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Heparin penetration into and permeation through human skin from aqueous and liposomal formulations in vitro

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Abstract

The transport of unfractionated (UH) and low molecular weight Heparin (LMWH) in human skin was investigated in vitro using heat separated epidermal membrane and dermis and the effect of liposomal formulations with Phospholipon® 80 (PL80) and Sphingomyelin (SM) was assessed. The distribution of Heparin within skin tissue was studied by the tape stripping method. Heparin concentrations were measured with a biological assay. Transepidermal water loss was determined to characterize barrier properties of skin. No consistent permeation of Heparin through epidermal membrane was detected. Penetration into the epidermal membrane was for LMWH significantly greater than for UH. Accumulation of UH was largely restricted to the outermost layers of the stratum corneum while LMWH penetrated into deeper epidermal layers. UH penetration into epidermis was detected for the PL80 liposomal formulation only. The extent of LMWH penetration was independent of the formulation, LMWH, however, showed a trend to accumulate in deeper epidermal layers for the PL80 compared to the aqueous formulation. Thus, molecular weight and liposomal formulations influenced the penetration pattern of Heparin in the epidermis. It can not be concluded whether the concentration of LMWH achieved at the blood capillaries is sufficient to exert a pharmacological effect. UH permeated readily through dermis irrespectively of formulation and its accumulation in the dermis was significantly enhanced and its lag time of permeation increased in the presence of SM liposomes.

Keywords: Heparin sodium salt; Liposome; In vitro study; Percutaneous penetration; Tape-stripped skin

1. Introduction

Unfractionated Heparin (UH), a sulfated glycosaminoglycan with a molecular weight between 6000 and 30 000, is pharmacologically an anticoagulant. In topical therapy, UH is used for the treatment of superficial thrombotic and increased vascular permeability symptoms. Low molecular weight Heparins (LMWH) have approximately one third the molecular weight of UH and are currently undergoing clinical trials for similar indications (Xiong et al., 1996). In systemic therapy,

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LMWH are typically administered subcutaneously and have the advantage of a longer therapeutic half life than the intravenously administered UH. Permeation of topically applied Heparin through the stratum corneum (SC) barrier of the skin is required in order to achieve a therapeutic effect.

Conflicting results have been reported about the skin tissue levels of Heparin after exposure to UH formulations in vivo. Investigations using radiolabelled UH (^{35}S) as a tracer, have shown that UH permeates across human, pig, and rat skin in vivo, although to a low extent (Schraven and Trottnow, 1973; Schaefer and Zesch, 1976; Zesch and Schaefer, 1976; Artmann et al., 1990). UH penetration into the skin was reported to be markedly dependent on the vehicle (Schaefer and Zesch, 1976), and a gel formulation was pointed out to be particularly suitable (Zesch and Schaefer, 1976). Schaefer and coworkers reported about no specific accumulation of UH in the skin tissue layer, while Artmann and coworkers found increased levels in the subcutaneous fat tissue. Zimmermann, 1982 showed a significant dependency of UH penetration rate on the concentration in a cream by establishing the bleeding time and antifactor Xa activity at the site of application of the cream whereas de Moerloose et al., 1992 did not observe any dose ranging effect. Rodemer et al., 1986 found a weak systemic LMWH anti FXa activity in four out of ten healthy volunteers following topical administration. Early reports have also assessed the penetration of UH into human skin in vitro using radiolabelled Heparin (^{35}S and ^{3}H) (Schaefer and Zesch, 1976; Stüttgen et al., 1990; Bonina and Montenegro, 1992, 1994a). Bonina and coworkers showed that soy bean lecithin was able to enhance UH skin permeation using the pretreatment technique. Xiong et al., 1996 claimed that the skin's LMWH accumulation capacity is independent of the type of enhancer utilized and that the SC is not the only major barrier for the permeation of LMWH.

In the in vitro permeation and penetration experiments performed so far, radiolabelled Heparin was employed and mass transport was determined based on a radioactivity assay. With this analytical method the label rather than the pharmacologically active entity is determined. Thus, radiochemical and biochemical processes which may occur within the skin and alter the labelling and the anticoagulative properties of Heparin are not taken into account. These processes may have a marked influence on the experimental findings when the transport rate is particularly small, as it is for Heparin. Remarkably, the presently available literature taken together does not provide a firm conclusion about the permeation behaviour of Heparin across human skin. Therefore, the objective of the present study was to clarify this question using a biological assay based on anti FXa activity. In this way, it can be confirmed that every detected Heparin molecule contains the antithrombin III binding site and therefore retains its anticoagulative properties. The smallest detectable molecular sequence with this assay is the pentasaccharide unit, that is responsible for antithrombin III binding. This approach has the essential advantage that measured penetration into skin layers and permeation across these layers unequivocally reflects the pharmacologically intact drug molecule. UH and LMWH were used in order to investigate the influence of molecular size on permeation and penetration and the effect of liposomal formulations on the skin transport of Heparin was studied.

