Available online at www.sciencedirect.com
ScienceDirect
European Journal of Pharmaceutics and Biopharmaceutics 67 (2007) 759–765
European Journal of Pharmaceutics and Biopharmaceutics
www.elsevier.com/locate/ejpb

Research paper

The role of the lymphatic system in subcutaneous absorption of macromolecules in the rat model

Leonid Kagan [a], Pavel Gershkovich [a], Avivit Mendelman [b], Sofia Amsili [b], Nathan Ezov [b], Amnon Hoffman [a,*]

[a] *Department of Pharmaceutics, The Hebrew University of Jerusalem, Jerusalem, Israel*
[b] *Harlan Biotech Israel Ltd., Rehovot, Israel*

Received 15 October 2006; accepted in revised form 2 April 2007
Available online 7 April 2007

Abstract

The purpose of this study was to assess the contribution of lymphatics to the systemic bioavailability of macromolecules following SC administration in a rat model. The rat model included continuous lymph collection from the thoracic lymph duct and concurrent serial blood sampling from freely moving animals. A thoracic lymph duct – jugular vein shunt produced by an implanted connective cannula, and maintained during the recovery period, enabled superior rat survival and prevented lymphatic cannula occlusion. The SC absorption of three macromolecules (bovine insulin, bovine serum albumin, and recombinant human erythropoietin alpha) was assessed in comparison to the non-lymph cannulated control group. For all tested molecules, only minimal amounts (less than 3%) of the SC administered dose were detected in the collected lymph. In the rat model, following SC administration, the macromolecules were absorbed mainly through the blood capillaries with minimal contribution of the lymphatic system to systemic bioavailability. The relatively small elevation in the lymphatic concentration, which occurred in all molecules, may be attributed to the redistribution of the molecules from the blood to the interstitial fluid compartment. These findings are important since rodents are commonly used in preclinical evaluation of macromolecular drugs.

Keywords: Pharmacokinetics; Lymphatic uptake; Therapeutic protein; Insulin; Erythropoietin

1. Introduction

Macromolecules, including hormones and antibodies, are an increasing part of the arsenal of compounds in clinical use. This is mainly due to constant advances in biotechnology and recombinant synthesis. Parenteral administration (i.e., injection) continues to be the primary route of delivery of these compounds due to their low and variable oral bioavailability.

The subcutaneous (SC) route provides important advantages over the intravenous (IV) mode of administration. These include: (1) convenience of injection for both the patients and the medical staff; (2) prolongation of the input kinetics of the drug into the systemic blood circulation, thus maintaining the desired blood levels of a drug for an extended time period and consequently, reducing the dosing frequency; and (3) targeting the drug or contrast agents to peripheral lymphatics [1].

Despite extensive clinical utilization of the SC route, the exact mechanism underlying SC absorption and factors influencing this process are not completely understood. This is particularly true with respect to lymphatic SC absorption kinetics, due to extreme technical difficulties of required surgical procedures, especially in small laboratory animals. Thus, our current ability to

* Corresponding author. Department of Pharmaceutics, School of Pharmacy, Faculty of Medicine, The Hebrew University of Jerusalem, P.O. Box 12065, Jerusalem 91120, Israel. Tel.: +972 2 6757014; fax: +972 2 6757246.
E-mail address: ahoffman@cc.huji.ac.il (A. Hoffman).

0939-6411/$ - see front matter © 2007 Published by Elsevier B.V.
doi:10.1016/j.ejpb.2007.04.002

predict the absorption rate following SC administration is poor [2].

The lymphatic system is a unidirectional flow system, which maintains homeostasis of fluid, proteins, and solutes in the body by their uptake from the extracellular matrix in tissues and transportation to the circulatory system. It is potentially a significant route for systemic absorption following SC administration of macromolecules [1,2] and nano- and microparticulates [3–5].

The main source of information currently available regarding factors affecting SC lymphatic uptake of macromolecules is data obtained from a sheep model [6]. The main conclusion from this series of experiments is that the percent of molecule uptake into the peripheral lymphatics following SC administration directly correlates with its molecular weight. This fact is attributed to differences between the permeability through two types of capillaries (blood and lymph) draining the SC space. While blood capillaries have a relatively "closed" structure, which limits the penetration of large molecules, the lymphatic capillaries are considerably more permeable (due to incomplete basolateral membrane and absence of inter-endothelial tight junctions [2,7]) and thus enable unrestricted drainage of macromolecules from the interstitial space. However, the data regarding the degree of macromolecule permeability through the capillary blood vessels wall are ambiguous. For example, in studies utilizing an immunocytochemical approach it was shown that insulin, and even molecules as large as albumin, can penetrate blood capillary endothelium by the transcellular route [8].

