Journal of Controlled Release 54 (1998) 243–250

journal of controlled release

Transdermal permeation of neutral molecules by skin electroporation

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Received 18 February 1997; received in revised form 2 July 1997; accepted 3 July 1997

Abstract

Electroporation of skin has recently been shown to enhance transport of charged molecules across skin by up to four orders of magnitude. This study demonstrates that high-voltage pulses can also increase transdermal permeation of two neutral model solutes, i.e. mannitol and water, up to 100-fold. The elevated flux results from the persistent increase in skin permeability following electroporation, rather than from electro-osmosis during pulsing. Control on transport was achieved by controlling the electrical parameters of the pulse, i.e. the pulse voltage, time constant and number.

Keywords: Skin electroporation; Transdermal transport; Neutral solutes; Diffusion; Electrical parameters

1. Introduction

Electroporation involves the creation of a transient elevation in the permeability of lipid bilayer membranes by applying short high-voltage pulses. It is believed that new aqueous pathways are created across the lipid bilayers [1,2].

Recently, electroporation of skin was shown to occur and increase transdermal transport by orders of magnitude [3–7]. Molecular transport occurs by electrophoretically driven transport during the pulses and diffusion through electropermeabilized skin during and after pulsing [3,4,6]. Increased transdermal delivery by electroporation has been shown for charged compounds: small ions [8], dyes such as calcein [3] and sulforhodamine [9], drugs such as metoprolol [4] and fentanyl [5,6], peptides such as LHRH [10], and macromolecules such as oligonucleotides [11] and heparin [12]. However, the effect of electroporation on the transdermal permeation of neutral molecules has never been studied.

The aim of this study was to check whether skin electroporation can increase transdermal permeation of neutral solutes. Two model permeants whose transdermal permeation has been extensively studied were used: mannitol and water [13–15]. The enhancement provided, the mechanisms involved and the factors affecting the neutral molecules permeation due to high-voltage pulses were investigated in vitro.

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0168-3659/98/$ – see front matter
PII: S0168-3659(97)00146-6

2. Materials and methods

2.1. Chemicals and animals

D-Mannitol (crystalline) was obtained from Sigma Chemical Co. (St. Louis, MO). D-1-[^{14}C]Mannitol and [^{3}H]water were obtained from New England Nuclear Research (Du Pont de Nemours, Brussels). Sodium phosphate and citrate salts, and *n*-butanol were supplied by Union Chimique Belge (UCB, Drogenbos, Belgium). Glucose was obtained from Merck (Darmstadt, Germany). All solutions were prepared in ultrapure water (Sation 900, Vel, Leuven, Belgium).

Skin for in vitro experiments was obtained from 2–3-month-old male hairless rats (mutant rat Iops hairless from Iffa Credo, Saint Germain les Arbreles, France).

2.2. In vitro model and procedures

Skin electroporation was performed in vitro in vertical chambers made of two compartments separated by skin (Makrolon, Obra, Liège, Belgium) [4]. Freshly excised full-thickness abdominal skin of hairless rats was mounted between the two compartments with stratum corneum facing the donor solution. The surface area of the membrane was 3 cm^2. Platinum electrodes (1×1 cm) (platinum pure, Aldrich Chemie, Bornem, Belgium) were immersed in the solutions (unless otherwise noted, the anode in the donor solution and the cathode in the receiver solution). The distances between the upper (donor) electrode and the under (receiver) electrode was 1 cm.

The receiver compartment (7.5 ml) was filled with phosphate buffer at pH 7.4 (0.048 and 0.024 M for mannitol and tritiated water studies, respectively), made isotonic with glucose. The receiver solution was continuously stirred magnetically and maintained at 37°C.

The upper reservoir was filled with 1.5 ml of donor solution. The mannitol solution was mannitol (1 mg/ml) and [^{14}C]mannitol (0.5 μCi/ml) in 0.06 M phosphate buffer at pH 7.4. The tritiated water solution was 0.01 M citrate buffer at pH 5 or ultrapure water, added with [^{3}H]water (1 μCi/ml).

