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# Lipid extraction and iontophoretic transport of leuprolide acetate through porcine epidermis

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**Abstract**

The purpose of this study was to explore the effect of lipid extraction by the simple alkyl acetates of increasing carbon chain lengths (e.g. methyl, ethyl, propyl, butyl, pentyl, hexyl, and octyl acetates) and iontophoresis on the in-vitro transport of leuprolide acetate through porcine epidermis. The extent of lipid extraction from the stratum corneum (SC) by alkyl acetates was studied by Fourier transform infrared (FT-IR) spectroscopy. Ethyl, propyl, pentyl, hexyl, and octyl acetates significantly increased ($P < 0.05$) the permeability of leuprolide acetate through the epidermis in comparison to the control (epidermis without alkyl acetate treatment). Iontophoresis further increased ($P < 0.05$) the permeability of leuprolide acetate for all the alkyl acetates studied, when compared to their corresponding passive permeability. Ethyl acetate produced the maximum passive (13.47 μg/cm$^2$/h) and iontophoretic (89.79 μg/cm$^2$/h) flux among all the alkyl acetates studied. The SC treated with alkyl acetates showed a decrease in peak heights and areas of asymmetric and symmetric C–H stretching absorbances in comparison to untreated SC. A greater percentage decrease in peak heights and areas was obtained by ethyl acetate. Chloroform:methanol(2:1) [C:M(2:1)] was used as a positive control for lipid extraction. Our findings provide evidence that alkyl acetates cause lipid extraction, which leads to an enhancement in the passive and iontophoretic permeability of leuprolide acetate.


*Keywords:* Iontophoresis; Lipid extraction; Leuprolide acetate; Permeability; Porcine skin

## 1. Introduction

The primary barrier to transdermal diffusion is the stratum corneum (SC) that is composed of a regular array of protein-rich cells embedded in a multilamellar lipid domain. The lipoid domain is an integral component of the transport barrier (Sweeny and Downing, 1970). Proteins and peptides, because of their hydrophilic nature and large molecular size, have a limited permeability in the skin.

Leuprolide acetate is a potent luteinizing hormone-releasing hormone (LHRH) agonist. It is used to treat many diseases, including prostate cancer, endometriosis, precocious puberty and metastatic breast cancer (Adjei and Garren, 1990).

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Leuprolide acetate is mostly ionized across a wide pH range of physiological interest, and it is not absorbed orally (Adjei and Hsu, 1993). This makes it a potential agent to be administered transdermally.

Possible routes of solute transport through the skin during iontophoresis are: (a) transappendageal, which involves following a path within sweat ducts and hair follicles, completely avoiding the intercellular lipid bilayers; (b) transcellular, which involves transport directly across the bulk of the SC, where a solute ion sequentially crosses keratinocytes; and (c) inter- or paracellular, which involves the movement of solute ions through the lipid pathways between the cells.

Literature supports the existence of intercellular and trans-appendageal routes and suggests the transcellular route as unlikely and speculative. Recently, Monteiro-Riviere et al. (1994) examined the ultrastructural patterns of staining of mercuric chloride in pig skin following iontophoresis. The electron-micrograph clearly revealed that mercuric chloride traversed the SC via an intercellular route. The authors concluded that the predominant pathway for percutaneous absorption is the intercellular route.

Scheuplein (1965) have suggested that appendageal transport is important only during the initial stages of percutaneous penetration. The visualization of fluorescent ions using confocal microscopy as they permeate through the skin has also demonstrated the presence of charged polar ions in intercellular and follicular spaces during iontophoretic permeation through mouse skin (Cullander and Guy, 1991a). Cullander and Guy (1991b), using a vibrating probe electrode, have detected the movement of ions in skin at locations where no appendageal structures were apparent, further suggesting a role of intercellular transport through the skin during iontophoresis.

With so much evidence to support that iontophoretic transport involves intercellular lipoidal pathways, it would be reasonable to hypothesize that removal of the intercellular lipids will help decrease the SC barrier resistance and increase passive and iontophoretic transport of solutes.

Many strategies have been suggested to overcome skin impermeability, such as iontophoresis and the use of penetration enhancers (Smith and Maibach, 1995; Bhatia and Singh, 1998). A synergism of iontophoresis and ethanol as enhancer was reported on the transport of peptides through human epidermis (Srinivasan et al., 1990). Iontophoresis in combination with chemical enhancers enhanced the transport of peptides by increasing lipid extraction and loosening and swelling of cell layers of the porcine SC (Bhatia et al., 1997a,b; Bhatia and Singh, 1998).