In addition to molecular weight, certain other factors that may affect lymph formation and propulsion have been suggested to influence the lymphatic SC absorption of macromolecule drugs. These include animal species, site of lymph cannulation, and the site of SC injection [7,9].

Since the SC route is commonly utilized for drug administration and because rodents are customarily used for preclinical biopharmaceutical drug evaluation, there is a need for definitive information regarding this absorption pathway in rodents and, in particular, data on the contribution of the lymphatic system to this route of absorption. It should be noted that data on lymphatic bioavailability (following both oral and SC administration) in rodents are highly limited since these experiments are time consuming and require very sophisticated surgical skills [10].

Consequently, the goals of this work were to produce a rat model that enables the investigation of SC lymphatic absorption in a freely moving animal and to use it to evaluate three model substances with difference in their molecular weights: bovine insulin (monomer molecular weight 5.6 kDa), recombinant human erythropoietin alpha (30.4 kDa), and bovine albumin (66 kDa).

The major finding of this work is that following SC administration all tested macromolecules were absorbed mainly by direct penetration into the blood capillaries without any significant contribution of the lymphatic system to the absorption process, despite the wide range of molecular weights.

2. Methods

2.1. Materials

Bovine insulin was purchased from Sigma Chemical Company, St. Louis, MO, USA. Lispro insulin (Humalog®, Eli Lilly & Co., France) and recombinant human erythropoietin alpha (EPO, Eprex®, Janssen-Cilag, Switzerland) were purchased from a local pharmacy. Bovine serum albumin – fluorescein conjugate (BSA) was purchased from Molecular Probes Inc., Eugene, USA.

2.2. Animals

All surgical and experimental procedures were reviewed and approved by the Animal Experimentation Ethics Committee of the Hebrew University Hadassah Medical School, Jerusalem. Male Wistar rats (Harlan, Israel), weighing 300–350 g, were used for all surgical procedures.

In the lymph-cannulated animal group the thoracic lymph duct was cannulated to allow for continuous lymph collection, as described below. Additionally, in this group, as well as in control group, the right jugular vein was cannulated for systemic blood sampling.

The anesthesia of animals for the period of surgery was initiated with 1 mL/kg solution of ketamine 20 mg/mL:xylazine 100 mg/mL (90:10 v/v) by intra-peritoneal injection and maintained by pure ketamine as needed.

The thoracic duct cannulation technique was based on previously reported methods [11,12], but was further improved to meet our requirements.

The thoracic duct is a very thin and transparent vessel, which lies dorsal to the abdominal aorta. For visualization of the vessel, peanut oil was administered by gavage (1.5, 1 and 0.5 mL at 2, 1 h and 2 min prior to anesthesia, respectively). A cannula made up of polyethylene tubing (PE50, Intramedic® Polyethylene Tubing, Becton Dickinson, MD) and silicone tubing (Silastic®, ID 0.02 in, OD 0.037 in) was used for lymphatic cannulation. The cannula was secured in place by 5–0 silk ligatures around the lymphatic duct and the cannula. The right jugular vein was cannulated using PE50 tubing. The venous and lymphatic cannulas were tunneled subcutaneously to the dorsal part of the neck of the animal and connected by a 2 cm loop of Tygon® (Thomas Scientific, ID 0.7 mm, OD 2.4 mm). The flow of lymph into blood circulation was verified and the skin on the dorsal part of the neck was closed by suture clips. The thoracic duct – jugular vein shunt mimics the physiological state where lymph empties into the circulatory system. It ensures continuous lymph flow thereby minimizing lymphatic catheter occlusion and prevents fluid and protein loss during the recovery period. An additional PE50 cannula was inserted into the duodenum of the lymph-cannulated animals. This cannula was used for infusion of

isotonic saline at a constant rate (1.5 mL/h) during the experiment to prevent lymphatic cannula occlusion.

After the surgery, animals were transferred to metabolic cages and stabilized overnight (12–16 h), during which the animals were fasted, but drinking solution (277 mM glucose and 3.4 mM KCl) was available *ad libitum*. Two hours prior to the experiment the drinking solution was changed to water.