Mannitol was present in the donor compartment during and after pulsing, only during pulsing, or only after pulsing. In those cases when the mannitol solution was present only during pulsing and then removed afterwards, the donor compartment was emptied, rinsed, and filled with phosphate buffer within 1 min after the last pulse. When mannitol solution was added only after the electrical protocol, pulsing was carried out with the phosphate buffer and replaced with mannitol solution within 1 min after the last pulse.

Tritiated water was present in the donor compartment either only during, or only after pulsing. In the cases when tritiated water was present only during pulsing and then removed afterwards, the donor compartment was filled with the 'citrate tritiated solution' and emptied, rinsed, and filled with buffer within 1 min after the last pulse. When tritiated water was added only after the electrical protocol, pulsing was carried out with the citrate buffer and replaced with the 'ultrapure water tritiated solution' within 1 min after the last pulse.

After electroporation, mannitol or tritiated water transport across skin was measured for 6 h. Measurement was carried out by removing samples (0.4 ml) from the receiver compartment at regular intervals. Samples were replaced with an equal volume of buffer. The amount of [^{14}C]mannitol or [^{3}H]water in each sample was determined by scintillation counting (liquid scintillation cocktail Ready Safe, Beckman, Belgium; liquid scintillation counter Wallac 1410, LKB, Pharmacia, Uppsala, Sweden).

The stability of mannitol and its ^{14}C-radiolabel during high-voltage pulsing were verified by thin layer chromatography using silica gel 60 sheets (Merck, Darmstadt, Germany). The mobile phase contained 85% *n*-butanol and 15% water by volume. Chemical detection of mannitol was performed with iodine vapor (UCB, Drogenbos, Belgium) [16]. Radioactivity was detected by scintillation counting (Bioscan Type AO 3000 A, System 200 Auto Changer and Imaging Scanner, Canberra-Packard, Washington, DC). Both mannitol and ^{14}C-labeled mannitol were shown to remain intact under the conditions of this study.

2.3. Electroporation

The electroporation device used was an Easyject

Plus (Equibio Ltd., Kent, UK) delivering exponential-decay capacitive discharge pulses. The pulse time constant (t) is defined as the length of time between the beginning of the pulse (maximum voltage) and the time when the voltage reaches 37% of its initial value. The voltage (50–250 V), time constant (80–330 ms) and number of pulses (5–15) used in this study were selected because they have been previously used for providing dramatic enhancement of transdermal transport of charged molecules [4–6]. The pulses were separated by 1 min. Voltages are expressed as voltages applied across the electrodes and not as transdermal voltages [4].

2.4. Statistical analysis

Results are expressed as mean±the standard error of the mean (S.E.M., $n=3\pm4$). The transdermal permeations of the molecules (0–6 h) were compared by a two-way analysis of variance (ANOVA type III, $P<0.05$), the ratios of the cumulative quantities detected in the receiver compartment to the membrane area and the molecular fluxes by the Student's t-test ($P<0.05$). The analysis of the factorial design was performed with the help of the Systat package on Macintosh microcomputer [23].

3. Results and discussion

3.1. Increase in neutral molecule transport caused by skin electroporation

We first investigated the ability of electrical pulses to enhance transport of the two neutral model molecules across skin. As reported previously, under passive conditions (no electric field), transdermal transport of mannitol and water were very low [13–15]. However, while applying high-voltage pulses to skin, transdermal permeation of these neutral molecules increased (Figs. 1 and 2).

It has been previously shown that electroporation of skin can increase transdermal permeation of charged compounds [3,4]. The present study shows that electroporation can also enhance transdermal transport of two neutral solutes. In contrast to the up to four orders of magnitude increase observed for charged compounds, high-voltage pulses caused an enhancement of only up to two orders of magnitude for the neutral molecules (Figs. 1, 2, 3 and 4).

