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Fish Skin as a Model Membrane to Study Transmembrane Drug Delivery with Cyclodextrins

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Abstract

The possibility of using fish skin as model membrane to study drug permeation and penetration enhancement by cyclodextrins was investigated. The permeability of the skin from four species of fish, *Anarhichas lupus* (catfish), *Pleuronectes platessa* (Plaice), *Hippoglossus hippoglossus* (Halibut) and *Anarhichas minor* (Spotted catfish), was compared in a Franz diffusion cell set-up using 1% hydrocortisone aqueous solution as a donor phase. The drug flux through fish skin was more than 100 times faster than the flux through hairless mouse skin and more than 10 000 times faster than through snake skin. Catfish skin was most easily accessible and was therefore used for further study. The octanol-water partition coefficient did not affect the transmembrane flux of small molecules whereas the aqueous diffusion coefficient could be correlated with the flux. The hydrocortisone flux of from aqueous hydroxypropyl-β-cyclodextrin solutions, which were saturated with the drug, increased with increasing cyclodextrin concentration. From these and other observations it was concluded that small molecules are transported through fish skin in aqueous channels. The properties of these channels resemble the properties of the aqueous diffusion layer present in human and animal skin and other types of biological membranes. Previous studies have shown that cyclodextrins will enhance drug delivery by increasing aqueous diffusion rate. Catfish skin can therefore be a good model membrane to study penetration enhancement by cyclodextrins.

Introduction

Bioavailability of topically applied drugs is generally rather low. Various types of penentration enhancers have therefore been employed to enhance drug availability from topically applied drug formulations. Chemical enhancers are usually hydrophobic or amphiphilic compounds that will be absorbed into the skin, where they will alter the solvent potential of the membrane barrier, and lower its viscosity [1].

Cyclodextrins have been used to enhance the permeation of drugs applied topically or in-vitro in an aqueous donor phase [2]. Cyclodextrins will form water-soluble inclusion complexes with lipophilic drugs. The solubility and total availability of drugs from aqueous drug formulations will therefore increase. Cyclodextrin molecules are relatively large (MW ranging from 1000 to 2000) and hydrophilic. Their penetration into lipophilic membranes is therefore very limited. The mechanism for the penetration enhancement must therefore be different from what is known for chemical enhancers. In addition it has been shown that cyclodextins can, depending on formulation composition, either enhance or retard permeation of drugs through skin and other types of biological membranes [2]. Recently we have proposed that primary mechanism for cyclodextrins induced drug permeation enhancement is an increased effective flux of the drug in an aqueous diffusion layer, at the membrane surface, preceding the partition of the drug into the lipophilic membrane [3]. The nature of the diffusion layer has not been determined but it is possible that the aqueous diffusion layer is partially present in the skin appendages and intracellular pores [2, 4].

Detailed understanding of the mechanism of cyclodextrin induced permeation enhancement requires a study with a suitable model membrane. Ideally human skin should be used for such studies. However human skin is not readily available and more importantly the intra-sample variation tends to be very large [5]. Human skin is therefore not suitable to study how small variations in formulation composition will affect the permeation. Artificial membranes such as silicone [6], poly(vinyl chloride) [7], semi-permeable cellophane membrane [8] have been used to study drug transport. These membranes are highly uniform and therefore suitable for mechanistic studies. However these membranes lack the complex structure of biological membranes. Mechanistic models of drug transport should therefore also be verified in studies with biological membranes. Animal skin, such as hairless mouse skin suffers from some of the same disadvantages as human skin, e.g., high inter-sample variation. Snake skin has been proposed as a model membrane for the stratum corneaum [9]. Snake skin lacks hair follicles, sweat glands or other type of appendages. The transmembrane flux should therefore by controlled by partition of the drug into

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the lipophilic part of the membrane. This membrane is therefore probably only suitable to study permeation that is purely membrane controlled, i.e. where the transmembrane flux is controlled by partition of the drug from the donor phase into the lipophilic membrane. Suitable biological membranes to study penetration enhancement with cyclodextrins have therefore not been available.

