Iontophoretic delivery of 5-aminolevulinic acid (ALA): effect of pH

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Iontophoretic Delivery of 5-Aminolevulinic Acid (ALA): Effect of pH

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Received November 13, 2000; accepted December 12, 2000

Purpose. To examine the iontophoretic delivery of ALA as a function of pH and to determine the principal mechanisms responsible for its electrotransport.

Methods. Anodal iontophoretic transport of ALA was measured as a function of its concentration and pH of the donor solution. Experiments were performed *in vitro* using skin excised from porcine ears as the membrane. To deduce mechanism, the concomitant transport of the electroosmotic marker, mannitol, was also assessed.

Results. ALA iontophoresis at pH 7.4 is a linear function of concentration over the range 1–100 mM. The mechanism was deduced to be electroosmosis. By reducing the pH from 7.4 to 4.0, the dominant mechanism of ALA transport was shifted from electroosmosis to electrorepulsion as the skin's net negative charge was progressively neutralized. However, the total delivery of the compound was not altered by lowering the pH suggesting that the increased electrorepulsive contribution was essentially balanced by the concomitantly reduced electroosmosis.

Conclusions. Significant ALA delivery at pH 7.4 can be achieved by increasing the drug concentration in the anodal formulation to 100 mM. Lowering the pH does not result in increased ALA transport. Alternative strategies are therefore required to maximize and optimize ALA delivery by iontophoresis.

KEY WORDS: iontophoresis; photodynamic therapy; 5-aminolevulinic acid; electroosmosis; pH.

INTRODUCTION

Skin cancer is the most common of all cancers. The American Cancer Society reports about 1.3 million cases of non-melanoma skin cancer each year resulting, it is predicted, in about 1,900 deaths this year. Fortunately, most basal cell (BCC) and squamous cell (SCC) carcinomas, which are the most common non-melanoma skin cancers, can be cured. However, the conventional methods used to treat these cancers, viz. surgery, radiotherapy, chemotherapy and electrodissection, elicit undesirable effects, including pain and scarring. Photodynamic therapy (PDT) represents a new and hopefully better tolerated approach for the treatment of this problem.

PDT is a "simple" therapy which destroys malignant cells by inducing cytotoxic reactions subsequent to the interaction of light with a photosensitive compound. The latter is administered to the patient by one of several possible routes and is allowed to accumulate in the target tissue (1). PDT, associated with the topical application of 5-aminolevulinic acid (ALA), is an experimental treatment for skin cancer that is under investigation in many laboratories (2–4).

ALA (Figure 1) is not itself a sensitizer; rather, it is an endogenous porphyrin precursor that stimulates the synthesis of the photosensitizer protoporphyrin IX (PpIX) in the pathway of heme biosynthesis. The exogenous ALA by-passes the regulatory step of the heme cycle and can therefore result in a temporary accumulation of PpIX, particularly in cells with higher metabolic turnover (5). A complete destruction of a tumor by PDT critically depends on a sufficiently high concentration and homogeneous distribution of PpIX in the malignant tissue. Cairnduff et al. (6) successfully treated areas of Bowen's disease with PDT using ALA but the treatment of BCCs was less successful. Fritsch et al. (4) showed by fluorescence microscopy that the major factor limiting the efficacy of topical ALA-PDT is the penetration depth of ALA and the resulting ALA-induced porphyrins, which are in turn dependent on the incubation time of the tissue with ALA. Recently, De Rosa et al. (7) enhanced the penetration of ALA through hairless mouse skin *in vitro* with a vehicle containing dimethylsulphoxide and ethylenediaminetetraacetic acid disodium salt. They also observed, by confocal microscopy, an increase in the production and accumulation of PpIX. However, in all previous work, high ALA concentrations were used (typically > 1 M) and it was necessary to wait 3–6 hours post-ALA application to obtain maximal PpIX fluorescence.