2. Materials and methods

2.1. Materials

Unfractionated Heparin as Sodium salt with a molecular weight between 6000 and 30 000 was obtained from commercial sources. Its declared anti FXa activity was 172.9 IU per mg as determined by the USP24 assay against the WHO 4th International Standard. Activity determinations in this laboratory using the same method deviated at the most 8% from the declared value. Low molecular weight Heparin as Sodium salt with an average molecular weight of 9000 was a kind gift from Kraeber GmbH&Co, Ellerbek, Germany. The anti FXa activity of LMWH was determined in this laboratory using the WHO Fourth International Standard to be 120.1 IU per mg. Phospho-

lipon 80® was a kind gift from Nattermann Phospholipid GmbH, Cologne, Germany. This is a soy bean lipid extract with 76% (w/w) phosphatidylcholine. Sphingomyelin was a kind gift from Lipoid AG, Ludwigshafen, Germany. It is obtained from egg yolk and has a purity of $>98\%$. The reagents for the determination of Heparin anti FXa activity were purchased from Pentapharm LTD, Basel, Switzerland (Pefachrome® FXa) and from Endotell AG, Allschwil, Switzerland (Factor Xa and Antithrombin III). The activity of FXa was 71 nkat and of human AT III 10 IU. All other chemicals and reagents purchased from commercial sources were of analytical grade.

2.2. Skin

Excised human cadaver skin from the abdominal region of female donors (mean age of the donors $\pm$ SD 78 ± 8, $n = 6$) was obtained from Pathologisches Institut, University of Basel, Switzerland. Biopsies were taken within 24 h post mortem and stored at -70 °C. Before use, the skin was thawed for 1 h at room temperature and the subcutaneous fat was removed with a scalpel. The epidermal membrane was separated from the dermis by first immersing the skin for 60 s in water at 60 °C (Kligmann and Christophers, 1963) and then peeling off the epidermal membrane, in the beginning by forceps and then with the finger tips. The thickness of the epidermal membrane and of the dermis was measured between two cover slides with a micrometer (Microselect, Etalon Pierre Roch GmbH, Rolle, Switzerland). Both, the epidermal membrane and the dermis were used in permeation experiments. They were mounted horizontally in diffusion cells (see below) with their outer surface facing the donor compartment.

Skin samples were characterized by transepidermal water loss (TEWL) measurements (Tewameter TM 210, Courage Khazaka electronic GmbH, Germany) which were carried out in the diffusion cells with the receiver compartment filled with Sörensen buffer pH 6.4 (Wissenschaftliche Tabellen Geigy, 1979). To evaluate the method, TEWL was measured with selected skin specimens as a function of time for up to 26 h. Routine TEWL measurements were carried out after a 4-h equilibration of the skin in the diffusion cell prior to the permeation experiments. A TEWL value was also obtained in a diffusion cell that contained the same buffer in the receiver compartment but no skin membrane. From the TEWL measurements with and without skin the reduction factor (RF) was calculated as follows:

$$\mathrm{RF} = \frac{\mathrm{TEWL}_{\text{no skin}}}{\mathrm{TEWL}_{\text{Skin}}} \tag{1}$$

2.3. Preparation and characterization of liposomes

Liposome formulations were prepared by a modified ethanol injection method described by Batzri and Korn, 1973. This is a well established method that was shown to produce phospholipid vesicles using electron microscopy (Kremer et al., 1977). The water phase containing Heparin was poured rapidly into the ethanol phase containing the lipid whereas liposomes were formed spontaneously. The liposome formulations contained 25% (v/v) ethanol 96%. As lipids, Phospholipon® 80 (PL® 80), Sphingomyelin (SM), and a mixture of both at a mass ratio 2:1 were used. The liposome formulations were homogenized by Polytron (Kinematica AG, Switzerland) at 13 000 rpm for 12 min. The composition and properties of the prepared formulations are shown in Table 1. The z-average mean and polydispersity of the liposomes were determined routinely by photon correlation spectroscopy using a Malvern Autosizer 2c (Malvern Instruments Ltd., Malvern, UK with Autocorrelator 8, Type 7032 CN). Size determination was performed at 25°C and the viscosity value of pure water was used in the calculations. The particle charge was assessed with ζ potential measurements, which were based on the electrophoretic mobility determined by Laser Doppler anemometry using a Zetasizer 3 (Malvern Instruments, Malvern, UK). All measurements were carried out in triplicate. The phase transition temperature was determined by differential scanning calorimetry using a Differential Scanning Calorimeter (Pyris 1, Perkin Elmer, US Instruments Division, Norwalk). Drug entrapment in

the liposomes was determined by separating the liposomes from the dispersion medium either by centrifugation or by cross flow ultrafiltration and measuring Heparin concentration in the dispersion medium.