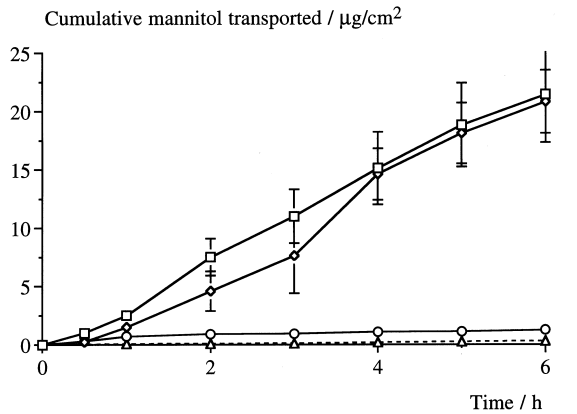

Fig. 1. Cumulative mannitol transported vs. time by passive diffusion (△), due to an electroporation protocol using five pulses of (150 V — 210 ms) and with mannitol present in the donor compartment only during (○), only after (◇), or both during and after pulsing (□).

3.2. Mechanisms of transdermal transport of neutral molecules by skin electroporation

Transdermal transport of molecules by electropo-

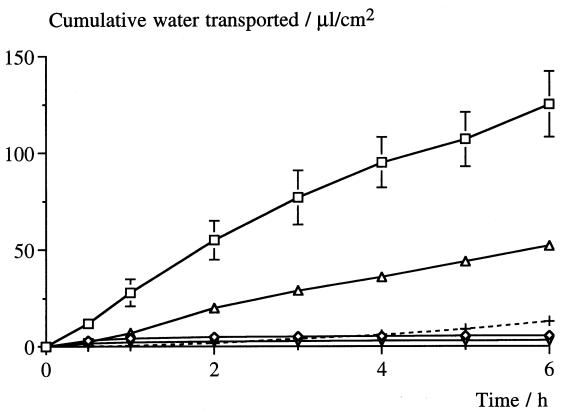

Fig. 2. Cumulative water transported vs. time by passive diffusion (+); due to an electroporation protocol using five pulses of (100 V — 125 ms) and with tritiated water present in the donor compartment only during (▽), or only after pulsing (△); or due to an electroporation protocol using five pulses of (250 V — 125 ms) and tritiated water present in the donor compartment only during (◇), or only after pulsing (□).

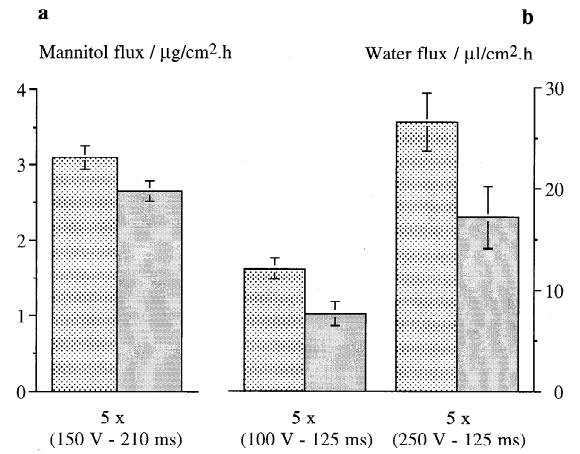

Fig. 3. Mannitol or water flux between 1 and 2 h ▨ and 5 and 6 h ▩ after different electroporation protocols. The neutral solutes were present only after pulsing.