Recent reports suggest a correlation between enhanced permeation of a polar solute (mannitol) and extraction of lipids from human skin in the presence of 75% (v/v) aqueous ethanol solution (Goates and Knutson, 1994). Decreases in the absorbances and the areas of C–H stretching peaks under the FT-IR spectrum of SC have been linked to the extraction of SC lipids (Bommannan et al., 1991). Extraction of the SC lipids with C:M(2:1) results in the reduction of the C–H stretching absorbances (Casal and Manstsch, 1984). Therefore, C:M(2:1) can be used as a positive control for lipid extraction. In this study, we investigated the effect of lipid extraction by alkyl acetates of increasing carbon chain-lengths (methyl, ethyl, propyl, butyl, pentyl, hexyl, and octyl acetates), and iontophoresis on the transport of leuprolide acetate through porcine epidermis. The extent of lipid extraction of the SC by above alkyl acetates was studied by Fourier transform infrared spectroscopy (FT-IR).

## 2. Materials and methods

### 2.1. Materials

Leuprolide acetate was a gift sample from TAP Pharmaceuticals Inc. (Deerfield, IL). Ethyl acetate and butyl acetate were purchased from Sigma Chemical Co. (St. Louis, MO). Propyl acetate, hexyl acetate and octyl acetate were obtained from Aldrich Chemical Company (Milwaukee, WI). Methyl acetate, pentyl acetate and HPLC grade acetonitrile were purchased from Fisher Scientific (Pittsburgh, PA). All other chemicals and reagents used were of analytical grade. Deionized water (Resistivity $\geq 18$ MΩ-cm) was used to prepare all solutions and buffers.

### 2.2. Preparation of epidermis

Porcine ear skin was used in the in-vitro transport studies. The ranking of skin permeability of different species in vitro have been determined by several investigators (Tregear, 1966; Marzulli et al., 1969; Wester and Maibach, 1989). Increasing evidence supports the contention that in-vitro permeability studies can accurately predict in-vivo absorption (Bronaugh, 1989). Skin from the pig generally approximates the permeability of human skin (Monteiro-Riviere, 1986, 2000; Bhatia and Singh, 1996; Monteiro-Riviere and Riviere, 1996). Porcine ears were obtained from a local slaughterhouse. Epidermal membranes were prepared by the heat-separation technique (Kligman and Christophers, 1963; William and Barry, 1991). The whole skin was soaked in water at 60°C for 45 s, followed by careful removal of the epidermis. The epidermis was then washed with water and used in the in-vitro transport studies.

### 2.3. Preparation of stratum corneum (SC)

The epidermis was incubated for 4 h in a 0.5% trypsin solution in phosphate-buffered saline (pH 7.4) at 37°C. The tissue was then smoothed out on a flat surface and the mushy epidermis removed by rubbing with a moistened cotton tipped applicator. The transparent SC obtained was briefly floated on water and lifted out onto aluminum foil, blotted dry, and used in FT-IR spectroscopic studies.

### 2.4. Fourier transform infrared (FT-IR) spectroscopy

After solvent treatment of the SC for 40 min, the samples were vacuum-dried (650 mmHg) at $21 \pm 1$°C for 3 days to evaporate the solvent (24). The samples were then subjected to FT-IR spectroscopic study. FT-IR (Nicolet 210, Nicolet Instrument Corporation, Madison, WI) was used to accomplish this study. For each SC sample, the peak heights and areas of C–H stretching absorbances were measured before and after the solvent treatment. This experimental strategy allowed each sample to serve as its own control. Attention was focused on characterizing the occurrence of peaks near 2848 and 2915/cm, which were due to the symmetric and asymmetric C–H stretching absorbances, respectively. OMNIC® FT-IR software (Nicolet Instrument Corporation, Madison, WI) was used to calculate the peak heights and areas of C–H stretching absorbances. FT-IR experiments with each condition were performed in triplicate.

### 2.5. In-vitro transport studies

Franz diffusion cells were used in the in-vitro transport studies. The solvent treated or untreated (control) epidermis was sandwiched between the cells with the SC facing the donor compartment. The maximum capacity of the donor and receiver compartments was 1 and 5 ml, respectively. The effective diffusional area was 0.785 $cm^2$. The donor compartment contained 1 ml solution (5 mg/ml) of leuprolide acetate in normal saline, and the receiver compartment was filled with 5 ml of normal saline. The cells were maintained at $37 \pm 0.5$°C by a PMC Dataplate® stirring digital dry block heater (Crown Bioscientific Inc., Somerville, NJ). Ag/AgCl electrodes and a Scepter™ iontophoretic power source were used in iontophoresis. Anodal iontophoresis was performed at 0.2 $mA/cm^2$ current density. The contents of the receiver compartment were stirred with the help of a magnetic bar at 100 revolution/min. At appropriate times, 0.5 ml samples were withdrawn from the receiver compartment. An equivalent amount of normal saline (0.5 ml) was added to the receiver compartment to maintain a constant volume. Experiments were performed in triplicate for each condition. The results were expressed as the mean $\pm$ S.D. of three experiments.