2.4. In vitro permeation and penetration experiment

Modified Franz-type diffusion cells with a surface area of transport of 1.7–1.8 cm^2 were used. The donor compartment contained 2.0 ml Heparin formulation (liposomal or aqueous) under occlusive conditions and the receiver compartment contained 8.5–9.0 ml Sörensen buffer pH 6.4. When a liposomal formulation was used in the permeation experiment, 25% (v/v) ethanol 96% was added to the Sörensen buffer. In a control experiment, a liposomal formulation with no ethanol addition in the receiver solution was used and the transport of ethanol from donor to receiver was monitored by gas chromatography. The diffusion cells were immersed in a water bath maintained at 37 °C, which resulted in a temperature on the skin surface of 33 °C. The receiver compartment was stirred by magnetic bar at 400 rpm.

In the beginning of each permeation experiment, a 100 μl sample was drawn from each, the receiver and the donor compartment in order to determine initial Heparin concentrations. The volume of these samples was not replaced. Permeation experiments were carried out for 140–170 h. Samples of 250 μl were drawn at first after 48 h and then every 24 h from the receiver solution and replaced with fresh Sörensen buffer which included 25% (v/v) ethanol where appropriate. The applied duration of the permeation experiments was as long as possible since a rather low skin permeability of Heparin was anticipated. Heat separated human epidermal membrane was shown to retain its permeation barrier properties for up to 150 h based on electrical resistance and mannitol permeability measurements (Peck et al., 1993). This result was confirmed in this laboratory (unpublished results). The likelihood of obtaining false positive permeation results due to the prolonged permeation experiment causing skin alteration could be easily eliminated, if necessary, at a reduced duration of the experiment. In a control experiment, 500 μl samples were drawn from the donor solution at 24, 48, 120 and 190 h and were used to characterize the liposome formulation.

At the end of the experiment, the contents of the donor compartment were quantitatively collected by rinsing this compartment four times with $\sim$3 ml Sörensen buffer pH 6.4 and combining the washings. These were diluted to an exact volume and used for determining the final Heparin concentration in the donor solution. Subsequently, the skin was taken out of the diffusion cell and pulverized in liquid nitrogen using a freezer mill 6750-230 (Spex Certiprep, Metuchen, NJ). Heparin was extracted from the pulverized skin with a total of 25 ml of Sörensen buffer pH 6.4. The recovery of Heparin from skin was tested by placing 100 μl of a Heparin solution with a known concentration on a piece of epidermal membrane, letting it dry and following the same milling and extraction procedure. As a control, a piece of paper filter was used instead of skin. The Heparin concentration was determined in all sam-

Table 1
Composition and properties of liposomal formulations (LF) of Heparin

	LF1	LF2	LF3	LF4
Phospholipid	PL 80	PL 80/SM	SM	PL 80
Final lipid concentration [mg/ml]	30	20/10	30	30
Heparin type	UH	UH	UH	LMWH
Heparin concentration [IU/ml]	2060	1400	1714	1744
pH	6.3	6.4	6.4	6.3
Osmolarity [mOsM]	3990.0	3893.0	3703.0	3580
±SD	±139.0	±5.8	±5.8	±42.4

ples based on anti FXa activity by a chromogenic substrate assay.

2.5. Tape stripping technique

With a separate number of epidermis specimens tape stripping after the permeation experiment was performed (Marttin et al., 1996). The skin was removed from the diffusion cell and fixed by pins on a soft surface. Tapes of Scotch 600, Crystal Clear Tape (3M France) were cut to a length of ~ 5 cm with a razor blade and ~ 1 cm was folded over for easy handling and labelling. The weight of the tapes was determined with an analytical balance (Mettler AT 261 Delta Range, Mettler Instruments AG, Switzerland). The tape with a surface area of approximately 4 cm^2 was applied to the stratum corneum surface of the epidermis specimen entirely covering the transport area and rubbed firmly with the finger with three horizontal movements. Immediately thereafter the tape was carefully peeled away with a steady, continuous pull. Each skin sample was stripped with 2–5 tapes until it was torn. After stripping, the tapes were equilibrated for at least 30 min at a constant relative air humidity and temperature (50%, 25 °C) and weighed again. The weight of the stratum corneum removed by stripping was calculated as the weight difference between the blank tape and the same tape after stripping. The stratum corneum weight was normalized by the surface area of transport. Heparin was extracted from each tape with 500 μl Sörensen buffer pH 6.4 according to Pershing et al., 1994, and assayed based on anti FXa activity. In contrast to Pershing et al., 1994 and Surber et al., 1999 the first tape was not discarded. The content of Heparin in the remainder of the epidermal membrane was determined after pulverizing the membrane in liquid nitrogen as described above.

