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Iontophoretic skin permeation of peptides: an investigation into the influence of molecular properties, iontophoretic conditions and formulation parameters

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ABSTRACT:

The transdermal route offers advantages for delivery of peptides and proteins. However, these polar and large molecules do not permeate the skin barrier well. Various enhancement methods have been employed to address this problem. Iontophoresis is one of the methods that showing promise but its application to peptide delivery has yet to be fully explored. This study investigates the effects of different molecular properties and iontophoretic conditions on the skin permeation of peptides.

In this study, the permeation of alanine-tryptophan dipeptide (Mw = 276), alanine-alanine-proline-valine tetrapeptide (MW = 355), Argireline® (Acetyl hexapeptide-3, MW = 889) and Triptorelin acetate (decapeptide, MW = 1311) through excised human skin under passive or iontophoretic current of 0.4mA was studied using Franz diffusion cells. The effects of pH (3.0-7.4, to provide different net negative, neutral and positive charges), donor concentration (1-10 mg/ml), background electrolyte (34-137 mM NaCl and/or 5-20 mM HEPES) and current direction (anodal vs cathodal) were also investigated. Peptides were analysed by HPLC or liquid scintillation counting.

Iontophoresis led to a 30 times increase in peptide permeation relative to the passive flux for the peptides. Electroosmosis was an important determinant of the total flux for and even high molecular weight charged peptides. Electrorepulsion was found to be considerable for low molecular weight charged moieties. Permeation flux was decreased at lower pH, possibly due to decreased electroosmosis. Results also showed that 10 times increase in donor peptide concentration increases flux of peptides by about 2-4 times and decreases permeability coefficients by about 2.5-5 times. The addition of extra background electrolyte decreased the permeation coefficient of peptides by 2-60 times.

This study shows that iontophoretic permeation of peptides is affected by a number of parameters that can be optimized for effective transdermal peptide delivery.

Key words: Percutaneous absorption; Peptide and proteins delivery; Iontophoresis; Molecular weight and charge, Electro-osmosis; Skin penetration

INTRODUCTION

Many proteins and peptides have been identified that have therapeutic potential for skin conditions [1], cardiovascular disease, Parkinson's disease, Alzheimer's disease, depression, anxiety, attention deficit hyperactivity disorder, and many other disorders. Poor oral bioavailability has led to ongoing research for a convenient and noninvasive delivery method. The transdermal route offers different advantages and potential for protein and peptide delivery if the considerable barrier to permeation presented by the stratum corneum can be overcome [2]. Proteins and peptides present a significant challenge to successful transdermal delivery due to the size and complexity of their molecular structure. In addition, most are hydrophilic and often exist in a charged state, thus passive skin diffusion across the highly structured lipid domains of the stratum corneum is unlikely to be effective. To overcome the skin's barrier to peptides and proteins delivery, different enhancement methods have been employed over the last decades, amongst which iontophoresis looks promising.

Iontophoresis involves the use of a mild electrical current (up to 0.5mA/cm^2) to drive charged and uncharged molecules across the skin. This method is considered non-invasive and delivers consistent quantities of drug through skin. The positive or negative drug ions act as charge carriers across the high impedance stratum corneum to enhance delivery of the drug into the skin. There have been a number of detailed analyses of the mechanistic aspects of iontophoresis and iontophoretic enhancement [3, 4]. Electrorepulsion (electromigration) is known to be the main mechanism by which iontophoresis exerts its enhancement effect on ionised solutes, but other factors including increased stratum corneum permeability in the presence of an electric current flow, and electroosmosis (movement due to convection flow) are also important, especially for uncharged molecules. Proteins and peptides are usually charged at physiological pH or can be rendered charged by altering pH and are therefore considered ideal candidates for iontophoretic delivery. Besides charge, other factors like molecular weight and mobility of molecules also affect the permeation of peptides. However, the iontophoresis of peptides and proteins requires further investigation, especially in the areas of the effects of molecular weight on the mechanism of iontophoretic permeation through the skin, that is the subject of the present investigation.

In this study the influence of molecular size of peptides, the direction of applied current and the effect of formulation parameters such as concentration, pH and presence of competing ions on the iontophoretic delivery of five peptides (Figure 1) across human epidermal

membrane in vitro were investigated. The peptides investigated were 5-Aminolevulinic acid (small peptide-like drug), a model dipeptide (L-Ala-L-Trp), a therapeutic tetrapeptide L-Ala-L-Ala-L-Pro-L-Val, a cosmetic hexapeptide (Acetyl-hexapeptide-3 or Argireline®) and a therapeutic decapeptide (Triptorelin acetate). These molecules provide a wide molecular weight range of 130-1300 and different net charges of negative, neutral and positive. Where possible peptides with potential therapeutic relevance have been chosen, as discussed below.

<Insert Figure 1>

5-Aminolevulinic acid (ALA) is a small molecular weight (131.1 g/mol), highly polar, hydrophilic (pKa 4.0 and 8.4) molecule. It is the intermediate in heme biosynthesis and is the precursor of protoporphyrin IX (PpIX), which is strongly fluorescent and undergoes photobleaching rapidly producing singlet oxygen. Consequently, ALA is used as an endogenous photosensitizer in photodynamic therapy for a range of non-melanoma skin cancers like basal cell carcinoma, squamous cell carcinoma, actinic keratoses and Bowen's disease, and in photo diagnosis [5]. ALA-PDT requires a long administration period of approximately 4h at the skin surface to provide homogenous delivery at the target site. Chemical and physical penetration enhancement techniques have been investigated to improve the skin penetration of ALA [6-9].

L-Ala-L-Trp (Alanine-Tryptophan, called Ala-Trp in this manuscript) is a model dipeptide molecule (molecular weight 275.9 g/mol), hydrophilic (alanine pKa 2.34 and 9.69, tryptophan pKa 2.28 and 9.39), log P 0.61 ± 0.52.

L-Ala-L-Ala-L-Pro-L-Val (Alanine-Alanine-Proline-Valine; AAPV) is a small molecular weight (355.4 g/mol), hydrophilic (logP -0.45 ± 0.65) tetrapeptide. It fits the P-P_1 subsites of elastase and inhibits human neutrophil elastase (HNE). HNE acts as a pro-inflammatory agent that is implicated in a number of conditions including rheumatoid arthritis, psoriasis, atopic dermatitis and allergic contact dermatitis. Peptidic HNE inhibitors have a common hydrophobic peptide sequence which partially mimics certain amino acid sequences found in elastin. [10].