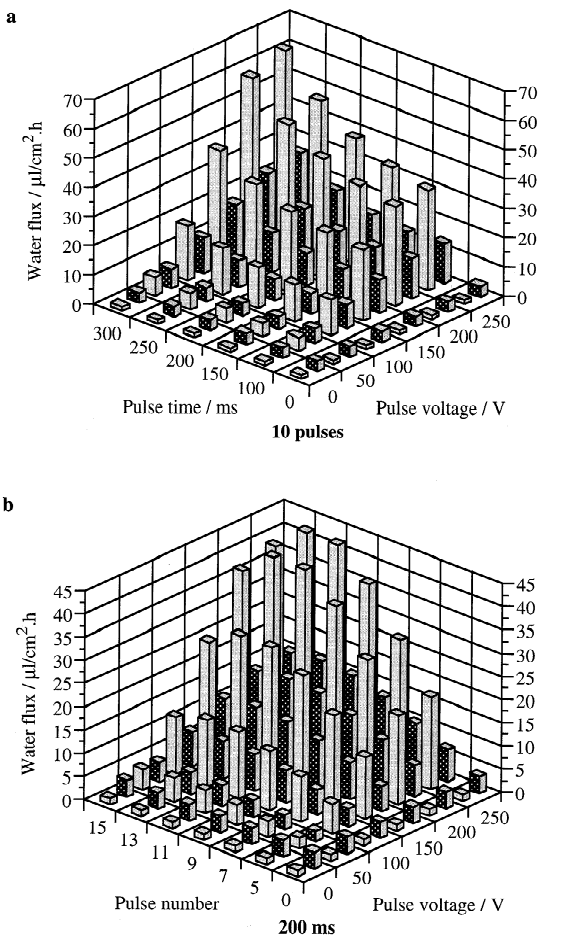

Fig. 4. Response surface plots obtained by fitting the experimental points. Water flux between 1 and 2 h ▨ and 5 and 6 h ▩, as a function of the pulse voltage and time constant (a) or as a function of the pulse voltage and number (b).

ration can occur during and/or after pulsing. During the pulse, the elevated transport can result from the increased permeability of the electroporated skin, from electrophoresis and/or less likely from electro-osmosis. After pulsing, the elevated transport can be caused by the changes in skin permeability which persist after pulsing and/or by releasing from a drug reservoir created within the skin during pulsing [3,4,6,12]. Experiments were performed to better understand the mechanisms of enhanced transport of mannitol and water by electroporation. For this purpose, mannitol or tritiated water were placed in the donor compartment only during, only after, or both during and after pulsing.

3.2.1. Transport of mannitol and water during pulsing

The transport mechanisms of neutral solutes occurring during pulsing were first investigated. Mannitol and water were placed in the donor compartment during pulsing and removed afterwards, transport out of the skin was then measured for 6 h. In this case, transdermal flux of mannitol or tritiated water was very low (Figs. 1 and 2).

The importance of electrophoresis in transdermal transport by electroporation has been previously demonstrated for charged molecules [3,4,6]. Because both water and mannitol are neutral, an increased transport due to electrophoresis during pulsing can be ruled out.

Even though electro-osmosis must take place during pulsing, its impact on transport could be low because of the short duration of current application. To test this hypothesis, the electrode polarity was reversed. Due to the net negative charge of the skin, the electro-osmotic flux is in the anodal to cathodal direction [14,17]. If electro-osmosis contributes significantly to mannitol or water transport by electroporation, a higher transport should therefore occur

Table 1
Assessment of electro-osmosis

Pulsing protocol	Anode in the donor	Cathode in the donor
Cumulative mannitol transported after 6 h ($\mu g/cm^2$)		
5×(150 V — 210 ms)	1.3±0.1	1.7±0.3
Cumulative water transported after 6 h ($\mu l/cm^2$)		
5×(100 V — 125 ms)	3.1±0.7	2.1±0.6
5×(250 V — 125 ms)	5.6±0.9	6.9±0.3

Mannitol or tritiated water were present in the donor compartment only during pulsing.

when the anode is in the donor compartment. As shown in Table 1, cumulative quantities of mannitol or water were equivalent whatever the electrode in the donor compartment (*t*-test, $P>0.05$). This indicates that indeed contribution of electro-osmosis is very low during electroporation. In contrast, electro-osmosis is the major mechanism involved in mannitol permeation by iontophoresis [13,14,17].

Hence, the low transport across skin of mannitol and water when present only during pulsing can be explained by the lack of electrophoretic movement and the negligible electro-osmosis. Due to creation of new aqueous pathways [2,18], some amount of solute is loaded into skin during and between pulses, creating a small reservoir effect (Figs. 1 and 2).