2.3. Experimental procedure

The animals were allocated into two groups: lymph-cannulated and control (without lymph collection). In the case of insulin, an additional control group that received monomeric lispro insulin SC (without lymph collection, $n = 3$) was used. In the lymph-cannulated group the lymph–blood shunt was disconnected immediately prior to drug administration and lymph was continuously collected. The systemic blood was sampled at predefined time intervals, as specified below. The SC injection of all materials was performed at the lateral upper part of the left hind limb of the animal.

The following dosing solutions were used in the study: (1) bovine insulin (1 IU/mL) in normal saline; (2) BSA (2 mg/mL) solutions in normal saline; (3) the commercially available insulin lispro solution (Humalog®, 100 IU/mL) was diluted with normal saline to achieve a final concentration of 1 IU/mL; (4) EPO was injected as a commercially available solution 2000 IU/mL. The following doses were used: 1 IU/kg for bovine insulin, 2.5 mg/kg for BSA, 0.5 IU/kg for lispro insulin, and 400 IU/kg for EPO.

Blood and lymph samples were collected to heparin (for insulin and BSA) or EDTA (for EPO) containing tubes. The volume of each lymph sample was recorded. The blood sampling times for insulin were 0, 5, 15, 30, 45, 60, 90, 120, 150, 180, 210, and 240 min. The lymph was collected in half hour intervals up to 4 h. The blood sampling times for EPO were 0, 2, 3, 4, 5, 6, 7, 8, 9, 10, and 12 h in control group; 0, 2, 4, 6, 10, and 12 h in lymph-cannulated groups. The lymph was collected in one hour intervals up to 12 h. The blood sampling times for BSA were 0, 1, 2, 3, 4, 6, and 8 h. The lymph was collected in one hour intervals up to 8 h. The fluid volume of each blood and lymph sample was replaced with normal saline containing heparin (50 IU/mL) through the jugular vein catheter.

Plasma was separated by centrifugation for 5 min at 1000*g*. Lymph and plasma samples were stored at −20 °C until analysis.

2.4. Analytical procedures

Quantification of bovine insulin and lispro insulin in rat plasma and lymph samples was performed using a commercially available Bovine Insulin ELISA kit and Iso-Insulin ELISA kit (Mercodia AB, Sweden), respectively. EPO samples were analyzed by erythropoietin ELISA kits (IBL, Germany). The linear ranges of ELISA kits are 6–155 mIU/L, 3–100 mIU/L, and 2.8–88 mIU/mL for Bovine Insulin, Iso-Insulin, and EPO ELISA kits, respectively. More concentrated samples were diluted according to instructions supplied by the kits' manufacturers.

The quantification of bovine serum albumin – fluorescein conjugate samples was performed by SPECTRAFluor Plus ELISA reader (Tecan®) using excitation and emission wavelengths of 485 and 535 nm, respectively. The calibration curves were prepared by spiking known concentrations of BSA solutions into rat plasma and lymph.

2.5. Data analysis

Area under plasma concentration-time curve (AUC) was calculated for all molecules of lymph-cannulated and control groups. For BSA and EPO, AUC was calculated up to 8 and 12 h, respectively. In addition, in the case of insulin, C_{max} and terminal half-life were calculated. The two-tailed *t*-test and ANOVA were used for comparison of two or more data sets, respectively. A p value of less than 0.05 was termed significant. All data are presented as means ± SEM, if not stated otherwise.

3. Results

The concentration vs. time profile of bovine insulin following SC administration is presented in Fig. 1. Insulin concentrations returned to a baseline level approximately 150 min following the administration. There was no significant difference between plasma AUCs of lymph-cannulated ($n = 4$) and control groups ($n = 4$), see Table 1. The insulin concentrations achieved in thoracic lymph and plasma of the lymph-cannulated groups were of the same order of magnitude. In addition, it should be noted that maximum concentration in lymph was reached later than in plasma. The cumulative amount of insulin collected in