AC-Glu-Glu-Met-Gln-Arg-Arg-NH_2 (EEMQRR; Acetyl hexapeptide-3; Argireline®) is a synthetic peptide (molecular weight 888.6 g/mol and log P -4.5 ± 0.95) that has been

incorporated into cosmeceutical products for anti-ageing properties [11]. It is designed from the N-terminal end of the protein SNAP-25 that inhibits Ca^{2+}-dependent catecholamine release from chromaffin cells [12]. It acts by down-regulation of muscle action and is applied to reduce wrinkles around the eyes.

p-Glu-His-Trp-Ser-Tyr-[D-Trp]-Leu-Arg-Pro-Gly-NH_2 (EHWSYWLRPG; Triptorelin available as acetate and pamoate) is a synthetic gonadotropin-releasing hormone (GnRH) agonist and a long-acting LHRH analogue (molecular weight: 1311.5 g/mol, pKa: 7.2, 9.5, 12 and log P 0.3 ± 1.41). It is marketed as a sustained release injection for treatment of infertility, endometriosis and hormone-responsive cancers.

MATERIALS AND METHODS

Materials

All chemicals and reagents listed below were used as supplied: 5-aminolevulinic acid hydrochloride (ALA) (A3785 ~98%), L-Alanyl-L-Trptophan (05400 ≥ 99%), N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid HEPES (H3375 ≥99.5% titration) and fluorescamine (F9015 ≥98%) were purchased from Sigma-Aldrich (Sydney, NSW, Australia). AAPV (AV-4-NH_2, P100401-SY121097, ≥96% purity), Acetyl-hexapeptide-3 (Ac-ER-6, 54947, ≥95% purity) and Triptorelin acetate (HG-9, 5773-63-4, ≥98% purity) were purchased from GL Biochem Ltd (Shanghai, China), Phosphate buffer saline was prepared according to United States Pharmacopeia. Sodium chloride (NaCl) from Asia Pacific Speciality Chemicals Ltd (Seven Hills, NSW, Australia), analytical grade potassium chloride (KCl) and sodium hydroxide (NaOH) were purchased from Merck Pty Ltd. (Darmstadt, Germany) and boric acid was purchased from Ajax Finechem (Taren Point, NSW, Australia). Analytical grade acetonitrile was purchased from JT Baker (Philipsburg, NJ, USA) and trifluoroacetic acid (TFA) from Sigma-Aldrich (Sydney, NSW Australia). Triethylamine (TEA) from Fluka Chemika (Switzerland) and orthophospohoric acid from Ajax Finechem (Australia). Silver (Ag)-wire (99.99%, 1 mm diameter) was obtained from Sigma-Aldrich and purified deionised Milli-Q water from Millipore (North Ryde, NSW Australia).

Quantitative Analysis

The quantitative analysis of all peptides was by reverse phase high performance liquid chromatography (HPLC) and liquid scintillation counting for ALA. The chromatographic system consisted of an Agilent 1100 series equipped with a quaternary pump (G1311A), autosampler (G1313A), solvent-degasser (G1312A) with a photo diode array detector/fluorescence detector (G1321A). Separation was achieved on a Phenomenex Jupiter C18 300A column (5 µm, 150mm×4.6 mm) with a guard column (wide pore C18). Peak integration was undertaken on a personal computer using Chemstation A08.01 software. All standards and samples were prepared at room temperature. The radiolabelled samples were analysed on a liquid scintillation counter (Packard LSC: Canberra, CT, AUS).

The HPLC mobile phase consisted of water and acetonitrile buffered with trifluroacetic acid (TFA), triethylamine or phosphoric acid. The ratio of aqueous phase to organic phase was dependent on the compound: 30:70 (0.1% TFA) isocratic system for ALA. ALA belongs to the class of alpha amino ketones with a weak chromophoric carbonyl group and is therefore unsuitable for quantification by ultraviolet (UV) absorption spectroscopy. Precolumn derivatisation step with fluorescamine generated a fluorescent compound that was detected using an excitation wavelength of 395nm and emission wavelength of 480nm [8]. For Ala-Trp a linear gradient system, aqueous (0.045% TFA): acetonitrile (0.036% TFA) from 10% to 100% (UV detection at 210nm), for AAPV a gradient system, aqueous (0.1% TFA): acetonitrile (0.1% TFA) from 5% to 20% (UV detection at 220nm), for acetyl hexapeptide 90:10 (0.05% TFA) isocratic system (UV detection at 210nm) and for triptorelin 75:25 (20mM triethylamine and 50mM phosphoric acid) isocratic system (UV detection at 280nm). The R.S.D of repeatability was less than 1% and the lowest limit of quantification was in the range of 12 to 320 *ng* for all the peptides assayed.

Radiolabelled ALA (0.5 µCi/diffusion cell) were mixed with scintillation cocktail (Emulsifier safe, Perkin Elmer, Waltham, MA, USA) and analysed for radioactivity.

Skin Preparation

Ethical approval for using human skin was obtained from the Human Research Ethics Committee of Curtin University prior to the study. Full thickness human skin (abdominal section) excised from female patients following abdominoplasty surgery at Perth hospitals was used. Skin was prepared by removing the subcutaneous tissue by dissection then

isolating the epidermal membranes from human skin by the heat separation method [13] where the full thickness human skin was immersed in water at 60°C for 1 min. The epidermal membrane was then teased off the dermis; placed onto aluminium foil with the stratum corneum layer facing upward, air dried and then stored at −20°C (for not more than 2 months) until required.

Iontophoretic in-vitro Permeation Studies

All iontophoresis experiments were conducted across human epidermal membrane using Pyrex glass side-by-side diffusion cells, enabling permeation across skin sections of cross sectional area 0.95 cm^2; donor and the receptor volume of 1.8 ± 0.1 mL. The membrane was allowed to equilibrate for 1 h in a water bath maintained at 35 ± 0.5°C. Membrane integrity was then determined by visual inspection over a bright light and by electrical resistance (kΩ) measurement using a digital portable LCR multimeter (TH2821/A/B, Changzhou Tonghui Electronic Co Ltd, Jiangsu Province, China). The measurements were taken by immersing the silver-silver chloride electrode attached to the probe lead tips, one each in the donor and receptor compartments. Membranes exhibiting an electrical resistance less than 20 kΩ were rejected.

The receptor compartment was always filled with electrolyte solution (20mM HEPES + 137mM NaCl). The donor compartment consisted of ALA, Ala-Trp, AAPV, acetylhexapeptide-3 or triptorelin acetate prepared with water or receptor electrolyte solution at various concentrations.

A constant iontophoretic current of 0.4mA was supplied by a custom-built power supply unit (Art Electro NSW, Australia) for 2 h unless otherwise stated. Passive diffusion cells were treated in the same way but without the electrodes. Samples were collected from the receptor compartment and replaced with fresh pre-warmed electrolyte solution throughout the experimental period. The data was plotted as mean cumulative amount permeating to the receptor compartment versus time. Transdermal permeation flux and permeability coefficient were calculated over the linear portion of the plot using Fick's first law.