Hence, despite the fact that ALA can permeate across the skin's barrier, the local bioavailability of the drug is normally insufficient for a complete therapeutic effect. We have therefore decided to examine the potential of iontophoresis for the enhancement of ALA delivery, and hence to obtain a better anti-tumor action with PDT. At pH 7.4 ALA is essentially zwitterionic and, given that the skin, at physiological pH, supports a net negative charge, the iontophoretic delivery of ALA at pH 7.4 can be anticipated to occur primarily via electroosmosis (8). With pK_a values of 4.0 and 8.4, it is clear that formulation of ALA at a lower pH would positively ionize the drug and offer the opportunity to take advantage of the skin's cation permselectivity (9). It should be said that the use of iontophoresis to improve ALA delivery has already been examined by Rhodes et al. (10), who found that PpIX production was related to the intensity of the current applied to the ALA "donor" formulation. However, this earlier investigation did not examine the manner in which the ALA formulation (specifically, the pH) may alter drug delivery, nor did it address the mechanism of electrotransport of this "prodrug". The object of this work, therefore, was to examine the iontophoretic delivery of ALA as a function of pH and to determine the principal mechanisms responsible for its electrotransport.

MATERIALS AND METHODS

Chemicals

5-aminolevulinic acid, N-2-Hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES) and D-mannitol were ob-

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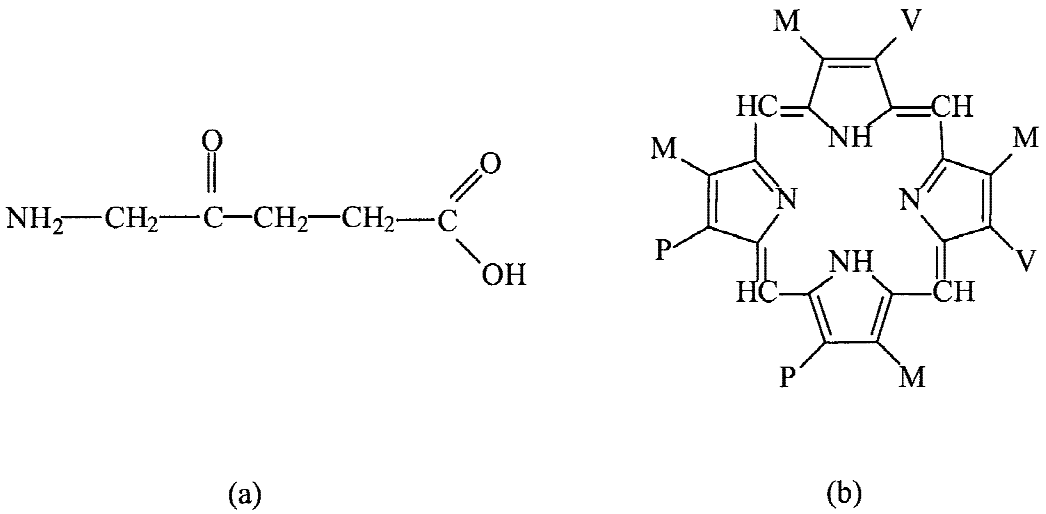

Fig. 1. Chemical structure of (a) ALA and (b) PpIX (M, methyl; P, propionate; V, vinyl).

tained from Sigma-Aldrich (Saint Quentin Fallavier, France). ^{14}C-Mannitol (56 mCi/mmol) was purchased from Amersham (Freiburg, Germany) and ^{3}H-ALA (0.5–3.0 mCi/mmol) was obtained from NEN (Paris, France). All other chemicals were analytical grade. Deionized water (resistivity $\geq$ 18 MΩcm) was used to prepare all solutions.

Skin

Dermatomed skin (~700 μm) from pigs' ears was obtained less than 2 hours after slaughter of the animal (Société d'Exploitation d'Abbatage, Annecy, France) and stored frozen for a maximum of 7 days before use.

Apparatus

Flux measurements were made *in vitro* using vertical, flow-through diffusion cells (Laboratory Glass Apparatus, Berkeley, CA) which have been previously described (11). The area of skin exposed in each electrode chamber was 0.8 cm^2. Ag/AgCl electrodes were prepared in the usual manner (12) and a constant current was passed between the electrodes from a custom-built power supply (Professional Design and Development Services, Berkley, CA) interfaced to a personal computer running Labview software (National Instruments Inc., Austin, TX).