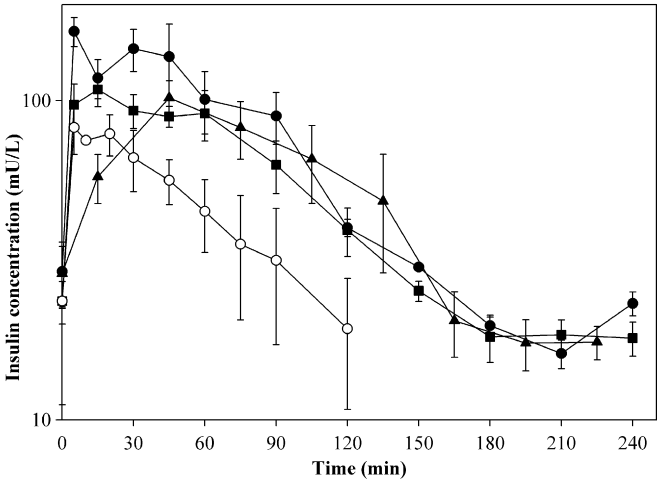

Fig. 1. Insulin concentration–time semi-logarithmic plot (means ± SEM) following SC administration of bovine insulin 1 IU/kg to control (● – plasma, $n = 4$) and lymph-cannulated (■ – plasma, ▲ – lymph, $n = 4$) group, or lispro insulin 0.5 IU/kg to control (○ – plasma, $n = 3$) group of rats.

Table 1
Pharmacokinetic parameters of insulin (bovine and lispro) following SC administration to rats

	Group			
	Bovine insulin			Lispro insulin
Sample:	Control	Lymph-cannulated		Control
Parameter	Plasma	Plasma	Lymph	Plasma
Baseline concentration, mIU/L	29.2 ± 6.9	23.5 ± 3.6	28.8 ± 6.2	23.6 ± 12.4
Half-life, min^{-1}	48.3 ± 6.8	55.6 ± 5.5	47.4 ± 13.5	49.0 ± 1.3
AUC, mIU·min/L	14,785 ± 2015	11,385 ± 963		
C_{max}, mIU/L	192 ± 20	121 ± 7		

the thoracic lymph of the lymph-cannulated group during the experiment was very low (0.072 ± 0.016% of the given dose). The half-life of insulin following intravenous administration in rats was reported to be 12–14 min [13,14]. The prolonged half-life of insulin obtained following SC administration is a result of absorption rate limited elimination and, accordingly, the terminal slope represents absorption kinetics. Since there was no difference between terminal half-lives of bovine insulin and monomeric lispro insulin (Fig. 1 and Table 1), it can be concluded that both insulins were absorbed at the same rate. It should be noted that non-zero baseline insulin concentration can be explained by cross-reaction of the utilized ELISA kit with the endogenous rat insulin.

Fig. 2 presents the pharmacokinetic profiles of BSA in plasma and lymph in the control ($n = 5$) and lymph-cannulated ($n = 4$) groups. In the case of albumin, the experiment was conducted 32 h for the control group. From the obtained profile it was evident that C_{max} is achieved at around 8 h with a subsequent decline of concentrations. Thus, it was concluded that it would be sufficient to collect the lymph up to 8 h to estimate the contribution of the lymphatic system to bioavailability of BSA. Analogous to the insulin results, the concentrations of BSA in lymph were very similar to corresponding plasma values. The plasma AUCs (up to 8 h) were not significantly different: 12.7 ± 0.9 and 16.5 ± 6.7 mcg·h/mL for control and lymph-cannulated groups, respectively. The cumulative amount of BSA collected in lymph for 8 h was 2.15 ± 1.08% of the administered dose. It should be noted that a trend for a slightly higher concentration of the BSA in the plasma of the lymph-cannulated group in comparison to the control rats can be explained by a minor dehydration due to lymph collection.

Fig. 3 presents pharmacokinetic profiles that were obtained following SC administration of EPO for lymph-cannulated ($n = 4$) and control ($n = 4$) groups. As in the two other molecules, EPO concentrations in lymph were similar to plasma concentrations and only a minimal amount of EPO was recovered in lymph during 12 h (1.44 ± 0.26% of the administered dose). However, in contrast to other tested molecules, for EPO a significantly lower plasma AUC was obtained in the lymph-cannulated group than in the control group (2304 ± 199 vs. 3581 ± 198 mIU·h/mL, respectively). As evidenced by zero level baseline concentration, the erythropoietin ELISA has no cross-reaction with the endogenous rat erythropoietin.