Influence of concentration: The donor compartment consisted of Ala-Trp at 3.6 and 36 mM (1 and 10 mg/ml), AAPV at 2.84 and 28.4 mM (1 and 10 mg/ml), acetylhexapeptide-3 at 1.13 and 11.3mM (1 and 10 mg/ml) and triptorelin at 0.9 and 9mM. The solutions were prepared

in water or 20 mM HEPES + 137 mM NaCl. The later electrolyte was also used as the receptor electrolyte solution.

Influence of pH: The donor solution pH was maintained at physiological pH 7.4 or at pHs 6.0, 5.5, 5.0, and 3 using 0.1M or 0.01M sodium hydroxide (NaOH) and/or 0.1 or 0.01M hydrochloric acid (HCl) solutions. The net electrical charges of peptides were calculated at all pHs using Calculator Plugins for structure property prediction and calculation, Marvin 6.0.0, 2013, ChemAxon (http://www.chemaxon.com) [14].

Influence of background electrolyte: The donor solutions of Ala-Trp, AAPV and triptorelin were prepared in water or receptor electrolyte solutions (20 mM HEPES + 137 mM NaCl). The donor solution for acetylhexapeptide-3 (EEMQRR) was prepared at six different combinations (20, 10, 5 mM HEPES + 137, 68.5, 34.2 mM NaCl respectively and 137, 68.5 and 34.2 mM NaCl alone).

Influence of direction of current: For anodal iontophoresis, active cells received the silver electrode in the donor compartment and silver chloride electrode in the receptor compartment, and vice versa for cathodal iontophoresis.

Statistical Analysis

The permeation data for ALA was analyzed using SPSS 16.0 for Windows (SPSS Inc., Chicago IL). The data for other peptides were analyzed using The Mixed Procedure in SAS version 9.1 for Windows (SAS Institute Inc., Cary, NC, USA). The Mann-Whitney U Test (1-tailed) was used to determine significant differences between all parameters.

RESULTS

Table 1 provides the passive and iontophoretic flux and permeability coefficients of peptides with neutral or positive net charges under anodal iontophoresis. The results show that iontophoresis has been able to increase permeability coefficient of these peptides by about 7-27 times. Table 1 shows that, except for ALA, irrespective of the molecular weight (300-1300 Da) and charge (0 to 1.1) of the corresponding peptides, the permeability coefficients of di, tetra, hexa and deca-peptides are very close (12-17 $\times$ 10^{-3} cm/h). This means that, at similar concentrations, these molecules permeate the skin at almost identical fluxes,

regardless of their differences in physicochemical properties. Such behaviour might indicate that electroosmosis is the main driving force for iontophoresis of these molecules. Consistent with this suggestion, the estimate electroosmotic volume flux, based on the peptide fluxes and concentrations, ranges from 10 to 17 µl/cm^2/h for these four peptides and the conditions used. However, as these peptides are zwitterionic and the skin is cation permselective, electromigration as a determinant of peptide permeation is a potential confounder.

<Insert Table 1>

Influence of charge and pH: Table 2 provides the effects of pH on charge and iontophoretic permeation of peptides. The iontophoretic permeability coefficient of ALA increased from 1.3×10^{-3} cm/h to 11×10^{-3} cm/h when the pH was increased from 5.5 to 7.4 [15]. In contrast, Ala-Trp showed a significant increase ($P < 0.0001$) in the permeability coefficient from 0.4×10^{-3} cm/h to 13×10^{-3} cm/h when the pH was decreased from 7.4 to pH 5.5. The permeability coefficient of AAPV reduced significantly from 18×10^{-3} cm/h at pH 7.4 to 0.4×10^{-3} cm/h ($P < 0.0001$) at pH 3.0 and no change at pH 5.5 (18×10^{-3} cm/h). Acetylhexapeptide-3 also demonstrated a significantly reduced permeability coefficient from 17×10^{-3} cm/h at pH 7.4 to 4.8×10^{-3} cm/h at pH 5.0 ($P < 0.0001$).

<Insert Table 2>

Influence of concentration: The effect of donor peptide concentration is presented in Table 3. A 10x increase in donor concentration of Ala-Trp, from 3.6 mM to 36 mM (from 1mg/ml to 10 mg/ml) at pH 5.5 resulted in a 4.2x increase in iontophoretic flux but reduced the permeability coefficient from 13.0×10^{-3} cm/h to 5.5×10^{-3} cm/h. A 10x increase in donor concentration of acetylhexapeptide-3 from 1mg/ml to 10 mg/ml at physiological pH, resulted in a 2.5x increase in the iontophoretic epidermal flux but decreased the permeability coefficient by about 4 times. The same trend was observed for triptorelin where a 2x increase in flux and 5x decrease in permeability coefficient was observed after a 10x increase in the peptide concentration (Table 3).

<Insert Table 3>

Influence of background electrolyte:

The presence of HEPES and Na^+ ions significantly reduced the iontophoretic delivery of all peptides (Table 4 and Table 5). A 14x reduction in permeability coefficient of Ala-Trp resulted from the donor solution of 20mM HEPES and 137mM NaCl at pH 5.5 compared to water. A less pronounced reduction of less than 2x in the permeability coefficient of AAPV, EEMQRR and Triptorelin occurred with 20 mM HEPES and 137 mM NaCl (Table 4).

To investigate the effects of electrolytes type and concentration, the influence of different HEPES (10 and 5 mM) + NaCl (68.5 and 34.2 mM) systems and different NaCl solutions alone (137, 68.5 and 34.2 mM) were studied for EEMQRR iontophoretic permeation. The permeability coefficient data for all donor solutions is presented in Table 5 in comparison to base system. The results clearly indicate that a high concentration of ions in the delivery vehicle reduces the iontophoretic permeability coefficient of peptides. The extent of reduction was more for NaCl alone (11-57x) in comparison to HEPES-containing systems (less than 2x reduction). The permeability reduction effects of background electrolytes were also concentration-dependent and the effect increased with increase in electrolyte concentration (Table 5).

<Insert Table 4 and Table 5>

Influence of direction of current: anodal v cathodal: The direction of the current can influence the iontophoretic skin permeation depending on the predominant charge carrier. The permeability coefficient of AAPV and acetylhexapeptide-3 that permeated human epidermis under the influence of anodal versus cathodal current is presented in Table 6. The direction of current did not influence iontophoretic delivery of AAPV. In contrast the iontophoretic permeability coefficient of acetylhexapeptide-3 under cathodal current was similar to passive delivery [$(1.2 \pm 0.3) \times 10^{-3}$ cm/h] compared to $(17 \pm 2) \times 10^{-3}$ cm/h with anodal current.