2.6. Determination of Heparin

2.6.1. Principle

The principle of the employed biological assay of Heparin is depicted in Fig. 1 (Teien et al., 1976; Teien and Lie, 1977). After forming a complex of Heparin with antithrombin III (AT III), the mixture is incubated with an excess of coagulation factor Xa (FXa). An amount of FXa is neutralized by the [Heparin-AT III] complex in proportion to the available amount of Heparin. The remaining amount of FXa catalyzes the splitting off of paranitroaniline (pNA) from the chromogenic peptide substrate (Pefachrome® FXa). After stopping the reaction with acetic acid, the intensity of colour is measured photometrically at 405 nm.

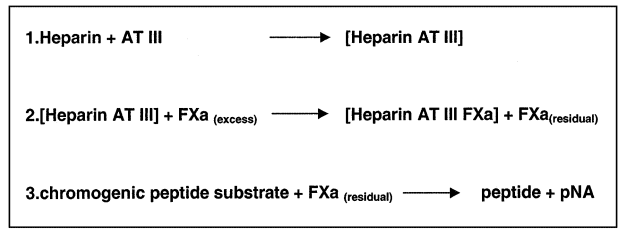

Fig. 1. Principle of chromogenic substrate assay.

2.6.2. Procedure

One hundred μl of a 1 IU/ml AT III solution were added to 100 μl of Heparin sample and the mixture was filled up to 1 ml with Sörensen buffer pH 6.4. Two hundred μl of this solution were first incubated for 3 min at 37 °C, and then mixed with 200 μl of a 7.1 nkat/ml solution of FXa and incubated for 30 s at 37 °C. Two hundred μl of a 1 mM solution of chromogenic peptide substrate were incubated with the above solution for 3 min, 300 μl of a 50% acetic acid solution were added, mixed with a vortex and the absorbance of the solution was measured with a Perkin Elmer Spectrometer Lambda 20 (Perkin Elmer, Überlingen, Germany). The concentration of Heparin was calculated using standards with known Heparin concentrations in the range between 0 and 2.0 IU/ml. The resulting standard curve was described by the following model:

$$y = a + e^{-b \cdot x + c}, \quad (2)$$

where, y is absorbance, x is Heparin concentration and a, b, and c are constants.

In the concentration range of 0–0.7 IU/ml the standard curve reduced to a straight line. A solution with no Heparin was always included in order to determine the maximum absorbance

value. The lowest detectable concentration of the standard solution was 0.069 IU/ml. Since UV absorbance decreased exponentially with increasing concentration, the precision of the method diminished at higher concentrations. The coefficient of variation of the assay was 9.4% at a Heparin concentration of 1.4 IU/ml.

2.6.3. Data analysis of the permeation experiments

In vitro steady-state drug fluxes, J_{ss} (IU/h/cm^2) were calculated using the linear portion of the cumulative permeating amount versus time curve, employing least square linear regression. The permeability coefficients, P (cm/s) were calculated from the drug fluxes and the average between the initial (C_{D0}) and the final donor concentration (C_{DE}) according to Eq. (3).

$$P = \frac{J_{SS}}{\frac{(C_{D0} + C_{DE})}{2}} = \frac{dm}{dt} \cdot \frac{1}{A} \cdot \frac{1}{\frac{(C_{D0} + C_{DE})}{2}}, \quad (3)$$

where, dm/dt is the slope of cumulative amount versus time line, and A is the diffusion area.

The diffusion coefficient (D) of the permeant in skin membrane can be estimated from the lag time (t_{lag}) (Flynn et al., 1974)

$$D = \frac{h^2}{6t_{lag}}, \quad (4)$$

where h is the thickness of the membrane.

3. Results

3.1. Characterization of liposomal Heparin formulations

The particle size, the polydispersity of size distribution and the ζ potential of the liposomes are shown in Table 2. Dilution of the sample with distilled water prior to photon correlation spectroscopy yielded for all formulations two to threefold smaller particles than dilution with the solvent that was used in the formulation and contained ethanol and 2000 IU/ml UH. The formulation with pure SM liposomes (LF3) showed larger particle size and higher polydispersity than the other compositions. Generally, the size of the liposomes increased with increasing phospholipid concentration. At ethanol concentrations $< 25\%$, the polydispersity of size increased (results not shown). The composition of the formulation used in the present study was optimized with respect to homogeneity and stability of the liposomes. The phase transition temperature of the PL® 80 containing liposomes was -30 °C and that of the pure SM liposomes was 37 °C. Heparin entrapped in the liposomes accounted for $< 10\%$ of the total concentration.