In the present work we have investigated the possibility of using fish skin as a model membranes to study transmembrane drug fluxes, and cyclodextrin enhanced drug delivery.

Materials and methods

Materials

Hydroxypropyl-β-cyclodextrin (HP-β-CD) was obtained from Pharmtec (FL, USA), β-cyclodextrin (β-CD) from Wacker Chemie (Germany), hydrocortison from INC Biomedicals (Ohio, USA), naproxene from Iceland Pharmaceuticals (Iceland), picric acid and sodium nitrated from Merck (Germany) and lidocaine-HCl, benzociane and diethyl stilbestrol from Norsk Medisinaldepot (Norway). All other reagents were of analytical or special reagent grade. The moisture content of HP-βCD was periodically determined and corrected for using Scaltec SMO 01 Moisture Analyzer (Germany).

Semi-permeable cellophane membrane (Spectra/Por® CE Dialysis membrane from cellulose esters) with MWCO 3500 was purchased from Spectrum Laboratories (Huston, USA) and hairless mice from Bommice (Copenhagen, Denmark). Shed snake skin was kindly donated by Venom supplies (Australia) and skins from freshly caught fish by Hamrafell (Hafnafjörður, Iceland).

Preparation of membranes

The semi-permeable cellophane membrane was washed with distilled water before use. The shed snake skin was stored dry in sealed plastic bags at room temperature. Before use it was immersed in distilled water for 30 minutes to soften it up and make it easier to cut. The fish used in the experiments were freshly caught and used within 24 hours. The skin was removed from the fish by skilled professionals at the fish factory and used within 2–3 hours. Residual fish meat was removed from the skin and the gel-like outer layer was washed of with water. Skin from a single fish was used in each experiment. Female hairless mice were sacrificed by cervical dislocation and their full-thickness skin was removed. The outer surface of the skin was gently rinsed with 35% (v/v) methanol in water to remove any contamination.

Permeation studies

The membrane to be tested was placed in a Franz diffusion cell of type FDC 400 15FF, diameter 1.5 cm (Vangard Internationl Inc. Neptune, NJ, USA), containing 11.9 ml receptor phase. Ther receptor phase consisted of phosphate buffer saline pH 7.4 (Ph.Eur., 2nd edn., VII. 1.3.) stirred with magnetic bar. The receptor phase contained 2.5% (w/v) HP-βCD to maintain sink contidion throughout the study. In case of hairless mouse skin the receptor chamber was kept at 37 °C by circulating water through an external jacket. Other membranes were studied at ambient temperature (about 23 °C). Two ml of donor phase was added to the donor chamber and 50 μl samples were withdrawn from the receptor phase at various time points up to 48 h and replaced by fresh receptor phase. The flux was calculated from the linear part of each permeability profile. Unless otherwise stated the donor phase was made from phosphate buffer saline pH 7.4, containing 2% HP-β-CD. Additional HP-β-CD (up to 7%) was added to the selected donor phases of poorly soluble compounds to maintain 10 mg/ml (1% w/v) concentration.

Electron microscopy

The samples of fish and mouse skin were chemically fixed by incubation in 2% gluteraldehyde, 0.025 M phosphate buffer, pH 7.0, for 16–24 hours at 4 °C. The samples were then washed twice for 20 minutes with 1 ml aliquots of phosphate buffer. The samples were then dried by critical point drying before sputter coating with gold in argon for 3 minutes.

