IV model. The absorption rate constants in the non-cannulated SC control groups were forced to be equal to the corresponding rate constants in the lymph-cannulated groups.

The model shown in Figure 1A was fit to the data from the interdigital injection site using first order input and elimination for both the non-cannulated and cannulated groups. Based on the results shown in Figure 3, the model provided a good fit to the experimental data for both the serum and the lymph.

When the model shown in Figure 1A was applied to the data for the abdomen group, the fits were poor. The lymph profile for the abdomen injection site appeared to be biphasic (Figure 4), and hence required a model with two first order inputs into both the lymph and blood and a single first order elimination process from the central compartment (Figure 1B). One of the two first order inputs for both the lymph and the

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blood also required a lag time to obtain a good fit to the data. Despite the incomplete data set, and realizing that there will be error in the estimated parameters, the model appeared to adequately describe the available data and estimated the lymph recovery of DA to be 67 ± 9% following injection into the abdomen.

Since the lymph data were found to be unreliable for the shoulder injection site, no attempt was made to fit the central lymph-cannulated data. The model shown in Figure 1C with first order input and elimination was fit to the non-cannulated SC control data for the shoulder group (Figure 2B).

DISCUSSION

Although the lymphatic absorption of protein drugs following SC injection has been previously reported (Supersaxo et al., 1988; Supersaxo et al., 1990; Charman et al., 2000; Charman et al., 2001; McLennan et al., 2003; McLennan et al., 2005), the injection site typically used has been the interdigital space in sheep, a site which is clearly quite different to clinically utilized injection sites in humans which typically include the thigh, shoulder or the abdomen. Previous studies with DA indicated a dramatic increase in the absorption rate following SC injection into the interdigital space in sheep (McLennan et al., 2006) compared with humans (Heatherington et al., 2001). The current studies were conducted to explore the role of injection site in dictating the rate of absorption of DA, and to further examine the contribution of the lymphatics to the absorption of DA from injection sites more similar to those used in humans.

The systemic availability of DA from the three SC injection sites was almost complete relative to the IV group with F_{abs} in the non-cannulated SC control groups being 106 ± 18%, 92 ± 13% and 85 ± 20% for the interdigital, shoulder and abdomen

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injection sites, respectively (Table 1). The rate of absorption from the three injection sites varied significantly with the abdomen group demonstrating the slowest rate of absorption (Figure 2, Panel A). The absorption rate constants obtained by compartmental modelling of the non-cannulated control groups were approximately 0.15, 0.08 and 0.02 h^{-1} for the interdigital, shoulder and the abdomen injection sites, respectively (Table 2).

The continuous collection of central lymph in the SC cannulated group led to a significant reduction in F_{blood} to 6.3 ± 4.2% for the interdigital injection site (Table 1 and Figure 3). Quantification of the fraction of DA in central lymph (F_{lymph}) accounted for 92 ± 15% of the injected dose. This value is similar to that previously reported when peripheral lymph was collected using the same injection site (McLennan et al., 2006). Summing F_{lymph} and F_{blood} for each animal, the value for F_{abs} was 99 ± 13% for the interdigital injection site which was not statistically different to F_{abs} for the non-cannulated SC control group (106 ± 18%). The comparable F_{abs} values in the non-cannulated and lymph-cannulated groups illustrates mass balance and confirms that the 24 h sampling period used for this group was sufficient. The model shown in Figure 1A provided good fits to the experimental data for both the non-cannulated and cannulated groups following injection into the interdigital space.

Absorption following injection into the abdomen was considerably slower than from the other injection sites with a T_{max} of approximately 48 h in comparison to approximately 8 h for the interdigital and shoulder injections sites (Table 1). Since it was not practical to sample beyond a maximum 48 h period in the cannulated animals due to the potential for dehydration and protein depletion, F_{abs} calculated using the data up to 48 h significantly underestimated the total absorption of DA following injection in this site. Further examination of the non-cannulated control data for this

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injection site indicated that the fractional AUC up to 48 h represented only 33% of the total AUC which was consistent with the total fraction absorbed in the lymph-cannulated group up to 48 h.