Iontophoresis Experiments

Two sets of experiments were performed. In the first series, ALA delivery by iontophoresis was measured as a function of concentration at pH 7.4. ALA transport from the anode compartment was followed over a period of 6 hours at a constant current of 0.5 mA/cm^2. The ALA formulation comprised a solution of the drug at 1, 15, 30 or 100 mM, "spiked" with ^{3}H-ALA (~1 μCi/ml), in a physiological buffer (133 mM NaCl, 25 mM HEPES) at pH 7.4. The cathodal and receptor chamber of the diffusion cell contained simply the buffer alone (HEPES-buffered NaCl) at pH 7.4. The receptor was perfused continuously at 2 mL/h, and samples were collected automatically every hour. At the end of the experiment, 5 ml of scintillation cocktail (Ultima Gold XR, Packard BioScience, Groningen, Netherlands) were added to each sample and transported drug was determined by measuring radioactivity in a liquid scintillation counter (Beckman LS 6500, Beckman Instruments Inc., Fullerton, CA). The disintegrations per minute were converted to molar flux by an appropriate mathematical transformation. "Passive" experiments were also performed with a donor solution containing 15 mM ALA. All conditions were identical to those described above except that no current was applied.

In the second series of experiments, anodal iontophoretic transport of ALA was measured at a fixed concentration (15 mM) from donor solutions at three different pH values (7.4, 5.5 and 4.0). However, the pH of the cathodal and receptor solutions was maintained at 7.4. The same current conditions and background electrolyte (HEPES-buffered NaCl) were employed as before and the ALA solution was again labelled with ^{3}H-ALA (~1 μCi/ml). In addition, in order that electroosmotic and electrorepulsive contributions to ALA delivery could be distinguished, the ALA formulation also included the electroosmosis marker, mannitol, dissolved at a concentration of 15 mM and "spiked" with the ^{14}C-labelled compound (~1 μCi/ml). Receptor phase samples, in this case, were assayed for both ^{3}H and ^{14}C, therefore. At the end of each experiment, the absence of significant pH changes in the electrode solutions was verified.

Statistics

The results were expressed as the mean ± standard deviation. Statistical comparisons between the ALA flux at different pH values were made using a one-way analysis of variance followed by the Student-Newman-Keuls Method ($p < 0.05$). Statistical comparison between ALA and mannitol flux at pH 7.4 used the t-test. In the first series of iontophoretic experiments, a linear correlation between ALA flux and the applied concentration was characterized by the regression coefficient.

RESULTS AND DISCUSSION

First, it should be noted that, in separate experiments without radiolabled ALA, we verified using a HPLC assay (13) that the iontophoretic transport at pH 7.4 was identical to

that determined by liquid scintillation counting (results not shown). Furthermore, in addition, we confirmed the stability of ALA when subjected either to 8 hours of passive diffusion or 8 hours of electrotransport (0.4 mA at pH 7.4). The results in Figure 2 demonstrate the stability of ALA and confirm that our measurements of radioactivity corresponded to measurements of the compound itself.

Figure 3 shows the passive and the anodal iontophoretic transport of ALA, after 6 hours, from a 15 mM donor solution at pH 7.4. Anodic iontophoresis of the zwitterionic ALA caused a significant (6-fold) enhancement over the passive flux. In a recent publication, De Rosa et al. (7) also improved passive ALA penetration (through hairless mouse skin) by including DMSO in the formulation. However, it was found that 20% DMSO in an oil-in-water emulsion only doubled the flux of ALA (relative to the emulsion without the penetration enhancer) after 24 hours. It follows that iontophoresis is a more efficient approach with which to increase ALA transport. It should also be said that (i) the use of DMSO may not be acceptable in man, and (ii) the enhancing effect of 20% DMSO on hairless mouse skin can be expected to be greater than that on human skin (14).