Analytical methods

Instrumentation for HPLC consisted of a ConstaMetric 3200 solvent delivery system (LDC Analytical, USA) operated at 1.5 ml/min, a SpectroMonitor 3200 variable wavelength detector (LDC Analytical, USA), an AS-2000A Intelligent Autosampler (Merck-Hitachi, Germany), and a D-2500 Chromato-integrator (Merck-Hitachi, Germany). Reversed-phase chromatography was conducted at ambient temperature using a Luna(2) C18, 5 μm, 150 × 4.6 mm column (Phenomenex, UK), with a Security Guard precolumn (Phenomex, USA). The mobile phases, retentiontimes and detection wavelength were as follows: lidocaine; methanol, water, triethylamine (80:19.5:0.5), 2.15 min, 254 nm, benzocaine; methanol, water, triethylamine (80:19.5:0.5), 1.9 min, 254 nm, naproxene; mobile phase acetonitrile, water, acetic acid (40:59:1), 5.11 min, 262 nm, diethyl stilbestrol; acetonitrile, sodium acetate buffer (pH 4.8) (68:32), 2.6 min, 236 nm detection wavelength, 1.8 min, hydrocortisone; acetonitrile, water, acetic acid (64:35:1) mobile phase, 11.8 min retention time and 254 nm detection wavelength.

The picric acid concentration was determined from absorption measurements with Lambda 35 UV-VIS (Perkin–Elmer, USA).

Quantitative determinations of β-CD was performed on HPLC system composed of a Waters Model 501 pump operated at 1.0 ml/min flow rate, a Rheodyne 7125 injector and a PAD-2 pulsed amperometric detector from Dionex (USA) with a gold working electrode and a silver-silver chloride reference electrode. The column was a CarboPac PA1 Analytical Column (4 × 250 mm) from Dionex. The eluent consisted of 150 mM sodium hydroxide and 300 mM sodium acetate in water. Duration times for detection were: E_1 = 100 mV (t_1 = 120 m s), E_1 = 100 mV (T_1 = 120 m s),

Table 1. Hydrocortisone transmembrane flux from 1% aqueous donor phase solutions containing 1% (w/v) hydrocortisone

Species/type		Flux
Scientific name	Common name	(μg × h^{-1} × cm^{-2})
Artificial membrane		
Cellophane membrane (MWCO 3500)		121 ± 14
Fish skin		
Anarhichas lupus	Catfish	71 ± 14
Pleuronectes platessa	Plaice	121 ± 14
Hippoglossus hippoglossus	Halibutt	34 ± 10
Anarhichas minor	Spotted catfish	76 ± 13
Snake skin		
Notechis scutatus	Tiger snake	0.012 ± 0.003
Mouse skin		
Mus domesticus (3CH/Tif h/h)	Hairless mouse	0.44 ± 0.15

$E_1 = 100$ mV ($t_1 = 120$ m s). The PAD response was set at 1 s.

Sodium nitrate electrode (Combination type from Cole Parmer, Illinois, USA) was used to determine the concentration of nitrate ions.

Partition coefficients and aqueous diffusion coefficients

The log $K_{o/w}$ values for octanol-water partition were obtained from literature. When available the partition coefficients for compounds at pH 7.4 was used. In the case of benzocaine and picric acid the lowest available partition coefficient was used as it is assumed that these compounds are primarily in the ionic form at pH 7.4. The partition coefficients for nitrate ions and cyclodextrin were not available. These are very hydrophilic compounds and it is therefore reasonable to expect that the log $K_{o/w}$ would be less than the log $K_{o/w}$ value for sucrose (log $K_{o/w} = -3.70$ [10]).

The aqueous diffusion constant (D_w) for the compounds was calculated according to the following equation [11].

$$D_w = (13.26 \times 10^{-5})/(\mu_w^{1.4} \times V_a^{0.589}),$$

where μ_w is the viscosity of water and V_a is the LeBas molar volume [12].

Results and discussion

Table 1 shows the transmembrane hydrocortisone flux for various types of membranes. As expected the highest observed flux was through the semi-permeable cellophane membrane. The flux was also high through skin from various salt-water fish species. The flux trough *Pleuronectes platessa* (plaice) skin was the same as the flux trough cellophane membrane, about 4 times the flux through *hippoglossus hippoglossus* (halibut) skin. The flux through *anarchias lupus* (catfish) and *anarhichas minor* (spotted catfish) was approximately 2/3 of the flux through cellophane membrane. The flux through mouse skin was more than 200 times slower than the flux through cellophane membrane and the flux through snake skin was about 10,000 times slower. The relative standard deviation in the measurements was also generally lower for fish skin (12–29%) than for mouse skin (34%) or snake skin (25%).