<Insert Table 6>

DISCUSSION:

Table 1 shows that, regardless of the molecular weight (300-1300 Da) and charge (0 to 1.1) of the peptides, the permeability coefficients are very similar (12-17 × 10^{-3} cm/h). This suggests that, irrespective of differences in their physicochemical properties, these molecules permeate the skin with effectively the same fluxes. Such behaviour is consistent with electroosmosis being the main determinant of the anodal iontophoretic flux for each of these molecules. The other mechanisms involved in the iontophoretic enhancement of skin permeation of drugs are: electro-repulsion (electromigration) and changes in skin permeability (skin damage) due to electrical current [16]. In contrast to passive skin permeation, where the unionised form of a drug molecule permeates better than its ionised form, it is the ionised solute that is transported across the skin in iontophoresis. Here, the iontophoretic electrical potential acts as an external force to push ionized drug molecules across the skin (electromigration). Cathodal iontophoresis is applied to repel a negatively charged drug from the negative electrode across an epidermal membrane, whilst anodal iontophoresis is used for positively charged drug molecules [17]. Electroosmosis produces bulk fluid flow in the membrane and occurs when a voltage difference is imposed across a charged membrane. This fluid stream carries all cargos (ions or neutral species) dissolved within it and always flows in the same direction as the flow of counterions (i.e. ions of opposite charge to the membrane charge) [16]. Human skin is negatively charged above about pH 4 and counterions are positive ions at this condition, thus electroosmotic flow occurs from anode to cathode [16]. Electroosmosis applies for both charged and uncharged drugs and its rate depends on solvent volume flux and concentration of the cargo. On the other hand, dependency of electroosmosis on molecular weight is expected to be lower than other mechanisms, as discussed later in this paper. Therefore, the similarity of permeability coefficients for different molecules (charged and uncharged with different molecular weight, Table 1) might show the important role of electroosmosis in peptides penetration in the present investigation. Considering this argument, the electroosmotic volume flux in the conditions used in the present study was calculated to be about 10-17 µl/cm^2/h. An electroosmotic flow of up to 37 µl/cm^2/h in hairless mouse skin, was reported by Pikal and Shah [18, 19]. However, as discussed later, the presence of other mechanisms, including electrorepulsion, cannot be dismissed specially for low molecular weight peptides.

In contrast to the above-mentioned argument, ALA showed a low permeability coefficient. This molecule is slightly positively charged and its low permeation cannot be justified based on electroattraction. Its low permeation might be due to its high concentration of 60 mM. As

ALA is a small molecule with a positive charge, the high concentration might cause neutralization of skin negative charge and therefore decrease the electroosmosis. Our studies show that the same drug at pH 7.4 [15], where it contains a net charge of -0.3, shows an anodal permeability coefficient of about 11 x 10^{-3} cm/h, while negative charges are expected to show lower permeability in anodal iontophoresis in comparison to neutral moities. Considering the above-mentioned volume flow and the fact that this compound is negative and small, these data might show that both electromigration and electroosmosis are playing important roles in permeation of this low molecular weight compound.

As shown in Table 1, triptorelin has a positive net charge of about +1 at pH = 7.4. Therefore, at least theoretically, a good contribution from electromigration is expected for this molecule for anodal iontophoresis. Thus a higher permeability is expected in comparison to those obtainable by electroosmosis only. As discussed by Pikal [16], electroosmotic flow increases in importance as the size of the drug ion increases. The 'ionic' or Nernst-Planck effect (electromigration) is the largest contributor to flux enhancement for small ions. Increased skin permeability or the skin 'damage effect', is a significant factor for both large and small ions, particularly for experiments at high current density. For monovalent ions with Stokes radii larger than about 1 nm, electroosmotic flow is the dominant flow mechanism [16]. This effect is in a way that transdermal delivery of a large anion (or negatively charged protein) from the anode compartment can be more effective than delivery from the cathode compartment [16]. The present data might show that permeation of triptorelin under the effects of iontophoresis is mainly ruled by electroosmosis.

Table 2 shows the effects of pH on iontophoretic permeation of peptides. pH affects permeation of drugs in iontophoresis in different ways. The pH of the donor solution plays an important role in ionisation of molecules (their electriomigration) and the charge of the skin (skin permselectivity) and therefore electroosmosis [20]. The pH of the solution can also affect the chemical stability of peptides and proteins, as was previously reported for ALA [7, 15]. Isoelectric point (pI) of skin is about 4-5 [21] and skin shows negative charges at pHs above this value and positive charges at lower pHs. Bath et al. [22] showed that electroosmotic flow is highly pH dependent and is from anode to cathode at pHs > 3.5 and from cathode to anode at pHs < 3.5. They also showed that anodal electroosmosis increases with pH increase above 3.5 and cathodal electroosmosis increases with pH decrease below 3.5.

As shown in Table 2, both Ala-Trp and AAPV are neutral/slightly positive at pH 5.5 and show similar permeability coefficients that are explainable by electroosmosis as discussed above. When the pH is increased to 7.4 both peptides become negative. In this condition, an electromigration in an opposite direction to that of electroosmosis is expected. This might explain the sharp decrease in permeability of Ala-Trp from 13 to 0.4, but cannot explain the similarity of permeation of AAPV at pHs 5.5 and 7.4. This might show the importance of electroosmosis for permeation of AAPV and electromigration for permeation of Ala-Trp. Such behaviour might be due to the difference in their molecular weight as discussed above, indicating that electroosmosis is important for higher molecular weights and electromigration for lower molecular weights. However, we cannot dismiss the importance and the role of electromigration at these conditions.

As discussed above, decreased pH will decrease electroosmotic flow from anode to cathode and can even reverse it when the pH becomes lower than 3.5. This might explain the decreased Kp of EEMQRR from 17×10^{-3} cm/h at pH = 7.4 to 4.8×10^{-3} cm/h (close to its passive permeation) when pH is decreased from 7.4 to 5. For AAPV, permeability coefficient decreases from 18×10^{-3} cm/h at 7.4 to 0.4×10^{-3} cm/h at pH 3.0. These reductions happen in spite of the positive charges of these molecules (0.2 and 0.9 for EEMQRR and AAPV respectively) at lower pHs (Table 2) that is expected to increase the flux through electrorepulsion. This again shows that electroosmosis is very important in the permeation of these higher molecular weight peptides. A similar finding was reported for thyrotropin-releasing hormone (TRH) [23]. Greater iontophoretic transport was achieved in its unionised form at pH 8 by electroosmosis than at pH 4 where it is 99% cationic; this was attributed to the reverse in skin permselectivity from cationic to anionic at acidic pH, and electroosmosis occurs from cathode to anode.