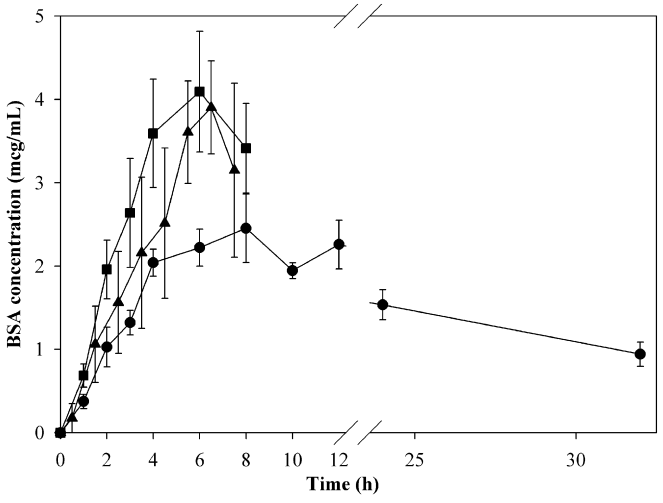

Fig. 2. BSA concentration–time plot (mean ± SEM) following SC administration of BSA 2.5 mg/kg to control (● – plasma, $n = 5$) and lymph-cannulated (■ – plasma, ▲ – lymph, $n = 4$) group of rats.

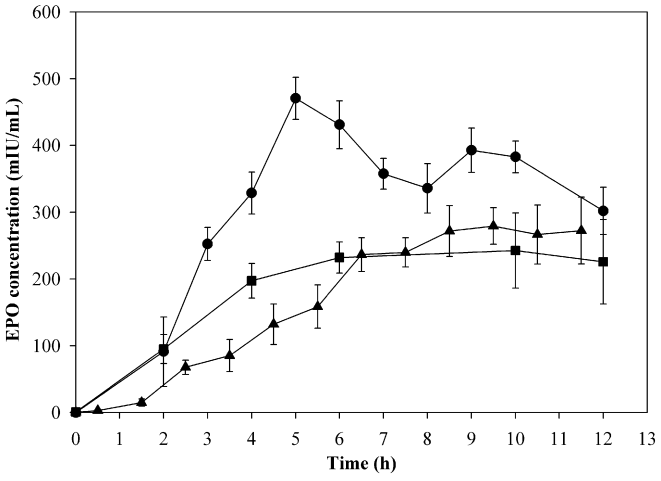

Fig. 3. EPO concentration–time plot (means ± SEM) following SC administration of EPO 400 IU/kg to control (● – plasma, $n = 4$) and lymph-cannulated (■ – plasma, ▲ – lymph, $n = 4$) group of rats.

4. Discussion

SC injection is a primary route of administration for many clinically used and experimental macromolecular drugs. Existing data on the role of the lymphatic system in SC absorption are very limited and based mostly on the sheep model [6]. The data from rodents are scarce due to the complexity of serial lymph sampling in freely moving animals.

There are two main technical difficulties in this kind of experiment: clogs in the lymphatic cannula indwelled in the thoracic duct and high mortality following surgery. To overcome these obstacles we have improved the previously reported methods for thoracic duct cannulation [11,12]. The main modification, that dramatically improved the yield of the surgical procedure, was the introduction of a *thoracic lymph duct-jugular vein shunt* to enable free flow of the lymph into the vein in a closed loop during the recovery time (up to 16 h) between the end of the surgery and the initiation of the pharmacokinetic study. In fact, no rat had survived the recovery period without the shunt, most probably, due to dehydration and protein loss. This shunt mimics the physiological state and, therefore, led to the significantly improved survival of rats and prevention of lymphatic catheter occlusion. Our rat model enabled continuous collection of the central lymph and concurrent serial blood sampling from unrestrained animals. The absence of restraint and/or anesthesia is important because muscle activity affects the natural pattern of lymph flow [2,7]. The advantage of the described rat model is that it enables direct measurement of the drug in the lymph fluid and does not solely rely on the drug concentration in the systemic blood circulation. It should be noted that despite the improved rat model the lymphatic absorption experiments in small laboratory animals continue to be a complex procedure and may contribute to the relatively high SEM values that were obtained in the study. Therefore, our data provide essential information for this research field.

The cascade of kinetic processes that occurs following SC administration is illustrated in Fig. 4. All the lymph from the injection site is drained to the thoracic duct cannula. Therefore, in the case of significant lymphatic absorption of the tested macromolecule it is expected that: (1) a significant amount of the dose will be recovered in lymph; (2) the concentration of a substance in lymph will be significantly greater (several orders of magnitude) than in plasma; and, (3) collection of the lymphatic component will result in a difference in the plasma AUC between the lymph-cannulated and control group.