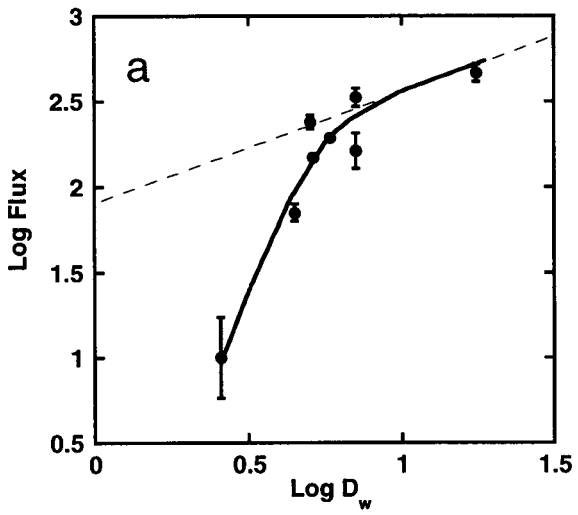
Catfish skin is free of scales, easy to handle and was more accessible than the other types of fish skin used in this study. Catfish skin was therefore used for further studies.

The drug permeation properties of fish skin were clearly different from the properties of biological model membranes that are commonly used to study transmembrane drug delivery. Due to high relative flux and low relative inter-sample variation fish skin may be a practical model membrane to study the contribution of formulation factors, e.g., cyclodextrin concentration, to transmembrane drug delivery.

It is generally accepted that permeability of human skin is correlated to the lipophilicity of the peremeating species. For example Potts and Guy [13] have constructed a mathematical model to predict skin permeability:

$$\text{Log } P - 0.71 \log K_{o/w} - 0.0061 \text{ MW} - 6.3,$$

where P is the permeability of the human skin for a given compound, $K_{o/w}$ is the octanol water partition coefficient and MW is the molecular weight of the compound. This model of delivery through lipophilic route correlates well with available data that from the scientific literature. Similar correlation can also be found for the permeability of cornea [10]. However other investigators have shown that for certain compounds, certain delivery systems and some other types of biological membranes the delivery through aqueous route, e.g., paracellular delivery or delivery through aqueous pores, is equally important [14–18]. This is for example the most important route when drug delivery through sclera and stroma is considered [10]. Thus two routes are possible in transmembrane delivery; an aqueous and a lipophilic route. The flux through catfish skin was determined for eight different compounds to investigate which route is more important in delivery through catfish skin. Table 2 shows the flux from 1% aqueous solutions of these compounds. Some cyclodextrin was added to the donor phase to solubilize some of the more lipophilic compounds. Figure 1 shows the log Flux



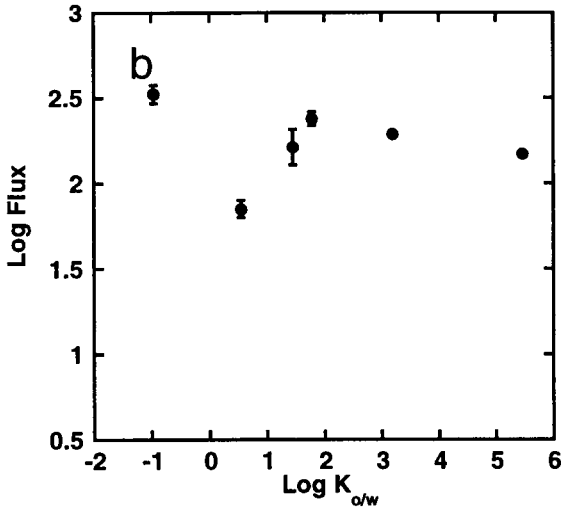


Figure 1. Graphical representation of flux data from Table 1, (a) log Flux vs. log D_w, (b) log Flux vs. log $K_{o/w}$.