Application of the simplified model shown in Figure 1A to the available serum and lymph data obtained following injection into the abdomen resulted in poor fits to the available experimental data. Visual inspection of the serum and lymph absorption profiles (Figure 4) suggested the possibility of multiple absorption processes which required two first order input pathways, one with a lag time and one without. The resulting model (Figure 1B) provided a reasonable estimation of the partial experimental profiles. Using the predicted estimates for F_{lymph} (67 ± 9%) and F_{blood} (16 ± 2%), F_{abs} was calculated to be approximately 83% which was in agreement with the experimental value obtained for the non-cannulated control group (85 ± 20%). Even though the estimates are likely to be associated with error given the paucity of experimental data, it is reasonable to conclude that the lymphatic pathway represents the main route of absorption following injection into the abdomen. Unfortunately, the practical limitations of further sample collection after 48 h precluded a more rigorous quantitative assessment of the lymphatic absorption process for this injection site.

At present, the basis for the very slow absorption profile evident following injection in the abdomen is not known. The later T_{max} for the abdomen group, however, is more consistent with the absorption profile seen in humans where T_{max} for DA is approximately 36-72 h following SC injection into the arm (Macdougall et al., 1999). The different absorption rates from different injection sites is likely to be related to regional differences in blood and lymph flow. It has been estimated that in “active” regions of the body (e.g. deltoid), one lymphatic junction in every 2 to 5 may be open, but in “motionless” regions (e.g. buttock) there may be only one junction in

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50 to 100 which is open (Ballard, 1968). Similarly, blood flow also varies in different regions of the body with blood flow being least in the buttock, intermediate in the thigh and greater in the deltoid region (Evans et al., 1975). These regional anatomical differences may be the basis for the different absorption rates observed for the different injection sites since the shoulder and interdigital regions would be associated with considerably more movement than the abdomen region.

Despite the very different rates of absorption for the different sites, the lymphatics represented the predominant absorption pathway for both the interdigital and abdomen injection sites. The contribution of the lymphatics in the shoulder group could not be determined using the described cannulation procedures. FITC dextran visualization studies revealed that the lymph draining the shoulder injection site entered the thoracic lymph duct at the junction of the thoracic duct and the jugular vein, which is in contrast to the entry points for most lymphatic ducts draining other regions of the body which join the thoracic duct at points significantly removed from this junction (Guyton and Hall, 1997). Since the cannula was inserted into the thoracic lymph duct approximately 2 cm cranial to the junction with the jugular vein, the lymph draining the shoulder injection site was not collected via the thoracic lymph duct cannula resulting in higher than expected concentrations of DA in the blood and lower than expected lymphatic recovery. This limitation in the cannulation procedure therefore precluded an estimation of the actual contribution of the lymphatics in the absorption of DA following SC injection into the shoulder.

The results described here are significant in that they provide further evidence for the role of the lymphatics in the absorption of protein drugs following SC administration using clinically relevant injection sites. The data indicate that even though the rate of absorption varied significantly with different injection sites, the

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contribution of the lymphatics was high for each site. The use of different injection sites, and species differences in the anatomical variation of different regions, is the most likely basis for different rates of absorption of DA and other protein drugs following SC administration.

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Legends For Figures

Figure 1. Proposed pharmacokinetic models for DA administered as a SC bolus dose in the interdigital space (A), the abdomen (B) and the shoulder (C). For the interdigital and abdomen groups, the central lymph-cannulated, non-cannulated control and IV models were fit simultaneously whereas for the shoulder group, the non-cannulated control and IV models were fit simultaneously. The dotted line is a transfer rate in the control group and was not included in the cannulated model.

Figure 2. DA serum concentration versus time profiles following IV administration at 0.5 μg/kg (Panel A) and SC administration to non-lymph-cannulated control animals at 2 μg/kg (Panel B) with injection in the interdigital space (circles), abdomen (squares) or the shoulder (triangles). The symbols represent the experimental data (mean ± SD, n=4) while the model predicted data are represented as a solid line.

Figure 3. DA serum concentration versus time profiles following SC injection in the interdigital space with the filled circles representing the non-cannulated control data and the filled triangles representing the central lymph-cannulated data (Panel A). Panel B illustrates the cumulative recovery (μg) of DA in lymph following SC injection in the interdigital space. Symbols represent the experimental data (mean ± SD, n=4) and the lines denote the model predicted data. In panel A, the discontinuous line has been used to indicate the approximate nature of the predictions for the cannulated group given the paucity of data. The horizontal discontinuous line in panel B is the mean administered dose in μg and the grey shaded area represents the SD.