Increasing the donor peptide concentration increased the iontophoretic flux of the peptides, but the flux increase was not equivalent to increase in concentration. A 10x increase in the peptide concentration resulted in only 2-4 times increase in flux, indicating 2.5-5x decrease in the permeability coefficients (Table 3). The effect of concentration on the iontophoretic permeation of drugs is complicated. Delgado-Charro et al. [24] showed that when drugs neutralize the skin charge, increased drug concentration decreases the permeability through decreased and even reversal of electroosmosis. This might explain in part the present data. However, the same group suggested that when electrorepulsion is the major mechanism, the

permeability coefficient is expected to stay constant (linear relationship between concentration and flux). Thus another explanation is the possible iontophoretic charge saturation, as was reported for LHRH and two analogues [25].

Marro et al showed that in the absence of background electrolyte the iontophoretic fluxes of the three drugs lidocaine, quinine and propranolol are essentially independent of donor concentration over a 100-fold range, while in the presence of background electrolyte lidocaine delivery increased linearly with concentration as it competed more and more effectively with Na+ to carry the charge across the skin. However, iontophoretic delivery of quinine and propranolol increased non-linearly with concentration [26].

The sum of the flux of individual ions must equal the current applied by the power source; in other words, there is competition among all the ions present to carry the charge. Physicochemical characteristics such as electrical mobility and concentration of the ion concerned, and the properties of the media through which the ion is moving, determine the probability of the ion being the major carrier, and eventually being efficiently transported across the skin [27, 28]. The concentration of an ionised species is said to influence the electro-migration component of the iontophoretic flux, when it is applied along with counterions and co-ions (buffers, electrolytes and preservatives in the formulation) [29]. In such cases, molar fraction of the drug molecule that plays a major role in iontophoretic delivery, as this is the concentration relative to that of the competing ions [26, 28]. The electro-diffusion model developed by Kasting and Keister based on single ion transport suggests that, in the absence of competing cations, iontophoretic flux is independent of concentration and dependent only on the ratio of mobilities of the cation and the main counterion (usually Cl^-) arriving from beneath the skin [30].

The present results show that the presence of 20 mM HEPES + 137 mM NaCl reduced the permeability coefficient of peptides (Table 4). The highest reduction was for Ala-Trp (about 14x). Other peptides showed about 2x difference regardless of their charges and molecular weight. To further investigate the effects of addition of background electrolytes, the effects of 5-20 mM HEPES and 34-137mM NaCl was investigated for the hexapeptide (EEMQRR: Table 5). The presence of co-ions and counterions revealed a linear trend in the permeation of acetyl hexapeptide-3, with the linear reduction in concentration of co-ions and counterions present (20, 10, 5 mM HEPES with 137, 68.5, 34.2 mM NaCl respectively). Significantly lower amounts of acetyl hexapeptide-3 permeated when the peptide donor solution was

prepared in NaCl (137, 68.5, 34.2 mM) alone. Results show that the reduction effects of added electrolyte is both electrolyte type and concentration dependent. In agreement with the present data, Bellantone et al [31] demonstrated a reduction in benzoate flux by more than half in the presence of equimolar amounts of sodium chloride. Cázares-Delgadillo et al [32] presented a similar observation with cytochrome C-A, a 12.4 kDa protein. The iontophoretic delivery of the macromolecule was reduced 3.9-fold when the sodium ion concentration increased to 170 mM. These effects can be related to the effects of added electrolytes on both electrorepulsion and also electroosmosis, as described below.

Addition of a background electrolyte, buffer or substance that could increase the ionic strength of the formulation increases the competition between the charge carriers and can affect iontophoretic drug flux. In practice, multiple ions exist as co-ions and counterions. Hence knowledge of the transport number of a drug molecule becomes essential. Transport numbers are complex functions of the concentration and mobility of all the ions present in a system. They are always less than unity due to the presence of relatively small and mobile endogenous ions such as sodium and chloride ions that have transport numbers of $t_{Na}^{+} = 0.6$ and $t_{Cl}^{-} = 0.4$ [29]. So, for electromigration, drug should compete with such ions. Smaller and more mobile ions (higher transport number) can carry a significant fraction of charge and reduce permeation of the drug.

Electrolytes can also affect electroosmotic flow. It has been demonstrated that increasing the electrolyte ionic strength in the electrode chambers (relative to the physiological level present beneath the tissue) decreased mannitol extraction (i.e., decreased electroosmosis) [33]. Santi and Guy [33] investigated the electro-osmotic extraction of mannitol (a non-ionizable, non-metabolizable model) in vitro using current density of 0.6 mA/cm^2 via Ag/AgCl electrodes and pH 7.4 HEPES-buffered saline (0.14 M). They found that iontophoresis of divalent ions from the anode chamber (i.e. an anode formulation with $CaCl_2$ or $MgCl_2$ instead of NaCl) increased electro-osmotic flow from beneath the skin surface towards the anode (i.e. modulation of the normal situation in which the skin's permselectivity dictates net electro-osmosis in the opposite direction). Shielding of the net negative charge on the skin was considered as a possible mechanism for this phenomenon. In the cathode chamber, to which electro-osmosis predominates, ~3-fold levels of enhancement were achieved by formulating the electrode bathing solution with either 2 mM calcein, 50–300 USP U/ml heparin, or 10 mM EDTA. It was postulated that this involves either binding of endogenous Ca^{2+} (and hence

decreased shielding of the negative charge on the skin), or simply the presence of more negativity in the membrane (or a combination of the two) [33].

The above mentioned data and discussion show that regardless of the mechanism, (electrorepulsion or electroosmosis), the concentration of the electrolyte should be at its optimum level to allow charge transfer whilst at the same time not neutralize the skin charge and not prevent the drug carrying the charge. A minimum amount of electrolyte, however, must be present in order to provide the required electrochemistry of the system.

Table 6 presents the effects of direction of the current on the permeation of two peptides. AAPV shows a similar flux from both anodal and cathodal concentration, in spite of its negative charge. This might show the importance of both electrorepulsion and electroosmosis for permeation of AAPV. However, acetyl hexapeptide-3 is benefiting from electroosmosis due to lack of charge and high molecular weight, therefore showing a high anodal flux and a low cathodal permeation (Table 6).