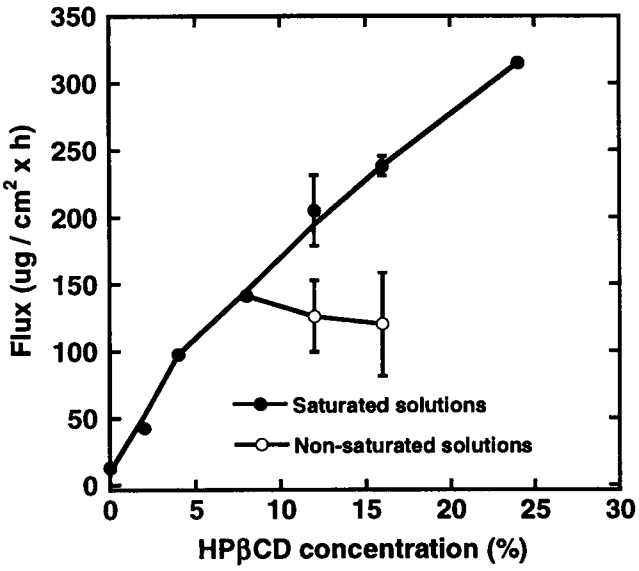


Figure 2. The relationship between HP-β-CD concentration and hydrocortisone flux through catfish skin from solutions saturated with the drug, (●) solutions with fixed hydrocortisone concentration (○).

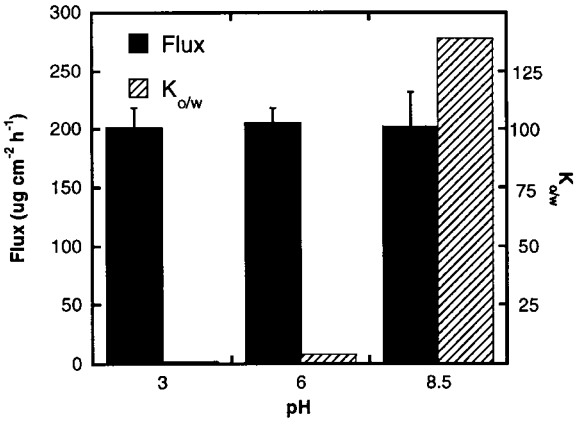


Figure 3. Lidocaine flux through catfish skin, and the $K_{o/w}$ of lidocaine, in relation to pH of the donor phase.

plotted against log D_w and log $K_{o/w}$. There is a correlation between log Flux and log D_w which is consistent with delivery through an aqueous layer. Delivery through an ideal aqueous layer should follow Fick's law and log Flux vs. log D_w should then be unity. This is consistent with the data for the six compounds with the highest flux (Figure 1a). For the largest compounds, hydrocortisone and β-cyclodextrin, there is a negative deviation showing less flux than could be expected from the calculated D_w value. In this case the diameter of the aqueous pores in the membrane may be a limiting factor.

Log $K_{o/w}$ values were available from literature for six of the compounds. In this case there was no correlation between log Flux and log $K_{o/w}$ (Figure 1b). Log $K_{o/w}$ values for the very hydrophilic compounds NO_3^- and β-CD were not available. Including data for NO_3^- and β-CD in Figure 1b would not have improved the correlation because these were the compounds that showed the highest and lowest flux, respectively .

The pKa value for lidocaine is 7.9 [19]. At high pH lidocaine is in its neutral form and at low pH lidocaine has a positive charge. The octanol water partition coefficient is therefore strongly dependent on pH, whereas the aqueous diffusion coefficient is almost independent of pH. The permeability of lidocaine through catfish skin was not dependent on pH (Figure 2). From these studies it could be concluded that this compound was transported through the membrane by an aqueous route and that partition was not a part of this process.