3.2. Thickness and TEWL value of skin layers

The thickness of the epidermal membrane was approximately 50 μm (Table 3). Histological studies (Woodley et al., 1983; Müller, 2000) have shown that using the heat separation method, the entire epidermis is removed and the lamina lucida and lamina densa remain on the dermis. The measured thickness of the epidermal membrane was smaller than reported values for the stratum corneum of 10–20 μm and the viable epidermis of 50–100 μm (Schaefer and Redelmeier, 1996). This difference is probably due to the method of measurement. The thickness of the dermis was consis-

Table 2
Characterization of liposomes

	Formulation			
	LF1	LF2	LF3	LF4
Dilution with double distilled water				
z-average mean [nm]	315.9	251.8	422.5	309.0
±SD	±16.1	±4.6	±39.9	±14.0
Polydispersity index	0.21	0.21	0.44	0.19
Dilution with 25% (v/v) ethanol in water containing 2000 IU/ml UH				
z-average mean [nm]	440.9	572.8	743.2	553.0[a]
±SD	±12.0	125.3	±104.0	±16.6
Polydispersity index	0.21	0.19	0.50	0.31[a]
ζ potential [mV]	−11.03	−7.88	−8.17	[b]

[a] Diluted with 25% (v/v) ethanol in water.
[b] Not determined.

Table 3
Thickness and TEWL of epidermal membrane and dermis

	Thickness [μm]	TEWL	
		RF	% Occurrence
Epidermal membrane	32–75	1–4	34
		4–20	57.5
		20–60	8.5
Dermis	1186–1263	1.1–2.7	100

tent with values from the literature of 1–2 mm (Schaefer and Redelmeier, 1996).

TEWL for the epidermal membrane reached its lowest value 4 h after the specimen was mounted in the diffusion cell and remained constant for at least 26 h. TEWL reflects the ability of skin to restrict the diffusion of water, and the measured values were roughly in the range of in vivo measurements given in the literature (Nangia et al., 1998; Pinnagoda et al., 1990), they showed, however, considerable variation. The values reported in Table 3 correspond to 40–50 skin specimens which were divided into three groups based on TEWL RF for clarity and to facilitate further discussion. The dermis represented no barrier to water diffusion.

3.3. Skin permeation from liposomal formulations with different lipid compositions

3.3.1. Permeation of ethanol from donor to receiver compartment

Ethanol which is included in the formulation, is shown to permeate into the receiver compartment when this contains a purely aqueous buffer, no equal concentration in both compartments, however, was reached within 89 h (Table 4). Therefore, ethanol had to be added to the receiver solution at the same concentration as in the drug formulation in order to attain the same osmotic conditions in both solutions. Approximately 45% of ethanol is not accounted for in the final mass balance indicating that notable evaporation took place during the experiment.

3.3.2. Permeation of Heparin across epidermal membrane

No Heparin permeation over the entire duration of the experiment could be detected with all formulations (LF1–LF4) for $n = 31$ epidermal membrane specimens which produced a RF of TEWL of at least four. This TEWL behaviour was exhibited by $\sim 70\%$ of all specimens, which were accordingly considered to possess adequate transport barrier properties. UH permeation was detected for LF 1 and the aqueous formulation with epidermal membrane specimens which produced a RF of TEWL between two and four. The calculated permeability coefficients for these specimens were in the range of $1.5E-7$ to $1.5E-11$ cm/s. Thus, the UH permeation across epidermal membrane showed a clear dependency on barrier integrity of the membrane as determined by TEWL. In the experiments with LMWH, a permeability of the order of $5E-8$ cm/s was measured only in three out of fourteen skin specimens with TEWL RF < 5. For all other specimens no steady permeation was detected. For the aqueous formulation only, a LMWH concentration of 71.5 IU/ml in average was detected after 48 h in the receiver solution. No increase of this concentration was observed, however, in the following 100 h of the experiment denoting that no permeation was taking place. In general, LMWH permeation

Table 4
Permeation of ethanol from the donor to the receiver compartment

Sampling time (h)	Ethanol in donor [mg]	Ethanol in receiver [mg]	Mass balance total [%]	
0	439.0	<0.94	439.0	100
40	273.6	33.5	307.1	70.0
65	229.5	80.0	309.5	70.5
89	139.6	105.6	245.2	55.9

Table 5
Permeation parameters of UH across dermis

	P [cm/s]	Lag time [h]	D [cm^2/s]
Aqueous UH formulation			
Mean	9.45E−8	10.9	6.80E−8
SD ($n=4$)	8.58E−8	3.6	1.90E−8
LF1			
Mean	7.07E−8	28.6	2.75E−8
SD ($n=6$)	5.59E−8	11.8	7.38E−8
LF2			
Mean	1.02E−7	52.1	1.25E−8
SD ($n=4$)	2.54E−8	2.5	6.10E−9
LF3			
Mean	1.34E−7	50.6	1.33E−8
SD ($n=4$)	1.59E−8	6.4	1.63E−9

across epidermal membrane was not found to correlate with TEWL. Heparin concentration in the donor solution remained constant within an $\sim 20\%$ error margin throughout the permeation experiment. The particle size of the liposomal formulations deviated at the most 6% from the original particle size throughout the experiment.