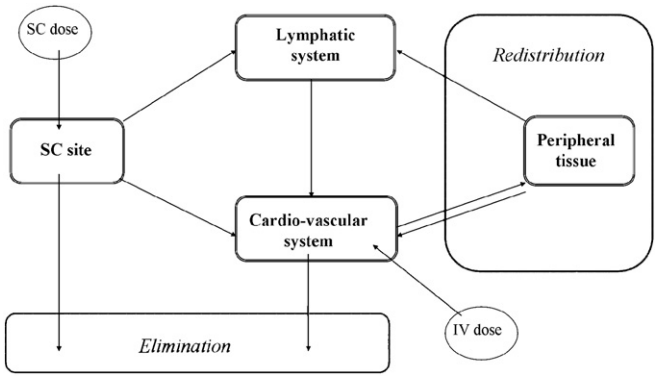

Fig. 4. Schematic presentation of the cascade of kinetic processes that occurs following SC administration of macromolecules.

The results showed a very low lymphatic recovery, less than 3% of the administered dose, and relatively low concentrations in lymph (in the same range as plasma concentrations) for all three tested molecules. Based on the pharmacokinetic profile of intravenous insulin in rats [13] the mean total bioavailability of the subcutaneously administered insulin in our experiments is about 81.5%, and the contribution of the lymphatic system to the bioavailability is 0.089%. Woo et al. [15] recently reported that the bioavailability of EPO following SC administration in rats is 59%. Consequently, the contribution of the lymphatic system to the bioavailability of EPO following SC administration as found in our work is 2.44%. Thus, it can be concluded that in the rat model SC absorption occurred mainly through direct uptake into the blood capillaries without any substantial involvement of the lymphatic pathway. An intriguing finding was that molecular weight had only limited effect on the degree of the lymphatic uptake (despite the wide molecular weight range of the tested molecules, 5.6–66 kDa).

The data concerning SC lymphatic absorption of macromolecules in small laboratory animals are scant and contradictory. In one limited report an increase of molecular weight (five undisclosed proteins, 20–78 kDa) was associated with a trend toward increased recovery in thoracic duct lymph [16]. However, specific information regarding the injection site and lymphatic contribution to the bioavailability was not provided. In another study results corroborating our finding were reported. In that work, recombinant human tumor necrosis factor (TNF, ~45 kDa) showed similar lymphatic and plasma concentrations, as well as very low recovery in thoracic lymph following SC administration to the back of the rats (0.03% of the administered dose) [17].

Our results appear to contradict the widely accepted perception derived from investigations in the sheep model. In this model, 48% of the systemic bioavailability of insulin following SC administration was contributed by the lymphatic system [18]. Likewise, erythropoietin demonstrated lymphatic uptake of 83.9% of the injected dose [19]. Similar results were reported also for other macromolecules in the sheep model [1,20,21].

The discrepancy between results, in our opinion, can be attributed mainly to two differing factors in the models: (1) utilization of different anatomical injection sites, and (2) amount of molecules administered at the injection site. It is known that the injection site has a major influence on the kinetics of subcutaneous absorption of proteins

[22,23]. In the sheep model, the dose was injected into the interdigital space of the hind limb. This very specific injection site was chosen probably as the most distal part of the limb to allow the collection of both peripheral and central lymph. On the other hand, in the rat model, we injected at lateral side of the thigh, which is, in our view, the most common SC injection site in rodents. It is known that the pressure gradient between the interstitium and the initial lymphatics is a very important factor in lymph formation [7]. The increased lymphatic flow causes higher lymphatic uptake of macromolecules that are distributed within the interstitial fluid. The main difference between the two sites of administration is that injection into the interdigital space may be associated with an elevation in hydrostatic pressure, which can enforce the significant lymphatic drainage. In contrast, SC injection at the thigh region (as in this work) does not produce a similar consequence due to the presence of anatomical space, which enables distribution of the injected volume. The influence of the SC injection site on the degree of lymphatic uptake following SC administration in rats was demonstrated by Oussoren et al. [24] in their research of biodistribution of liposomes. They found that 52 h following SC administration of large (0.1 μm) radio-labeled liposomes, $93 \pm 1\%$ of the injected dose was still present at the injection site when injected into the flank compared to $60 \pm 6\%$ and $54 \pm 1\%$ when injected into the dorsal side of the foot and the footpad, respectively. Thus, the anatomical site of the SC injection may have a great influence on the outcome of the lymphatic absorption experiment and this issue should be further investigated.