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Figure 4. DA serum concentration versus time profiles following SC injection in the abdomen with the filled circles representing the non-cannulated control data and the filled triangles representing the central cannulated data (Panel A). Panel B represents the cumulative recovery (µg) of DA in lymph following SC injection in the abdomen. Symbols represent the experimental data (mean ± SD, n=4) and the lines denote the model predicted data. In panels A and B, the discontinuous line has been used to indicate the approximate nature of the predictions for the cannulated group given the paucity of data. The horizontal discontinuous line in panel B is the mean administered dose in µg and the grey shaded area represents the SD.

Figure 5. Schematic representation of the lymphatic absorption and transfer processes following injection of 21 kDa FITC dextran into the shoulder as indicated by visualization studies in two anesthetised sheep.

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Table 1. Non-compartmental pharmacokinetic parameter estimates for DA following intravenous (IV, 0.5 µg/kg) and subcutaneous (SC, 2 µg/kg) administration in sheep. Values represent the mean ± SD for n=4 animals.

Parameter	IV Bolus	SC Control (non-cannulated)		
		Interdigital	Abdomen	Shoulder
C_{max} ($ng.mL^{-1}$)	-	20.7 ± 5.8	7.1 ± 1.0	12.6 ± 3.0
T_{max} (h) range	-	6-8	18-72	6-12
$T_{1/2}$ (h)	26.3 ± 3.4	30.5 ± 2.0	36.1 ± 9.3	29.0 ± 2.0
V_{ss} ($ml.Kg^{-1}$)	69.5 ± 11.9	-	-	-
CL ($mL.h^{-1}.Kg^{-1}$)	2.24 ± 0.31	-	-	-
F_{abs} (%)	-	106 ± 18	85 ± 20	92 ± 13
		SC Central Lymph-cannulated		
F_{lymph} (%)	-	92 ± 15	28 ± 4[a]	c.n.c.
F_{blood} (%)	-	6.3 ± 4.2	8.2 ± 3.4[a]	c.n.c.
F_{abs} (%)	-	99 ± 13	37 ± 7[a]	c.n.c.

[a] incomplete profile up to 48 h

c.n.c. – could not calculate since the lymph from this region emptied into the thoracic duct at a point distal to the cannulation site.

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Table 2. Compartmental pharmacokinetic parameter estimates for DA following intravenous (IV, 0.5 µg/kg) and subcutaneous (SC, 2 µg/kg) administration in sheep. Models were simultaneously fit to the mean data for n=4 animals in each group; the SD reflects the confidence in the parameter estimates.

Parameter	IV Bolus	SC Control (non-cannulated)		
		Interdigital	Abdomen	Shoulder
V_c (mL kg^{-1})[a]	44.8 ± 2.5	44.8 ± 2.5	44.8 ± 2.5	44.8 ± 2.5
k_{12} (h^{-1})[a]	0.206 ± 0.083	0.206 ± 0.083	0.206 ± 0.083	0.206 ± 0.083
k_{21} (h^{-1})[a]	0.290 ± 0.097	0.290 ± 0.097	0.290 ± 0.097	0.290 ± 0.097
k_{10} (h^{-1})[a]	0.0493 ± 0.0030	0.0493 ± 0.0030	0.0493 ± 0.0030	0.0493 ± 0.0030
T_{lag}_lymph (h)	-	0.19 ± 0.11	53.6 ± 13.6	-
T_{lag}_blood (h)		-	-	-
F_{abs} (%)	-	92 ± 1	83 ± 9	99 ± 5
k_a (h^{-1})	-	0.151 ± 0.003	0.0240 ± 0.0021	0.0815 ± 0.0101
		SC Central Lymph-cannulated		
		Interdigital	Abdomen	Shoulder[b]
$k_{lymph}1$ (h^{-1})	-	0.204 ± 0.008	0.0456 ± 0.0084	-
$k_{lymph}2$ (h^{-1})	-	-	0.0211 ± 0.0005	-
$k_{blood}1$ (h^{-1})	-	0.00481 ± 0.00060	0.00281 ± 0.00021	-
$k_{blood}2$ (h^{-1})	-	-	0.0133 ± 0.0020	-
T_{lag}_lymph (h)	-	0.38 ± 0.05	100 ± 15	-
T_{lag}_blood (h)		-	102 ± 12	-
F_{lymph} (%)	-	90 ± 1	67 ± 9	-
F_{blood} (%)	-	2.0 ± 0.3	16 ± 2	-

[a] The parameters k_{12}, k_{21}, k_{10}, and V_c were constrained to be equal in each group. The rate constants k_{lymph} and k_{blood} were constrained to be equal in the non-cannulated and cannulated SC groups.