The iontophoretic flux of ALA at pH 7.4, as a function of the initial concentration is shown in Figure 4. ALA transport increased linearly with drug concentration over the range 1 to 100 mM. In terms of the potential clinical relevance of the iontophoretic delivery observed, it should be noted that De Rosa et al. (7) applied a 1.5% (90 mM) solution of ALA containing 20% DMSO and measured a flux of less than 20 nmol/cm^{2}h. In addition, the same authors showed that increasing the ALA concentration to 10% elicited the function of PpIX *in vivo* (7). Figure 4 shows that iontophoresis delivery ALA from a 100 mM (1.7%) solution at more than 200 nmol/cm^2 h. Clearly, (a) it would be straightforward to increase this flux proportionally by increasing the concentration of ALA in the donnor anode formulation, and (b) given the results of De Rosa et al. (7), the fluxes achieved would be expected to result in the clinically relevant production of PpIX.

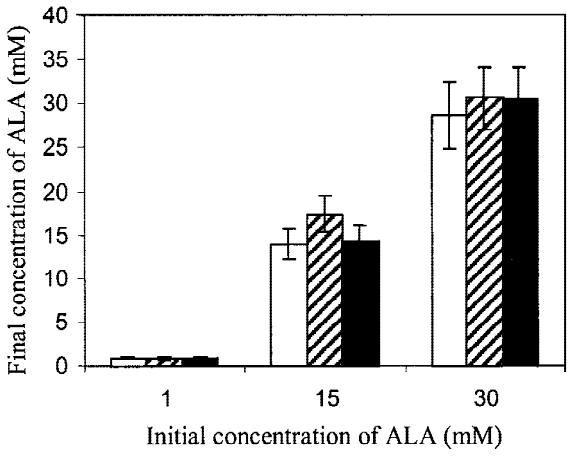

Fig. 2. Stability of ALA as a function of initial donor concentration at pH 7.4 following 8 hours of either passive diffusion (hatched bars) or iontophoretic transport (0.4 mA) (filled bars), compared to the "pre-treatment" control (0 h) (open bars). The results represents the mean ± SD of 4 replicates. ANOVA reveals no statistical difference between the results at any concentration.

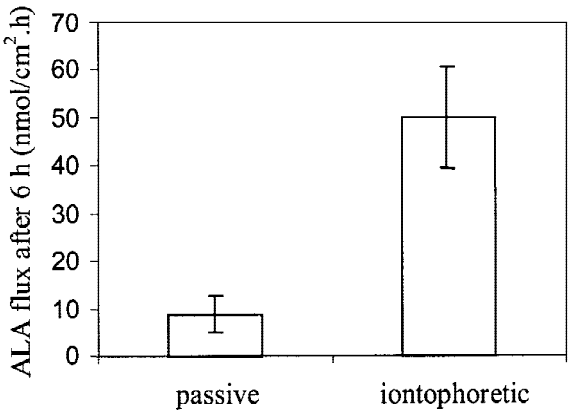

Fig. 3. Comparison between the passive and anodal iontophoretic fluxes of ALA from a 15 mM donor solution at pH 7.4 (mean ± SD; n ≥ 3).

As mentioned above, given the primarily zwitterionic nature of ALA at pH 7.4, the mechanism of electrotransport was expected to be electroosmosis. To confirm this prediction, the simultaneous delivery of mannitol, a classic marker of convective flow (9), was assessed. We know that these compounds are both highly water-soluble and polar, and of similar molecular size (molecular weights of ALA and mannitol are 168 and 180, respectively). Figure 5 demonstrates that mannitol and ALA, when present together in the anodal chamber at the same concentration (15 mM), are "delivered" at the same rate, a result completely consistent with their electroosmotic transport (given that the flux via electroosmosis equals the product of solvent velocity and permeant concentration). This finding, of course, completely agrees with similar observations in the literature, for example, (i) the reverse iontophoretic extraction of phenylalanine, a zwitterionic amino acid (net charge = 0 at pH 7.4) is a linear function of its subdermal concentration (15) and (ii) reproducibility and linearity of the glucose extraction process by reverse iontophoresis (16).