3.2.2. Post-pulse transport of mannitol and tritiated water

In another set of experiments, post-pulse transport of the neutral molecules was assessed. The skin was not exposed to mannitol or tritiated water during pulsing, but the solutes were added immediately after pulsing. As shown in Figs. 1 and 2, mannitol and water transport across skin was much greater when the compounds were present after rather than during pulsing (ANOVA, $P<0.05$). Whether mannitol was present during and after, or only after pulsing, its transport across skin was equivalent (ANOVA, $P>0.05$). These results clearly demonstrate that transport of the model neutral molecules occurs mainly after pulsing. The elevated transport results from the electroporation-induced alterations in skin permeability which persist after pulsing [3,6,19].

The reversibility of the elevated skin permeability caused by electroporation was evaluated. In the case when mannitol and tritiated water were present only after pulsing, the transdermal fluxes were measured between 1–2 h and between 5–6 h (Fig. 3). Water fluxes between 5–6 h were lower than corresponding fluxes between 1–2 h (*t*-test, $P<0.05$). However, the decrease in mannitol transport over time was not significant (*t*-test, $P>0.05$). These results indicate that the elevated permeability is partially reversible, as reported previously [3,4,6,19]. The persistent elevated transport of mannitol between 5–6 h is probably due to the releasing from a reservoir created within skin.

Overall, the data indicate that electroporation of skin enhances transdermal permeation of neutral compounds because of an increase in skin permeability which persists for several hours.

3.3. Control of neutral molecule transport by the electrical parameters of the pulses

The electrical parameters of the pulses, i.e. pulse voltage, time constant and number, have been shown to allow control of charged molecule delivery across skin [3–5,9,12]. Since the mechanisms involved in transdermal transport by electroporation are partially different for neutral vs. charged molecules, the influence of these electrical parameters on tritiated water transport by electroporation was analyzed.

The relative influence of the pulse voltage, time constant and number on water permeation was evaluated using a Fractional factorial design of three factors at three levels [5,20]. The low (level -1), middle (level 0) and high (level $+1$) levels of the pulse voltage (U) were 50 V, 150 V and 250 V, respectively. The low, middle and high levels of the pulse time constant (t) were 80, 200 and 330 ms, respectively. Those of the pulse number (N) were 5, 10 and 15, respectively. The combination of the

factor levels performed are shown in Table 2a. Table 2a also presents the results for each run: the water fluxes transported in the receiver compartment between 1–2 h and 5–6 h.

After logarithm transformation, the results were analyzed by General Linear Model (Macintosh Systat program) (Table 2b) [5]. Table 2b gives a coefficient for each factor studied representing the mean change of the dependent variable (log of the water flux) induced by an increase of the factor (U, t, or N) from its level -1 to its level 0, or from its level 0 to its level $+1$; and the significance (P value: <0.05 is significant, <0.01 is highly significant) of each factor, interaction term or quadratic term studied.

Table 2b indicates that changes in all three factors have significant effects on the increase in water flux, occurring both immediately or several hours after pulsing. The table also shows the significance of some interaction ($U\times t\times N$) and quadratic (U^2 and N^2) terms. Significance in quadratic terms indicates the non-linear dependence of the variable with U and N: at high values of U and N, the water flux did not further increase but reached a plateau. This was also

Table 2
Influence of the electrical parameters of the pulses on transdermal water transport

a. Factor levels in experimental units and results for each run of the factorial design

Run	U (V)	τ (ms)	N	Water fluxes±S.E.M. (μl/cm^2.h) between	
				1 and 2 h	5 and 6 h
1	−1	−1	−1	2.5±0.7	3.4±0.6
2	−1	+1	−1	4±1	4±1
3	+1	−1	−1	17±7	7±2
4	+1	+1	−1	39±6	24±9
5	−1	−1	+1	3.9±0.8	4.4±0.5
6	−1	+1	+1	6.7±0.9	10±1
7	+1	−1	+1	35±3	18±2
8	+1	+1	+1	66±15	31±6
9	0	0	0	29±4	11±2
10	0	−1	+1	23±3	15±3
11	0	+1	−1	22±3	12±3
12	−1	+1	0	8±2	9.1±0.9
13	+1	−1	0	32.5±0.8	14±2