3.3.3. Permeation across dermis

UH permeation through dermis was measured consistently and permeability coefficients were independent of vehicle composition (Table 5). In the presence of SM the lag time increased and the diffusion coefficient decreased compared to the aqueous formulation. This was less evident for the PL® 80 formulation.

3.3.4. Heparin accumulation in skin

The Heparin concentration in skin at steady state was determined from the amount of Heparin extracted from the tissue and the volume of the tissue specimen calculated based on its surface area and thickness (Table 6). The recovery of the extraction method was at least 60% as compared to the control. The results of skin extraction reported herein reflect the measured values that have not been corrected for recovery. UH penetration into the epidermal membrane from the PL® 80 formulation (LF1) under occlusive conditions was notable, resulting in an accumulated amount in the tissue of 144.6 IU/cm^3. For the aqueous UH formulation and the other lipid formulations (LF2–LF3) accumulation was in general non-detectable. LMWH penetration into the epidermal membrane resulted in an accumulated amount in the tissue of 337.8 and 443.1 IU/cm^3 for the PL® 80 formulation (LF4) and the aqueous formulation, respectively. The average concentration of LMWH in the epidermal membrane did not differ significantly (Student's t-test, $P=0.4$) between the two formulations. The concentration of LMWH in the tissue was 2- to 3-fold greater than that of UH, this difference being statistically significant (Student's t-test, $P=0.0005$). Accumulation of UH in the dermis was 3- to 8-fold greater for the SM containing formulations than for the aqueous and the PL® 80 formulation, this difference being statistically significant (Student's t-test, $P=0.0008$ and 0.004, respectively).

3.4. Tape stripping

The weight of skin removed per strip decreased with progressing number of tape strips. Likewise, the amount of Heparin recovered from the tapes progressively decreased (Fig. 2). Assuming a den-

Table 6
Accumulation of Heparin within epidermal membrane and dermis

Formulation	Heparin concentration, mean ± SD [IU/cm^3]	
	Epidermal membrane	Dermis
UH aqueous	Non-detectable	113 ± 128.6 $n=3$
LMWH aqueous	443.1 ± 173.3 $n=7$	−[a]
LF1 (UH)	144.6 ± 36.5 $n=9$	280.2 ± 324.1 $n=6$
LF2 (UH)	Non-detectable	887 ± 161.6 $n=4$
LF3 (UH)	Non-detectable	927.7 ± 364.7 $n=4$
LF4 (LMWH)	337.8 ± 175.3 $n=4$	−[a]

[a] Not determined.

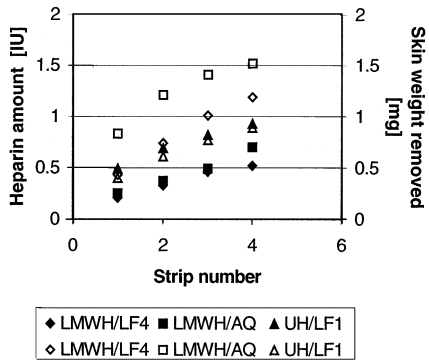

Fig. 2. Cumulative skin weight and corresponding cumulative amount of Heparin removed by tape stripping following incubation with liposomal (LF) and aqueous (AQ) formulations; cumulative numbers calculated from averages of all skin samples for each strip number. Open symbols denote skin weight; closed symbols denote Heparin amount.

sity for the stratum corneum of 1 g/cm^3 (Yogeshvar et al., 1996), it is estimated that UH penetrated only in the upper 6 μm of the SC. Even though the exact depth of penetration depends on the assumed tissue density, it is evident that UH penetrates primarily into the outermost epidermis layers, since no UH was found in the deeper layers of the epidermal membrane (Table 7). In contrast, LMWH was observed to penetrate into deeper layers of the epidermal membrane. The outermost roughly 10 μm of the stratum corneum contained 62% of the total detected amount of LMWH for the aqueous formulation and 38% for the liposomal formulation (LF4) (Table 7). Larger amounts of LMWH were found to penetrate into deep layers of the epidermal membrane for the liposomal formulation than for the aqueous formulation, the difference, however, was not statistically significant (Student's t-test, $P = 0.2$).

Table 7
Heparin amount in tape strips and in residual epidermal membrane

Heparin amount [IU], Mean ± SD ($n = 4$–8)		
	All strips of each skin sample, cumulatively	Residual epidermal membrane of each skin sample
LMWH aqueous	0.42 ± 0.22	0.26 ± 0.05
LMWH LF4	0.50 ± 0.08	0.83 ± 0.64
UH LF1	0.83 ± 0.61	Non-detectable

In order to compare the results of the tape stripping procedure with the results of skin accumulation of the previous section (Table 6), the data of tape stripping are expressed in terms of concentration in Table 8. The rather high concentration of UH is localized in the outermost layers of the epidermis. The total membrane concentrations obtained by tape stripping were smaller than those of the total accumulation experiment. This is probably because of loss of Heparin occurring during the large number of preparation steps involved in the tape stripping method. This appears to be more profound for the aqueous LMWH formulation. The larger total membrane concentration of LMWH LF4 in Table 8 originates from the larger Heparin content of the residual membrane for LF4 compared to the other formulations.