The second series of experiments, performed at lower pH values, were designed to explore whether ALA delivery could be improved by increasing the fraction of drug present

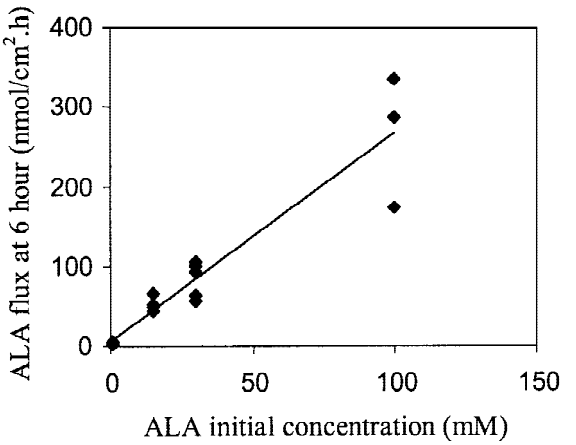

Fig. 4. Anodal ALA flux, after 6 hours of current passage (0.5 mA/cm^2), as a function of the initial donor concentration at pH 7.4. The line of linear regression ($y = 4.7 + 2.6x$) through the data is shown ($r^2 = 0.900$; $F = 182$; $p < 0.001$).

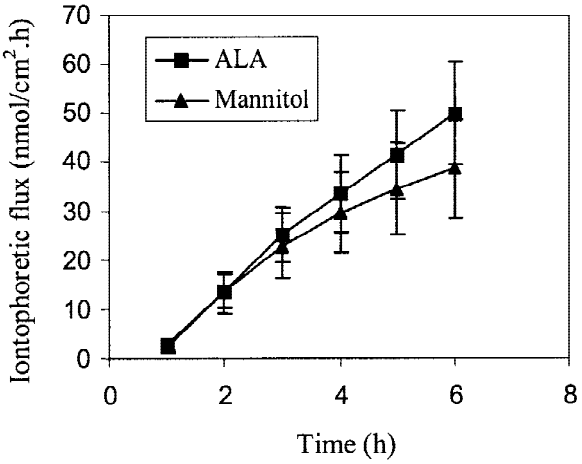

Fig. 5. Anodal iontophoretic flux of ALA and mannitol, as a function of time, across pig ear skin *in vitro* at pH 7.4. The initial donor concentration of each molecule was 15 mM. Each data point represents the mean (±SD) of six determinations.

in cationic form. This strategy has, of course, been used to enhance the transport of other compounds, such as lignocaine, histidine, different peptides, and so on (17–20). To separate electrorepulsive and electroosmotic contributions to ALA delivery at the lower pH values, mannitol was again incorporated into the anodal formulations (at the same, 15 mM, concentration as ALA).

At pH 4.0, it can be calculated that ALA is about 50% in the protonated form and that an important electrorepulsive flux should be evident. However, the results in Figure 6 show that there is no significant difference between total ALA delivery at pH 4.0 and pH 7.4. The reason for this similarity is revealed by the mannitol transport results at pH 4.0 (also shown in Figure 6). It is observed that, compared to pH 7.4, mannitol electrotransport, that is electroosmosis, is dramatically reduced (by about an order of magnitude). Thus, for ALA, it is clear that while electrorepulsive transport at pH 4.0 has become an important, and indeed dominant, contribution, the electroosmotic component has fallen to a very low level.

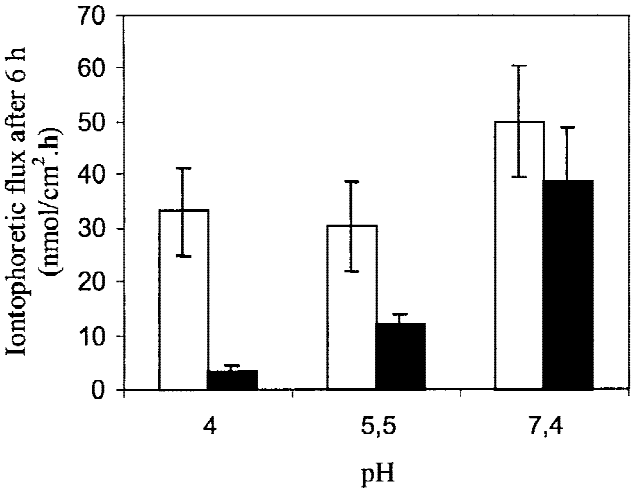

Fig. 6. Anodal iontophoretic fluxes of ALA (open bars) and mannitol (filled bars), from a donor solution in which both were initially present at 15 mM, as a function of pH.