b. General linear model analysis

	Log (water flux 1–2 h)		Log (water flux 5–6 h)	
Factor	Coeff.±S.D.	P value	Coeff.±S.D.	P value
Constant	1.45±0.09	0.000	1.04±0.09	0.000
U	0.47±0.03	0.000	0.26±0.03	0.000
τ	0.14±0.03	0.000	0.14±0.03	0.000
N	0.14±0.03	0.000	0.15±0.03	0.000
$U\times\tau$	0.03±0.03	0.297	0.04±0.03	0.192
$U\times N$	0.01±0.03	0.768	0.01±0.03	0.714
$t\times N$	−0.01±0.03	0.759	0.00±0.03	0.890
$U\times t\times N$	−0.03±0.03	0.350	−0.08±0.03	0.020
U^2	−0.29±0.08	0.001	−0.15±0.08	0.064
τ^2	0.1±0.1	0.644	0.2±0.1	0.176
N^2	−0.18±0.08	0.030	−0.14±0.08	0.081

U is the pulse voltage, τ the pulse time constant and N the pulse number. -1 is the low-, 0 the middle- and $+1$ the high-level of the factors. $n=3$ for each run.

The coefficient (±S.D.) of each factor studied corresponds to the mean change of the dependent variable (log of the water flux) induced by an increase of the factor (U, τ or N) from its level -1 to its level 0, or from its level 0 to its level $+1$. P value is the significance (P value: <0.05 is significant, <0.01 is highly significant) of the factors, interaction terms and quadratic terms.

reported in other electroporation experiments with skin and cells. Plateaus in transport at high field strengths were explained by the plateau observed in transmembrane voltages, plateaus with additional pulses by saturation in the permeabilization [1,3,5,7,9,19,21,22]. Using their coefficient, the effects of the factors on increasing skin permeability to water can be classified: the higher the coefficient, the greater the effect. The pulse voltage was the most important factor, followed by the pulse length and number. Similarly, this was reported for fentanyl transport [5]. The response surface plots (obtained by fitting the experimental points) allow to visualize the results of the analysis performed with the General Linear Model, and to better point out the reversibility observed in the water flux (Fig. 4) [5].

A study was performed to assess the influence of the electrical parameters of the pulses on mannitol transport as well. The data showed similar results (data not shown).

These data demonstrate that the electrical parameters of the pulses i.e. the pulse voltage, time constant and number allow control of neutral molecule transport. This agrees with previous results using charged molecules of different molecular weight [3–5,9,11,12]. The data also show that skin alteration caused by high-voltage pulsing is related to the electrical protocol. The higher the pulse voltage, time constant and/or number, the higher the prolonged increase in skin permeability. Long-lived changes in skin permeability could open new opportunities in transdermal delivery of drugs: electroporation might be used as long-acting penetration enhancer for sustained transdermal drug delivery by passive diffusion.

4. Conclusion

Electroporation of skin has recently been shown to enhance and control the transport of charged molecules across skin. This study provides the first demonstration that high-voltage pulses can increase and control the transdermal permeation of neutral molecules as well. The elevation in transdermal flux resulted from the prolonged permeabilization of skin induced by electroporation. No electro-osmosis emerged.

Acknowledgements

We thank Equibio for lending the electroporation device. This work was supported in part by the Fonds National de la Recherche Scientifique (FNRS, Belgium). Prof. V. Préat is a Senior Research Associate of the FNRS (Belgium).