4. Discussion

The larger size of liposomes measured in the solution containing ethanol and Heparin compared to double distilled water is consistent with the finding of Kirjavainen et al., 1999, who suggested that the increased liposome size in the presence of ethanol might be due to a decrease in interfacial tension or the induction of interdigitation. Therefore, measurements in 25% (v/v) ethanol including Heparin reflect the particle size of the formulations. The pure SM liposomes were in the gel state at room temperature and at the temperature of the permeation experiment and tended to undergo aggregation which was visible to the naked eye. These phenomena were reversible above the phase transition temperature. This explains why this formulation showed a roughly 2-fold higher particle size polydispersity than the ones with PL 80, which contained liposomes in the liquid crystalline state.

In the transport experiments, not detecting Heparin in the receiver compartment is considered to be due to lack of permeation rather than

Table 8
Concentration of Heparin in various skin layers

	Concentration, Mean ± SD [IU/cm^3]		
	All strips, cumulatively	Residual epidermal membrane	Total epidermal membrane
LMWH aqueous	316.7 ± 185.6	59.9 ± 13.4	118.0 ± 45.2
LMWH LF4	381.0 ± 126.2	169.1 ± 129.1	217.0 ± 85.9
UH LF1	1098.0 ± 887.0	Non-detectable	104.0 ± 76.0

because of metabolic processes taking place within the skin tissue. This is because no UH degradation was observed in stability tests, in which UH was incubated with epidermal membrane for periods of time at least as long as the permeation experiment (results not shown) and because biologically active Heparin was extracted from within the skin.

In all, roughly 80% of all specimens showed no consistent Heparin (UH and LMWH) permeation. Measurable UH permeation was associated with a compromised diffusion barrier function of the epidermal membrane in the onset of the experiment as detected by TEWL. This is in agreement with Stüttgen et al., 1990 who also reported a dependency of UH permeation across skin on the barrier function of the stratum corneum. The epidermal membrane is shown to be the main barrier for Heparin permeation across the skin, since UH permeation across dermis was readily determined. The initial surge of LMWH levels in the receiver compartment for the aqueous formulation can not be presently explained, this result, however, did not attest to a steady permeation across epidermal membrane as a function of time because subsequent receiver concentrations remained constant.

Contrary to UH, permeation of LMWH, or lack thereof, did not exhibit a dependency on the barrier function of the SC. It appears, therefore, that TEWL can only be used as a qualitative criterion to classify epidermal membrane specimens based on their barrier properties and does not correlate with the skin permeability of Heparin. This may possibly be because TEWL merely reflects the diffusion of water across the tissue which may have different permeation characteristics, for instance, follow a different permeation pathway than the solutes in question. The variation of TEWL was considered to be partly due to interindividual differences between skin donors and partly due to the preparation of the skin as performed by different experimenters.

The lack of UH and LMWH permeation through SC is attributed to their high average molecular weight of 17 000 and 9000, respectively, and their hydrophilic properties. It has been suggested that large polar molecules with low diffusion coefficients in the SC and low SC/water partition coefficients pass through the skin mainly by means of the transappendageal route (shunt diffusion) (Barry, 1986). Furthermore, Heparin, which has a high negative charge because of its sulfate groups, could be subject to repulsion by the skin which has been demonstrated to be also negatively charged at pH 6.4.

The liposome formulations did not have any positive effect on UH or LMWH permeation across human epidermal membrane. This is in contrast to Bonina and Montenegro, 1994b, who, using ^{3}H UH, reported 15-fold increased permeation rates in vitro after pretreatment of skin with soy bean lecithin, owing to increased diffusion coefficient rather than an effect on partitioning into the stratum corneum. Also, using young pigs Artmann et al., 1990 found elevated fat tissue levels and increased liver content and renal elimination with a liposomal Heparin formulation in vivo as compared to an aqueous formulation.

Penetration and accumulation of Heparin into epidermal membrane was reproducibly measured. Although some loss of Heparin occurred during the extraction from the tissue resulting in incomplete recovery, significant differences were iden-

tified. The amount of LMWH penetrating into the tissue, irrespective of formulation, was statistically significantly larger than the amount of UH. Moreover, tape stripping showed that UH was accumulated exclusively in the outermost layers of the stratum corneum while LMWH was also localized in deeper tissue layers. These results demonstrate that the molecular size of the Heparin molecule plays an important role in skin penetration, the smaller size favouring transport into the skin. Penetration of UH was detected only for the liposomal formulation with PL 80. Penetration of LMWH, on the other hand, was observed to equal extents for both the aqueous and the PL 80 formulation, accumulation, however, tended to be larger in deep tissue for the liposomal formulation compared to the aqueous formulation. Thus, in general, the PL 80 liposomal formulation appears to positively affect Heparin penetration into epidermal membrane.