The explanation lies, of course, in the fact that lowering the pH from 7.4 to 4.0 "neutralizes" much of the negative charge on the skin present under physiological conditions. This net charge, in turn, is at the origin of the normal anode-to-cathode convective flow across the membrane - without the charge, there is no electroosmosis.

This conclusion is well-supported by the literature (19, 21–22) (and also by the intermediate results seen at pH 5.5 - Figure 6), including recent experiments which have determined, in slightly different ways, the isoelectric point (pI) of the porcine skin model used here (23–24). This parameter, when measured under 'symetrical' conditions (i.e., the somewhat artificial situation in which the pH of solutions on both sides of the membrane are systematically changed), is in the range of 4.0–4.5. That is, at pH values in this region, the skin becomes a membrane with a net charge of zero across which, therefore, little or no convective flow will be observed.

A simple calculation shows that, at pH 4.0, ALA in its cationic form transports less than 0.2% of the total charge measured across the skin in one hour. The majority of the charge, of course, is being carried by other, more mobile, ions in the system, including H_3O^+, Na^+ and Cl^-. To improve the iontophoretic delivery of ALA, therefore, one might envisage, on the one hand, formulations at pH 3, where more than 99% of the molecule will be cationic and, on the other, reducing as far as possible the "competing" cations in the anodal chamber. However, the extent to which this will be possible remains unknown and a number of potential problems are foreseeable, including: (i) a formulation at pH 3 by definition incorporates a significant concentration of H_3O^+, a highly efficient and competitive charge carrier, (ii) the skin at pH 3 will support a net positive charge and convective flow will oppose the direction of ALA delivery, and (iii) prolonged contact between the skin and an acidic formulation may provoke unacceptable irritation.

CONCLUSION

Significant ALA delivery at pH 7.4 can be achieved by increasing the drug's concentration in the anodal formulation to 100 mM. The mechanism of delivery is almost exclusively electroosmotic. Lowering the pH to increase the fraction of the drug in cationic form, while maintaining all other variables constant, does not result in increased ALA transport because the improved electrorepulsive component is offset by the reduced electroosmotic flow as the skin loses its net negative charge. Alternative strategies are therefore required to maximize and optimize ALA delivery by iontophoresis.

ACKNOWLEDGMENTS

This work was supported in part by Fundação Coordenaç ão de Aperfeiçoamento de Pessoal de Nível Superior - CAPES grant (Brasil), and by the Fonds National Suisse de la Recherche Scientifique.

REFERENCES

1. A. Marti, N. Lange, H. Van den Bergh, D. Sedmera, P. Jichlinski, and K. Kucera. Optimization of the formation and distribution of protoporphyrin IX in the urothelium: an in vitro approach. *J. Urology* **162**:546–552 (1999).
2. K. Svanberg, T. Anderson, D. Killander, I. Wang, U. Stenram, S. Andersson-Engels, R. Berg, J. Johansson, and S. Svanberg. Pho-