The peptides were chosen here to provide a series with increasing molecular weight and different charges. Figure 2 show the relationship between iontophoretic permeability coefficient and molecular weight and molecular charge for the peptides used in the present investigation. As is seen, the relationship between these variables looks complex. As discussed above, different mechanisms are involved in iontophoretic drug delivery including passive diffusion, electrorepulsion and electroosmosis. Whatever the mechanism, it has been shown that iontophoretic permeation of drugs decreases with increased molecular size [34-37]. However, the size-dependency for iontophoretic permeation under these mechanisms is different and the size-dependency of electroosmosis is expected to be lower than that of passive diffusion or electrorepulsion [3]. Also pH-dependency and charge-dependency of iontophoretic permeation under different mechanisms are also expected to be different. Using the ionic mobility-pore model, Roberts et al [35] correlated the molecular weight of the peptides with their iontophoretic permeability coefficient. The same has been reported with experimental evidence using small molecular weight local anaesthetics. The ionic mobility pore model identified ionic mobility and molecular size to be the major predictors of iontophoretic permeability. Ionic mobility of a molecule depends on its p*K*a, molecular weight and conductivity.

<Insert Figure 2>

Here and on the basis of the mechanisms described above, iontophoretic permeation of higher weight molecular weight peptides, AAPV (tetrapeptide), Argirelin (hexapeptide) and Triptoreline (decapeptide), were analysed against their molecular weight using linear regression and a line with a negative slope of 0.0002 was observed (Figure 3). Such relationship has been reported in the literature, with Green et al [34, 38] reporting a negative slope of 0.002 for cathodal iontophoresis of a series of amino acids. Similarly DelTerzo et al [39] reported a negative slope of 0.006 for alkanoic acids and Phipps et al [40] reported a negative slope of 0.003 for inorganic cationic drugs. Based on the free volume model by Yoshida et al [41, 42] a mean slope of 0.0032 was interpreted to give an average free volume equivalent to the molecular volume of an ionised solute with molecular weight of 135.

<Insert Figure 3>

CONCLUSION:

This investigation showed that transdermal permeation of peptides is a complex process and is affected by different factors including molecular weight, charge, donor pH, donor drug concentration, background electrolytes and direction of the iontophoresis. Results show that although both electroosmosis and electrorepulsion play important roles in iontophoretic permeation of peptides, the importance of each depends on the molecular weight of the peptide, pH of the environment and molecular charge. Electroosmosis plays a very important role in permeation of higher molecular weight peptides, even when they are charged, while lower weight peptides are affected more by electromigration. The present data clearly shows that these different parameters need to be adjusted for optimized peptide delivery.

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Conflict of Interest Statement

All authors declare that have no conflict of interest related to this research.

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Figure Legends:

Figure 1. Molecular structures, molecular weight and some other properties of peptides used in the present investigation

Figure 2. Relationship between molecular weight, molecular charge and iontophoretic permeability coefficient of peptides through human skin.

Figure 3. Linear relationship between molecular weight and iontophoretic permeability coefficient of AAPV (tetrapeptide), Argirelin (hexapeptide) and Triptorelin (decapeptide) at pH 7.4 through human skin

Tables:

Table 1. Permeation of peptides across human skin under 0.4 mA/cm^2 anodal iontophoresis in relation to their molecular properties. Data are mean ± SEM.

Peptide	MW	Conc (mM)	pH	Net charge	Iontophoretic flux (µg/cm^2/h)	Permeability coefficient (cm/h × 10^3)			
						Iontophoresis	Passive	Pure (Ionto-Passive)	ER
ALA	131	60	5.5	0.06	12.5 ± 1	1.3 ± 1.1	0.08 ± 4.8	1.22	16
Ala-Trp	276	3.6	5.5	0.04	13 ± 2.8	13 ± 3.0	ND	~13	>13
AAPV	355	28.4	5.5	0.03	175 ± 7.1	18 ± 0.1	ND	~18	>18
EEMQRR	889	1.13	7.4	0.0	15.7 ± 2.3	17 ± 2.0	2.5 ± 0.05	14.5	6.8
Triptorelin	1311	0.9	7.4	1.1	12.1 ± 3.6	12 ± 3	0.4 ± 0.0	11.6	27.3

ND: Not detectable
ER: Enhancement ratio; Iontophoresis/passive

Table 2. Effect of pH on permeability coefficient (cm/h × 10^3) of peptides across human skin under 0.4 mA/cm^2 anodal iontophoresis. Data are mean ± SEM.

Peptide	MW	pH = 7.4		pH =5.5		pH = 5		pH = 3	
		Charge	Kp	Charge	Kp	Charge	Kp	Charge	Kp
ALA	131	-0.27	11 ± 10	0.06	1.3 ± 1.1	-	-	-	-
Ala-Trp	276	-0.11	0.4 ± 0.1	0.04	13 ± 3.0	-	-	-	-
AAPV	355	-0.2	18 ± 0.7	0.03	18 ± 0.1	-	-	0.9	0.4 ± 0.1
EEMQRR	889	0.0	17 ± 2	-	-	0.18	4.8 ± 1.4	-	-

Table 3. Effect of concentration on flux and permeability coefficient (Kp) of peptides across human skin under 0.4 mA/cm^2 anodal iontophoresis. Data are mean ± SEM.

Peptide	MW	Donor pH	Charge	Flux (μg/cm^2/h)		Kp (cm/h × 10^3)	
				1 mg/ml	10 mg/ml	1 mg/ml	10 mg/ml
Ala-Trp	276	5.5	0.04	13 ± 2.8	52 ± 11	13 ± 2.8	5.5 ± 1.2
EEMQRR	889	7.4	0	16 ± 2.3	41 ± 6.1	17 ± 2	4.3 ± 0.6
Triptorelin*	1311	7.4	1.1	12 ± 3.6	21 ± 6.5	12 ± 3	2.3 ± 0.6

Table 4. Effect of added background electrolyte on permeability coefficient (Kp, cm/h) of peptides across human skin under 0.4 mA/cm^2 anodal iontophoresis. Data are mean ± SEM.

Peptide	No added electrolyte (Control)			Added 20/137mM HEPES/NaCl			Kp reduction ratio
	pH	Charge	Kp (× 10^3)	pH	Charge	Kp (× 10^3)	
Ala-Trp	5.5	0.04	13 ± 3.0	5.5	0.04	0.9 ± 0.06	14.4
AAPV	7.4	-0.2	18 ± 0.7	6.0	0	10 ± 1	1.8
EEMQRR	7.4	0.0	17 ± 2	6.0	0.02	9.3 ± 2.4	1.8
Triptorelin	7.4	1.1	12 ± 3	7.4	1.1	6.8 ± 2.6	1.8

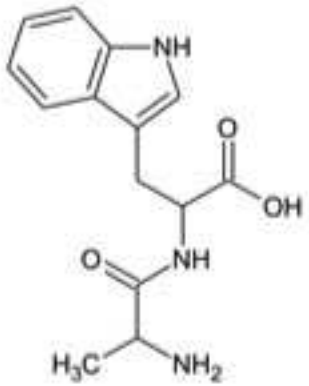
Table 5. Effect of added background electrolyte on permeability coefficient (Kp) of EEMQRR across human skin under 0.4 mA/cm^2 anodal iontophoresis. Data are mean ± SEM.