The other important issue regarding SC administration is dose normalization per kg body weight. In this mode the tissue surrounding the injection site "sees" 100-fold more active molecules in sheep in comparison to the rat. This phenomenon may have an influence on the mechanism and kinetics of absorption from the SC site to the systemic circulation. The insulin case is an example that demonstrates this phenomenon. It is known that insulin may associate to form aggregates (e.g., hexamers) at high concentrations and exist as monomers when diluted. The most prevalent forms of insulin existing in the 100 IU/mL (used in sheep) and 1 IU/mL (used in rat) solution are hexamer and monomer, respectively [25]. We assume that in the sheep, insulin was absorbed mostly in the aggregated form via the lymphatic pathway whereas, in the rat, insulin was absorbed as monomers via direct uptake into the blood capillaries. To clarify this assumption we compared the absorption rate of the bovine insulin with lispro insulin (which has a much lower tendency to aggregate). The similarity in the absorption kinetics confirms that bovine insulin was absorbed into the blood by the same pathway(s) as monomeric lispro insulin.

A relatively small elevation in the lymphatic concentration that occurred for all molecules in the rat model can be explained by the possible redistribution of the absorbed molecule to the lymphatics (Fig. 4). Following absorption (through either the blood or the lymphatic route) a substance reaches the central compartment (blood), after which it can be redistributed through other tissues exiting from the blood capillaries into the interstitial fluid, and entering the lymphatic capillaries. The thoracic duct cannula collects the lymph from most parts of the body. The major parameter that affects this process is the permeability of vascular capillaries. Many reports have shown that blood capillaries are permeable for a variety of macromolecules (insulin, albumin, transferrine, etc.) [8,26]. For example, we found that 1.5 h following IV administration of EPO (400 IU/kg) the plasma and lymph concentrations were 6.6 and 5.2 IU/mL, respectively. The concentration found in the lymph is much higher than that detected following SC administration. This fact emphasizes that EPO is capable of redistributing from systemic circulation to lymph. Our data are corroborated by Bocci et al. who demonstrated redistribution of INF-b from blood to lymph following IV injection in rabbits [27].

In summary, the freely moving rat model was used in this study for assessment of SC lymphatic absorption. The absorption of three model macromolecules following SC administration occurred mainly through the blood capillaries with minimal contribution of the lymphatic route to the systemic bioavailability. These findings are of considerable importance in view of the fact that rodents are commonly used in the preclinical evaluation of newly developed macromolecular drugs.

Acknowledgments

This study was partially supported by Israeli Consortium of Pharmalogica. Prof. Amnon Hoffman is affiliated with the David R. Bloom Center for Pharmacy at The Hebrew University of Jerusalem. We thank the members of the Israeli Consortium of Pharmalogica and Dr. Josh Backon for their constructive comments.

References

[1] D.N. McLennan, C.J.H. Porter, S.A. Charman, Subcutaneous drug delivery and the role of the lymphatics, Drug Discov. Today: Technol. 2 (1) (2005) 89–96.
[2] C.J. Porter, S.A. Charman, Lymphatic transport of proteins after subcutaneous administration, J. Pharm. Sci. 89 (3) (2000) 297–310.
[3] A.E. Hawley, S.S. Davis, L. Illum, Targeting of colloids to lymph nodes: influence of lymphatic physiology and colloidal characteristics, Adv. Drug Deliv. Rev. 17 (1) (1995) 129–148.
[4] C. Oussoren, G. Storm, Liposomes to target the lymphatics by subcutaneous administration, Adv. Drug Deliv. Rev. 50 (1–2) (2001) 143–156.
[5] P. Gershkovich, L. Kagan, A. Hoffman, in: A.J. Domb, Y. Tabata, M.N.V. Ravi Kumar, S. Farber (Eds.), Nanoparticles for Pharmaceutical Applications, American Scientific Publisher, 2006.
[6] A. Supersaxo, W.R. Hein, H. Steffen, Effect of molecular weight on the lymphatic absorption of water-soluble compounds following subcutaneous administration, Pharm. Res. 7 (2) (1990) 167–169.
[7] M.A. Swartz, The physiology of the lymphatic system, Adv. Drug Deliv. Rev. 50 (1–2) (2001) 3–20.
[8] M. Bendayan, Morphological and cytochemical aspects of capillary permeability, Microsc. Res. Tech. 57 (5) (2002) 327–349.