References

[1] D.C. Chang, B.M. Chassy, J.A. Saunders, A. Sowers (Eds.), Guide to Electroporation and Electrofusion, Academic Press, New York, 1992.
[2] J.C. Weaver, Electroporation theory: concepts and mechanisms, in: J.A. Nickoloff (Ed.), Molecular Biology Methods, 1995, pp. 3–28.
[3] M.R. Prausnitz, V.G. Bose, R. Langer, J.C. Weaver, Electroporation of mammalian skin: a mechanism to enhance transdermal drug delivery, Proc. Natl. Acad. Sci. USA 90 (1993) 10504–10508.
[4] R. Vanbever, N. Lecouturier, V. Préat, Transdermal delivery of metoprolol by electroporation, Pharm. Res. 11 (1994) 1657–1662.
[5] R. Vanbever, E. Le Boulengé, V. Préat, Transdermal delivery of fentanyl by electroporation I. Influence of electrical factors, Pharm. Res. 13 (1996) 559–565.
[6] R. Vanbever, N. De Morre, V. Préat, Transdermal delivery of fentanyl by electroporation II. Mechanisms involved in drug transport, Pharm. Res. 13 (1996) 1360–1366.
[7] M.R. Prausnitz, Electroporation, in: B. Berner, S.M. Dinh (Eds.), Electronically Controlled Drug Delivery, CRC Press, Boca Raton, FL, (in press).
[8] U. Pliquett, T.E. Zewert, T. Chen, R. Langer, J.C. Weaver, Imaging of fluorescent molecule and small ion transport through human stratum corneum during high voltage pulsing: localized transport regions are involved, Biophys. Chem. 58 (1996) 185–204.
[9] U. Pliquett, J.C. Weaver, Electroporation of human skin: simultaneous measurement of changes in the transport of two fluorescent molecules and the passive electrical properties, Bioelectrochem. Bioenerg. 39 (1996) 1–12.
[10] D. Bommannan, J. Tamada, L. Leung, R.O. Potts, Effect of electroporation on transdermal iontophoretic delivery of luteinizing hormone releasing hormone (LHRH) in vitro, Pharm. Res. 11 (1994) 1809–1814.
[11] T. Zewert, U. Pliquett, R. Langer, J.C. Weaver, Transdermal transport of DNA antisense oligonucleotides by electroporation, Biochem. Biophys. Acta. 212(2) (1995) 286–292.
[12] M.R. Prausnitz, E.R. Edelman, J.A. Gimm, R. Langer, J.C. Weaver, Transdermal delivery of heparin by skin electroporation, Biotechnology 13 (1995) 1205–1209.
[13] A. Kim, P.G. Green, G. Rao, R.H. Guy, Convective solvent flow across the skin during iontophoresis, Pharm. Res. 10 (1993) 1315–1320.

[14] M.B. Delgado-Charro, A.M. Rodriguez-Bayon, R.H. Guy, Iontophoresis of nafarelin: effects of current density and concentration on electrotransport in vitro, J. Control. Release 35 (1995) 35–40.
[15] S. Thysman, C. Tasset, V. Préat, Transdermal iontophoresis of fentanyl: delivery and mechanistic analysis, Int. J. Pharm. 101 (1994) 105–113.
[16] K. Randerath, Chromatographie sur couches minces, Gauthier-Villars, Paris, 1964.
[17] M.J. Pikal, The role of electroosmotic flow in transdermal iontophoresis, Adv. Drug. Deliv. Rev. 9 (1992) 201–237.
[18] M.R. Prausnitz, Do high-voltage pulses cause changes in skin structure?, J. Control. Release 40 (1996) 321–326.
[19] U. Pliquett, R. Langer, J.C. Weaver, Changes in the passive electrical properties of human stratum corneum due to electroporation, Biochim. Biophys. Acta. 1239 (1995) 111–121.
[20] C. Morgan, Chemometrics: Experimental Design, John Wiley, 1991.
[21] M.R. Prausnitz, C.D. Milano, J.A. Gimm, R. Langer, J.C. Weaver, Quantitative study of molecular transport due to electroporation: uptake of bovine serum albumin by erythrocytes ghosts, Biophys. J. 66 (1994) 1522–1530.
[22] M.P. Rols, J. Teissié, Electropermeabilization of mammalian cells: quantitative analysis of the phenomenon, Biophys. J. 58 (1990) 1089–1098.
[23] Systat Inc., Statistics, Version 5.2 Edition, Systat Inc., Evanston, IL, 1992, 724 p.