[b] Lymph-cannulated data for the shoulder injection site were not fit to a model

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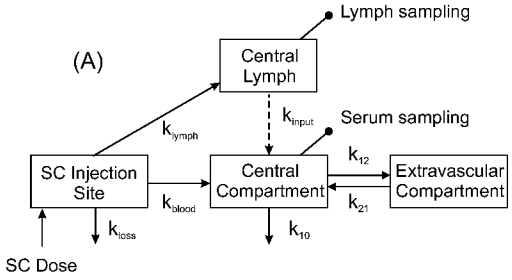
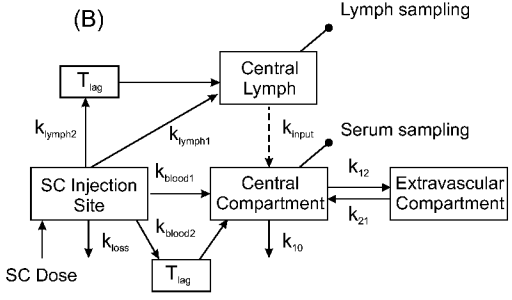
Figure 1.


(A)
Lymph sampling
Central Lymph
k_{input}
Serum sampling
k_{lymph}
k_{12}
SC Injection Site
Central Compartment
Extravascular Compartment
k_{21}
k_{blood}
k_{loss}
k_{10}
SC Dose


(B)
Lymph sampling
T_{lag}
Central Lymph
k_{lymph2}
k_{lymph1}
k_{input}
Serum sampling
k_{blood1}
k_{12}
SC Injection Site
Central Compartment
Extravascular Compartment
k_{21}
k_{blood2}
k_{loss}
k_{10}
SC Dose
T_{lag}

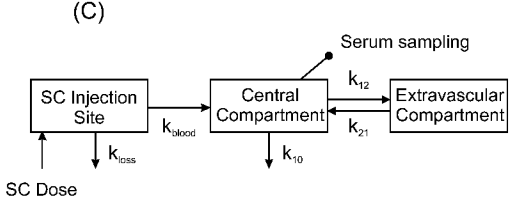

(C)
Serum sampling
k_{12}
SC Injection Site
Central Compartment
Extravascular Compartment
k_{21}
k_{blood}
k_{loss}
k_{10}
SC Dose

Figure 2.

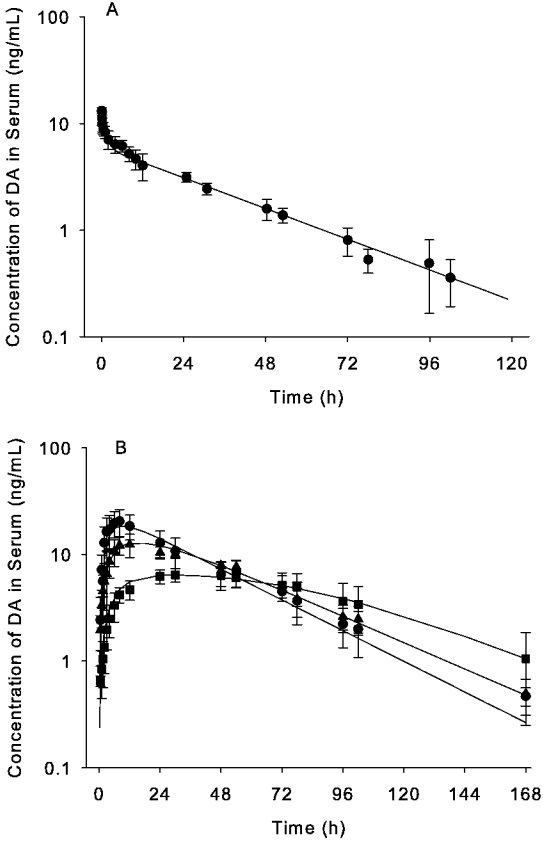

A
Concentration of DA in Serum (ng/mL)
100
10
1
0.1
0
24
48
72
96
120
Time (h)
B
Concentration of DA in Serum (ng/mL)
100
10
1
0.1
0
24
48
72
96
120
144
168
Time (h)

Figure 3.