Addition of HP-β-CD increased the transmembrane flux of hydrocortisone, through catfish skin, when donor phase was saturated with the drug (Figure 3). The increase was almost linear with a slight negative deviation. This is similar to what has been observed for hairless mouse skin [3] and semi-permeable cellophane membrane [20]. However in the case of fish skin there is no apparent saturation of the drug flux at high cyclodextrin concentration. When the hydrocortisone concentration was fixed at 12 mg/ml the drug flux decreased only slightly with increasing cyclodextrin concentration. These observations are consistent with theoretical models of cyclodextrin enhanced drug delivery

Table 2. Transmembrane flux of various compounds through catfish skin from 1% aqueous donor phase solution containing 1% (w/v) of the compounds

Drugs/compound	Conc. (mg/ml)	HP-β-CD conc.	Flux (μg/h cm^2)	MW (g/mole)	LeBas vol. (cm^3/mole)	D_w (cm^2/s)	Log K_{ow}
NO_3^-	10	2	466 ± 55	62.0	40.5	17.54	–
Picric acid	10	2	333 ± 42	229.1	189.9	7.08	−0.94 [21]
Lidocaine	10	2	238 ± 21	234.3	319.6	5.02	1.78 [19]
Benzocaine	10	7	163 ± 38	165.2	189.2	7.08	1.44 [22]
Naproxene	11	7	194 ± 5	230.3	262.1	5.84	3.18 [23]
Diethyl stilbestrol	10	7	148 ± 3	268.3	352.2	5.14	5.46 [24]
Hydrocortisone	10	7	71 ± 8	367.0	410.8	4.48	0.54 [10]
β-cyclodextrin	10	0	10 ± 5	1135.1	1059.8	2.56	–

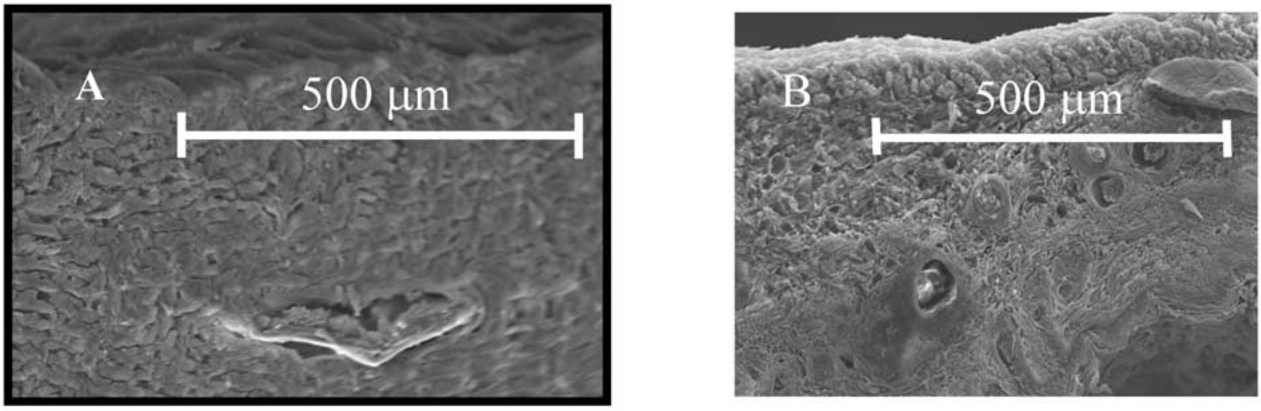


Figure 4. Electron microscopy images of the cross-section of catfish skin (A) and hairless mouse skin (B).

through membranes [2, 3] if it assumed that drug is transported by aqueous diffusion and that partition is not a part of the process.

From these observations it is clear that aqueous pores or channels are present in fish skin and that small molecules readily pass through these channels. Investigation with electron microscopy (Figure 4) revealed that the structure of mouse skin is much denser than the structure of catfish skin. The intercellular space in catfish skin is larger, which is consistent with the observation that small molecules can rapidly pass through this membrane via an aqueous route.

Conclusion

Cyclodextrins have been used as penetration enhancers for drug delivery through human skin, animal skin and other type of biological membranes. They enhance the diffusion in an aqueous layer from which the drug will partition into the lipophilic part of the membrane. Catfish skin is highly permeable for small molecules. The compounds are transported via an aqueous route. The properties of the aqueous channels in fish skin resemble the properties of the aqueous diffusion layer present in human and animal skin and other types of biological membranes. Catfish skin can therefore be considered as a good model membrane to study penetration enhancement with cyclodextrins.

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