[9] C.J. Porter, G.A. Edwards, S.A. Charman, Lymphatic transport of proteins after s.c. injection: implications of animal model selection, Adv. Drug Deliv. Rev. 50 (1–2) (2001) 157–171.
[10] P. Gershkovich, A. Hoffman, Uptake of lipophilic drugs by plasma derived isolated chylomicrons: linear correlation with intestinal lymphatic bioavailability, Eur. J. Pharm. Sci. 26 (5) (2005) 394–404.
[11] J.L. Bollman, J.C. Cain, J.H. Grindlay, Techniques for the collection of lymph from the liver, small intestine, or thoracic duct of the rat, J. Lab. Clin. Med. 33 (1948) 1349–1352.
[12] H.E. Gallo-Torres, O.M. Miller, A modified Bollman's technique for cannulation of the rat's thoracic duct: lymph flow standardization, Proc. Soc. Exp. Biol. Med. 130 (1969) 552–555.
[13] R.J. Schilling, A.K. Mitra, Pharmacodynamics of insulin following intravenous and enteral administrations of porcine-zinc insulin to rats, Pharm. Res. 9 (8) (1992) 1003–1009.
[14] A. Plum, H. Agerso, L. Andersen, Pharmacokinetics of the rapid-acting insulin analog, insulin aspart, in rats, dogs, and pigs, and pharmacodynamics of insulin aspart in pigs, Drug Metab. Dispos. 28 (2) (2000) 155–160.
[15] S. Woo, W. Krzyzanski, W.J. Jusko, Pharmacokinetic and pharmacodynamic modeling of recombinant human erythropoietin after intravenous and subcutaneous administration in rats, J. Pharmacol. Exp. Ther. 319 (3) (2006) 1297–1306.
[16] D.D. Xie, V. Hale, Factors affecting the lymphatic absorption of macromolecules following extravascular administration, Pharm. Res. 13 (1996) S396.
[17] K. Kojima, T. Takahashi, Y. Nakanishi, Lymphatic transport of recombinant human tumor necrosis factor in rats, J. Pharmacobiodyn. 11 (10) (1988) 700–706.
[18] S.A. Charman, D.N. McLennan, G.A. Edwards, C.J. Porter, Lymphatic absorption is a significant contributor to the subcutaneous bioavailability of insulin in a sheep model, Pharm. Res. 18 (11) (2001) 1620–1626.
[19] D.N. McLennan, C.J. Porter, G.A. Edwards, S.W. Martin, A.C. Heatherington, S.A. Charman, Lymphatic absorption is the primary contributor to the systemic availability of epoetin Alfa following subcutaneous administration to sheep, J. Pharmacol. Exp. Ther. 313 (1) (2005) 345–351.
[20] D.N. McLennan, C.J. Porter, G.A. Edwards, M. Brumm, S.W. Martin, S.A. Charman, Pharmacokinetic model to describe the lymphatic absorption of r-metHu-leptin after subcutaneous injection to sheep, Pharm. Res. 20 (8) (2003) 1156–1162.
[21] S.A. Charman, A.M. Segrave, G.A. Edwards, C.J. Porter, Systemic availability and lymphatic transport of human growth hormone administered by subcutaneous injection, J. Pharm. Sci. 89 (2) (2000) 168–177.
[22] S.A. Beshyah, V. Anyaoku, R. Niththyananthan, P. Sharp, D.G. Johnston, The effect of subcutaneous injection site on absorption of human growth hormone: abdomen versus thigh, Clin. Endocrinol. (Oxf) 35 (5) (1991) 409–412.
[23] E. Ter Braak, J. Woodworth, R. Bianchi, B. Cerimele, D. Erkelens, J. Thijssen, D. Kurtz, Injection site effects on the pharmacokinetics and glucodynamics of insulin lispro and regular insulin, Diab. Care 19 (12) (1996) 1437–1440.
[24] C. Oussoren, J. Zuidema, D.J.A. Crommelin, G. Storm, Lymphatic uptake and biodistribution of liposomes after subcutaneous injection I. Influence of the anatomical site of injection, J. Liposome Res. 7 (1) (1997) 85–99.
[25] J. Brange, A. Volund, Insulin analogs with improved pharmacokinetic profiles, Adv. Drug Deliv. Rev. 35 (2–3) (1999) 307–335.
[26] M. Simionescu, A. Gafencu, F. Antohe, Transcytosis of plasma macromolecules in endothelial cells: a cell biological survey, Microsc. Res. Tech. 57 (5) (2002) 269–288.
[27] V. Bocci, G.P. Pessina, L. Paulesu, M. Muscettola, A. Valeri, The lymphatic route. V. Distribution of human natural interferon-beta in rabbit plasma and lymph, J. Interferon. Res. 8 (5) (1988) 633–640.