The lipids of this formulation were in a liquid crystalline state in which the bilayer is more flexible than in the gel state, promoting an interaction with stratum corneum lipids. Also, it was shown that phosphatidylcholine and ethanol can act synergistically loosening the structure and increasing the fluidity of skin lipid bilayers (Kirjavainen et al., 1999; Touitou et al., 2000). Thus, it is suggested that the enhanced Heparin penetration into epidermal membrane observed in the present study may be the result of interaction of the liposomes and the ethanol with the skin. Schmid and Korting, 1994, proposed that liposomes should be localizers rather than transporters and that the deposition of liposomes into the stratum corneum could create a drug reservoir. This suggestion is consistent with the findings of the present work. Studies on the interaction of fluorescence labelled liposomes with the present phospholipid compositions with skin are presented elsewhere (Betz et al., 2001). Using radiolabelled UH with a variety of formulations (Schaefer and Zesch, 1976; Zesch and Schaefer, 1976; Stüttgen et al., 1990), penetration into deeper layers of the epidermis was typically found to be maximally 5% of the amount determined in the stratum corneum, this being qualitatively in agreement with the present work. Tape stripping of the separated epidermal membrane could be carried on for two to five tape strips before it was torn because of its being quite fragile. Other authors (Schaefer and Zesch, 1976; Artmann et al., 1990) achieved ~ 20 tape strips using full thickness skin. The tape stripping technique is shown to afford a precise localization of Heparin within the skin.

The Heparin concentrations determined in the epidermis are well above the lowest pharmacologically active concentration in blood that is reported to lie around 3 USP-U/ml (Pratzel, 1991). These concentrations, however, are average values of the respective tissue layers, while the concentration distribution, for instance, of LMWH in the deeper epidermis is not known. Therefore, it can not be unequivocally concluded whether the concentration reached in the blood capillaries at the epidermis/dermis interface, that constitutes the site of activity of Heparin is sufficiently high to exert a pharmacological effect. The absence of consistently measurable transepidermal permeation of Heparin may at first be taken as an indication that no pharmacologically relevant levels reach the site of activity. Yet for local therapy, momentarily reached drug concentrations without a continuous flux might suffice for a pharmacological effect. It is finally mentioned that Heparin has an antiinflammatory activity which may occur at concentrations not detected in the present study.

The isolated dermis was not a major barrier for UH permeation compared to the epidermis, exhibiting permeability coefficients of the order of 1×10^{-7} cm/s independently of the used formulation. Heparin did not lose its anti FXa activity during permeation across dermis and no endogenous Heparin was released from the dermis in control permeation experiments. The result of Xiong et al. (1996), that LMWH permeation rates remain the same after removing the SC from full thickness skin by tape stripping suggesting that the SC is not the major barrier for the permeation of LMWH could not be confirmed for UH in the present study.

Interestingly, the presence of SM in the liposome formulations increased the lag time for UH permeation across dermis 5-fold compared to the aqueous formulation; for pure PL 80 this increase was 3-fold. Accordingly, the calculated UH diffusion coefficient in dermis decreased. The accumulation of UH in the isolated dermis was much greater for the SM compared to the other formulations. This is evidence that the longer lag times are caused by binding of Heparin in the dermis. This binding is enhanced by phospholipids, especially sphingomyelin. Preferential accumulation of Heparin in dermis tissue has previously been reported (Zesch and Schaefer, 1976). The mechanism by which SM enhances UH binding in dermis is not clear, it may be, however, that this binding is mediated by the phospholipids' own affinity for the dermis which is demonstrated in an upcoming manuscript (Betz et al., 2001).

5. Conclusion

The employed bioassay provides the assurance that Heparin detected to be present in tissue or to permeate across tissue compartments reflects the pharmacologically active drug. Human cadaver skin did not alter the anticoagulative properties of Heparin during permeation and penetration. Heparin is found not to permeate human epidermal membrane which represents the major skin permeation barrier. Penetration into the epidermis is shown to depend on the molecular weight of Heparin and to be greater for LMWH than for UH. Also, LMWH penetrated deeper into the epidermis than UH, the latter being largely restricted to the upper stratum corneum layers. Results are not conclusive as to whether levels of LMWH reached in the epidermis can exert a local pharmacological effect at the blood capillaries. The present liposomal formulations influence the penetration but not the permeation of Heparin. Thus, penetration of UH took place only from the PL 80 formulation and the epidermal distribution of LMWH may be influenced by this formulation. Phospholipids seem to enhance the binding of Heparin to the isolated dermis.

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