Added electrolyte	pH	Kp (cm/h × 10^3)	Kp reduction ratio
No added electrolyte (Control)	7.4	17 ± 2	1
137 mM NaCl + 20 mM HEPES	6.0	9.3 ± 2.4	1.8
68.5 mM NaCl+ 10 mM HEPES	6.0	12 ± 0.2	1.4
34.2 mM NaCl + 5 mM HEPES	6.0	14 ± 1	1.2
137 mM NaCl	6.0	0.5 ± 0.1	34
68.5 mM NaCl	6.0	1.6 ± 1.2	11
34.2 mM NaCl	6.0	0.3 ± 0.0	57

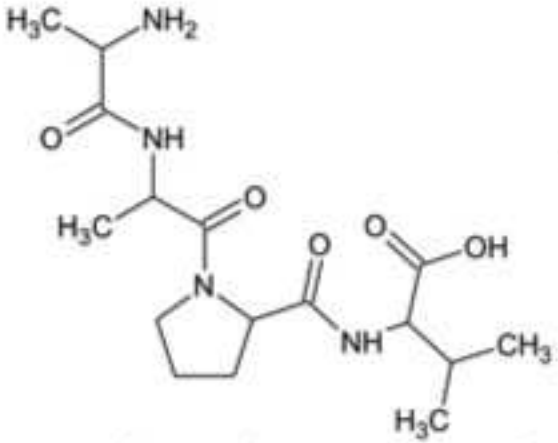
Table 6. Effect of current direction on permeability coefficient (Kp) of tetra and deca-peptides across human skin under 0.4 mA/cm^2 iontophoresis at pH 7.4. Data are mean ± SEM.

Peptide	MW	Charge	Kp (× 10^3) and [Charge]	
			Anodal	Cathodal
AAPV	355	-0.2	18 ± 0.7	17 ± 1
EEMQRR	889	0	17 ± 2	1.2 ± 0.3

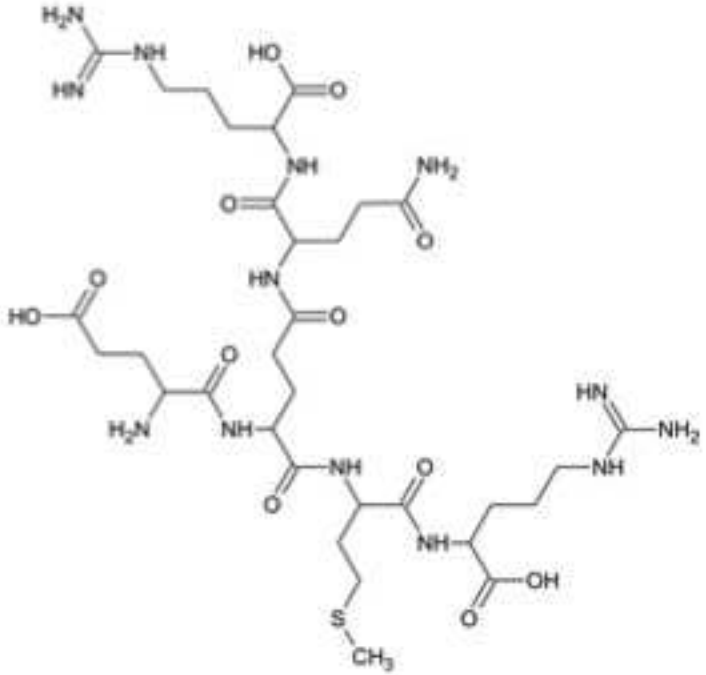
Figure 1



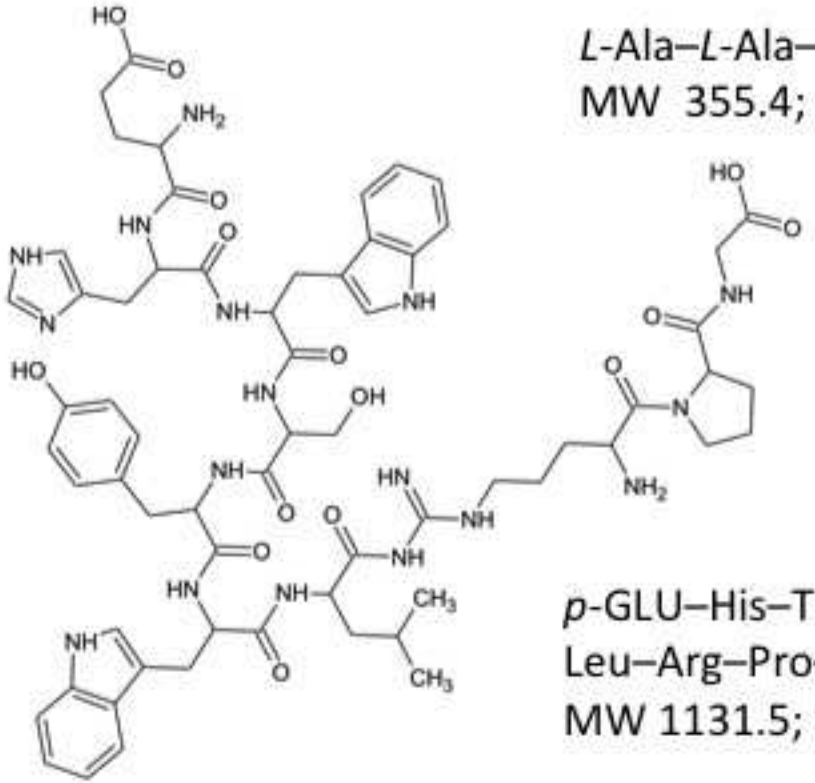
L-Ala–L-Trp MW 275.9; logP 0.6



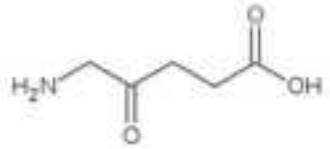
L-Ala–*L*-Ala–*L*-Pro–*L*-Val MW 355.4; logP -0.45