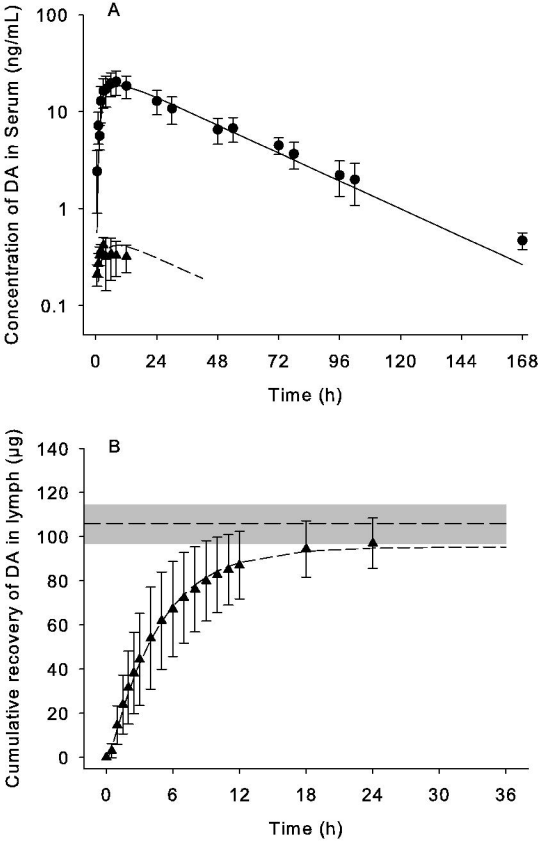

A
Concentration of DA in Serum (ng/mL)
100
10
1
0.1
0
24
48
72
96
120
144
168
Time (h)
B
Cumulative recovery of DA in lymph (µg)
140
120
100
80
60
40
20
0
0
6
12
18
24
30
36
Time (h)

Figure 4.

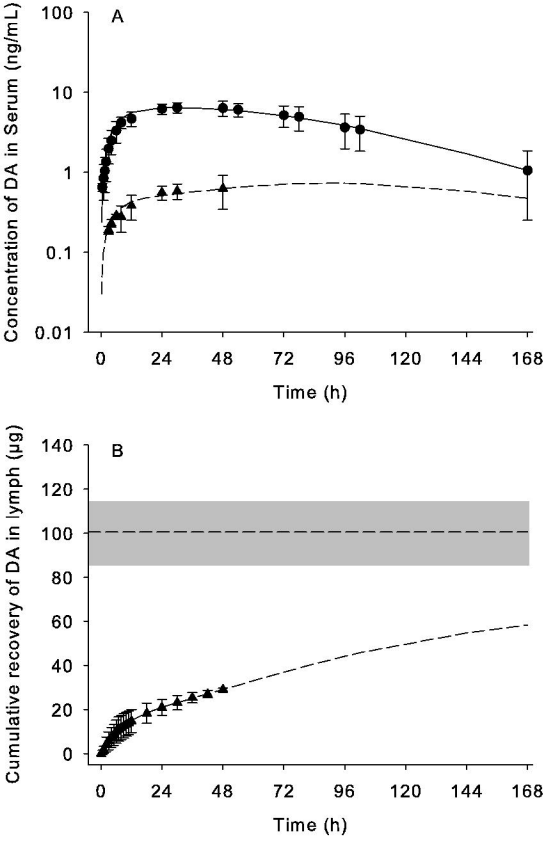
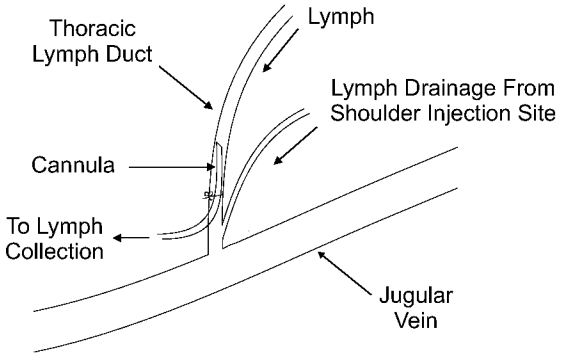

A
Concentration of DA in Serum (ng/mL)
100
10
1
0.1
0.01
0
24
48
72
96
120
144
168
Time (h)
B
Cumulative recovery of DA in lymph (µg)
140
120
100
80
60
40
20
0
0
24
48
72
96
120
144
168
Time (h)

Figure 5.


Thoracic
Lymph Duct
Lymph
Lymph Drainage From
Shoulder Injection Site
Cannula
To Lymph
Collection
Jugular
Vein