todynamic therapy of non-melanoma malignant tumours of the skin using topical δ-amino levulinic acid sensitization and laser irradiation. *Br. J. Dermatol*. **130**:743–751 (1994).
3. S. Lang, R. Baumgartner, R. Struck, A. Leunig, R. Gutmann, and J. Feyh. Photodynamic diagnosis and therapy of neoplasms of the facial skin after topical administration of 5-aminolevulinic acid. *Larynorhinootologie* **74**:85–89 (1995).
4. C. Fritsch, P. Lehmann, W. Stahl, K. W. Schulte, E. Blohm, K. Lang, H. Sies, and T. Ruzicka. Optimum porphyrin accumulation in epithelial skin tumours and psoriatic lesions after topical application of γ-aminolaevulinic acid. *Br. J. Cancer* **79**:1603–1608 (1999).
5. J. C. Kennedy, R. H. Pottier, and D. C. Pross. Photodynamic therapy with endogenous protoporphyrin IX: Basic principles and present clinical experience. *J. Photochem. Photobiol. B.* **6**: 143–148 (1990).
6. F. Cairnduff, M. Stringer, E. Hudson, D. Ashand, and S. Brown. Superficial photodynamic therapy with topical 5-aminolevulinic acid for superficial primary and secondary skin cancer. *Br. J. Cancer* **69**:605–608 (1994).
7. F. S. De Rosa, J. M. Marchetti, J. A. Thomazini, A. C. Tedesco, and M. V. L. B. Bentley. A vehicle for photodynamic therapy of skin cancer: Influence of dimethylsulphoxide on 5-aminolevulinic acid in vitro cutaneous permeation and in vivo protoporphyrin IX accumulation determined by confocal microscopy. *J. Control. Release* **65**:359–366 (2000)
8. A. Kim, P. G. Green, G. Rao, and R. H. Guy. Convective solvent flow across the skin during iontophoresis. *Pharm. Res*. **10**:1315–1320 (1993).
9. R. R. Burnette and B. Ongpipattanakul. Caracterization on the permeselective properties of excised human skin during iontophoresis, *J. Pharm. Sci*. **76**:765 (1987).
10. L. E. Rhodes, M. M. Tsoukas, R. R. Anderson, and N. Kollias. Iontophoretic delivery of ALA provides a quantitative model for ALA pharmacokinetics and PpIX phototoxicity in human skin. *J. Inv. Dermatol.* **108**:87–91 (1997).
11. P. Glikfeld, C. Cullander, R. S. Hinz, and R. H. Guy. A new system for in vitro studies of iontophoresis. *Pharm. Res.* **5**:443–446 (1988).
12. P. G. Green, R. S. Hinz, C. Cullander, G. Yamane, and R. H. Guy. Iontophoretic delivery of amino acids and amino acid derivatives across the skin in vitro. *Pharm. Res*. **8**:1113–1120 (1991).
13. H. Oishi, H. Nomiyama, K. Nomiyama, and K. Tomokuni. Fluometric HPLC determination of δ-aminolevulinic acid (ALA) in the plasma and urine of lead workers: Biological indicators of lead exposure. *J. Anal. Toxicol.* **20**:106–110 (1996).
14. R. C. Wester and H. I. Maibach. In vivo animal models for percutaneous absorption. In R. L. Bronaugh and H. I. Maibach (eds.), *Percutaneous Absorption,* Marcel Dekker, Inc, New York and Basel, 1989 pp. 221–238.
15. V. Merino, A. López, D. Hochstrasser, and R. H. Guy. Noninvasive sampling of phenylalanine by reverse iontophoresis. *J. Control. Release* **61**:65–69 (1999).
16. G. Rao, P. Glikfeld, and R. H. Guy. Reverse iontophoresis development of a noninvasive approach for glucose monitoring. *Pharm. Res*. **10**:1751–1755 (1993).
17. O. Siddiqui, M. S. Roberts, and A. E. Polack. The effect of iontophoresis and vehicle pH on the in-vitro permeation of lignocaine through human stratum corneum. *J. Pharm. Pharmacol*. **37**:732–735 (1985).
18. P. G. Green, R. S. Hinz, A. Kim, F. C. Szoka, Jr., and R. H. Guy. Iontophoretic delivery of a series of tripeptides across the skin in vitro. *Pharm. Res*. **8**:1121–1127 (1991).
19. R. R. Burnette and D. Marrero. Comparison between the iontophoretic and passive transport of thyrotropin releasing hormone across excised nude mouse skin. *J. Pharm. Sci*. **75**:738–743 (1986).
20. J. B. Phipps and J. R. Gyory. Transdermal ion migration. *Adv. Drug Del. Rev*. **9**:137–176 (1992).
21. M.J. Pikal The role of electroosmotic flow in transdermal iontophoresis. *Adv. Drug Del. Rev*. **9**:201–237 (1992).
22. M. B. Delgado-Charro and R. H. Guy. Characterization of convective solvent flow during iontophoresis. *Pharm. Res*. **11**:929–935 (1994).
23. V. Merino, A. López, Y. N. Kalia, and R. H. Guy. Electrorepulsion versus electroosmosis: effect of pH on the iontophoretic flux of 5-fluorouracil. *Pharm. Res*. **16**:758–761 (1999).
24. D. Marro, R. H. Guy, and M. B. Delgado-Charro. Characterization of the iontophoretic permselectivity properties of human and pig skin. *J Control. Release* **70**:213–217 (2001).