Ac-Glu–Glu–Met–Gln–Arg–Arg-NH_2 (Acetyl hexapeptide-3) MW 888.6; logP -4.5

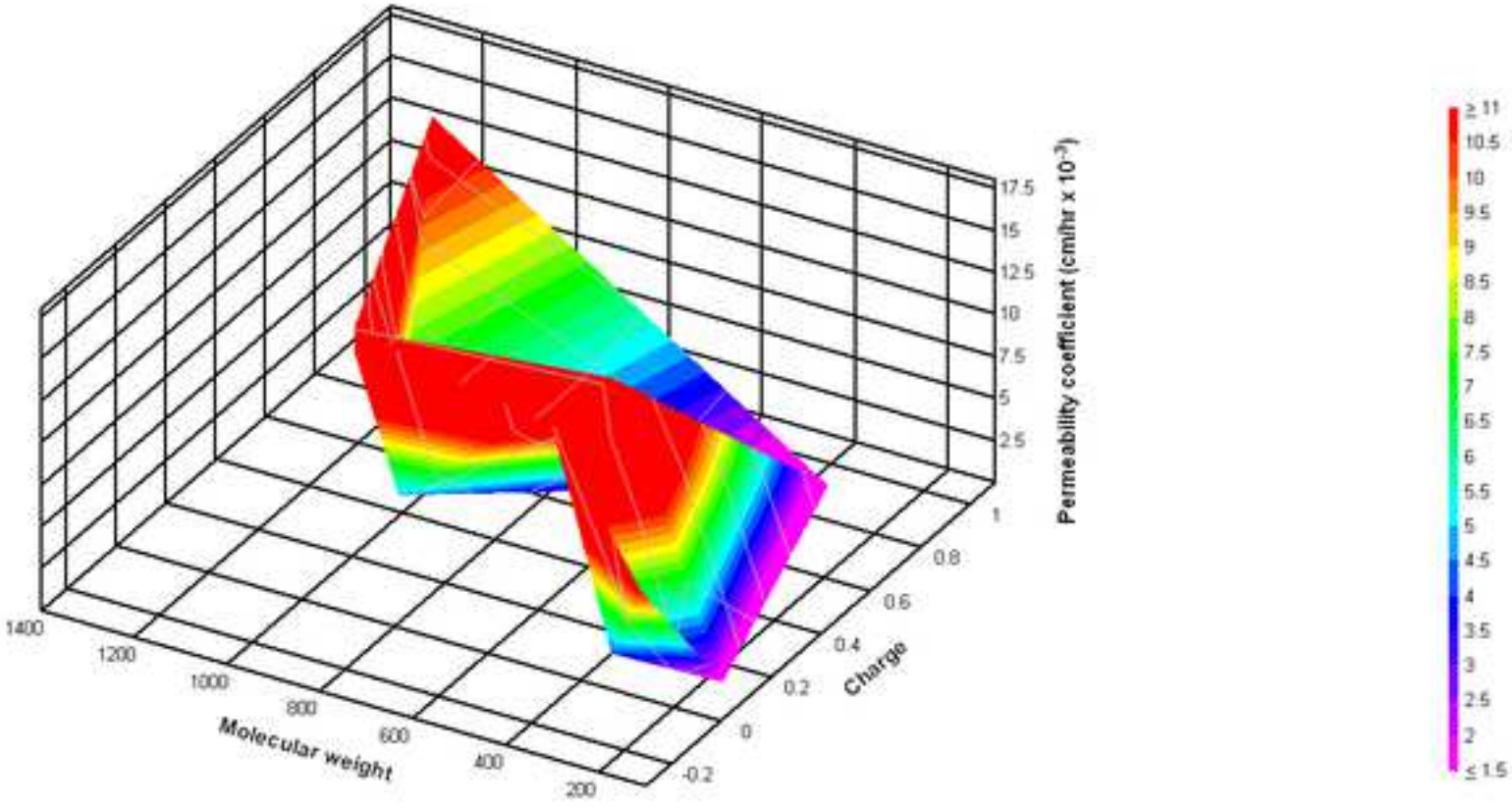


p-GLU–His–Trp–Ser–Tyr–[*D*-Trp]–Leu–Arg–Pro–Gly-NH_2 (Triptorelin) MW 1131.5; logP -0.3



5-Aminolevulinic acid MW 131.1; logP -1.3

Figure 2


Permeability coefficient (cm/hr x 10^{-3})
17.5
15
12.5
10
7.5
5
2.5
1
Charge
0.8
0.6
0.4
0.2
0
-0.2
Molecular weight
1400
1200
1000
800
600
400
200
≥ 11
10.5
10
9.5
9
8.5
8
7.5
7
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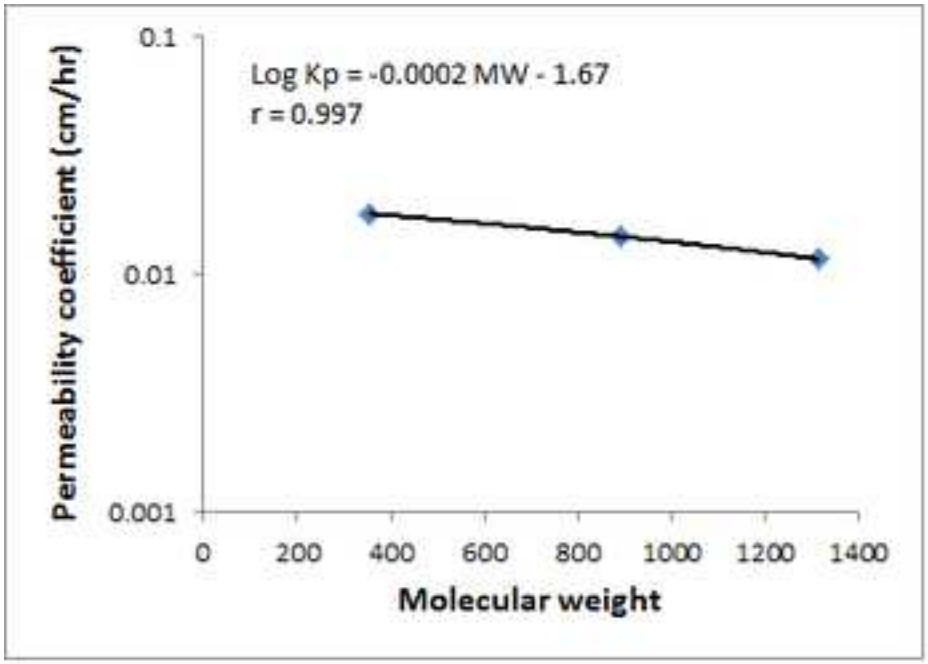
6
5.5
5
4.5
4
3.5
3
2.5
2
≤ 1.5

Figure 3


Log Kp = -0.0002 MW - 1.67
r = 0.997
Permeability coefficient (cm/hr)
0.1
0.01
0.001
0
200
400
600
800
1000
1200
1400
Molecular weight