### 2.6. Assay of Leuprolide acetate

Each sample containing leuprolide acetate was analysed by high-performance liquid chromatography (HPLC) using a 220 nm UV detection, $C_{18}$ MICROSORB-MV™ column (4.6 mm $\times$ 15 cm), mobile phase (0.03 M, 70% dibasic ammonium phosphate buffer: 30% acetonitrile), and 2.0 ml/min flow rate (Singh et al., 2000). The detection

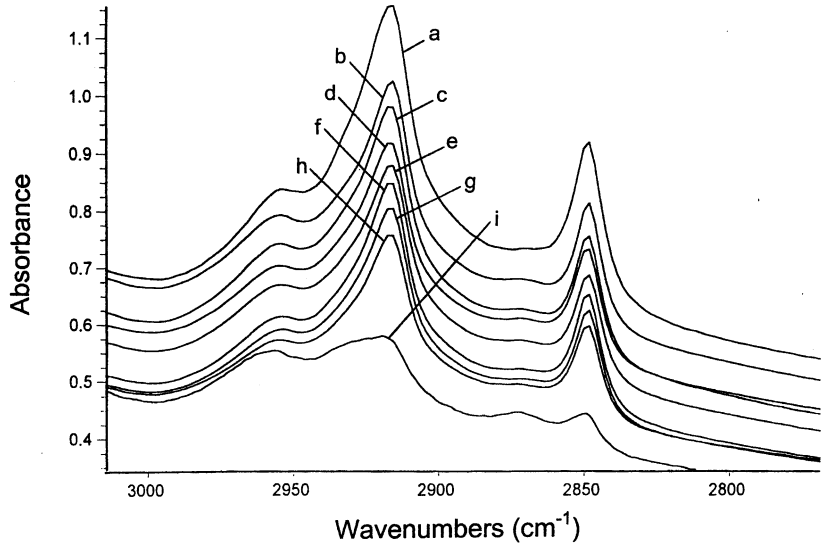


Fig. 1. FT-IR spectra (3000–2800/cm) of porcine stratum corneum. The SC samples were soaked in solvent in vials. After 40 min, the SC samples were taken out from the vials and vacuum-dried (650 mmHg) at 21 ± 1°C for 3 days. The samples were then subjected to FT-IR spectroscopic study. Key: (a) control; (b) methyl acetate; (c) ethyl acetate; (d) propyl acetate; (e) butyl acetate; (f) pentyl acetate; (g) hexyl acetate; (h) octyl acetate; (i) C:M(2:1).

limit for leuprolide acetate in this method, at a signal-to-noise ratio of 3:1, was 100 ng/ml.

### 2.7. Data treatment

The receiver compartment concentration of leuprolide acetate was corrected for sample removal (Hayton and Chen, 1982). The cumulative amount of leuprolide acetate permeated per unit skin surface area was plotted against time, and the slope of the linear portion of the plot was estimated as the steady state flux ($J_{ss}$). The permeability coefficient, $K_p$, was calculated as (Scheuplein, 1978):

$$K_p = \frac{J_{ss}}{C_v} \quad (1)$$

Table 1
Changes in symmetric and asymmetric C–H stretching absorbance peak heights after treatment with alkyl acetates[a]

| | Peak height (mean ± S.D., $n = 3$) | | | | | |
|---|---|---|---|---|---|---|
| | Asymmetric | | | Symmetric | | |
| Treatment | Control | Treatment | Percentage decrease | Control | Treatment | Percentage decrease |
| Methyl acetate | 0.349 ± 0.028 | 0.285 ± 0.011 | 18.07 | 0.164 ± 0.010 | 0.140 ± 0.003 | 14.35 |
| Ethyl acetate | 0.353 ± 0.005 | 0.248 ± 0.011 | 29.82 | 0.173 ± 0.002 | 0.130 ± 0.009 | 25.19 |
| Propyl acetate | 0.412 ± 0.009 | 0.318 ± 0.009 | 22.60 | 0.200 ± 0.017 | 0.164 ± 0.010 | 18.03 |
| Butyl acetate | 0.372 ± 0.010 | 0.298 ± 0.009 | 19.72 | 0.183 ± 0.009 | 0.145 ± 0.003 | 20.51 |
| Pentyl acetate | 0.353 ± 0.058 | 0.301 ± 0.035 | 14.06 | 0.178 ± 0.022 | 0.144 ± 0.006 | 18.11 |
| Hexyl acetate | 0.367 ± 0.013 | 0.306 ± 0.008 | 16.58 | 0.188 ± 0.012 | 0.149 ± 0.010 | 20.41 |
| Octyl acetate | 0.380 ± 0.011 | 0.282 ± 0.052 | 25.83 | 0.192 ± 0.032 | 0.150 ± 0.008 | 20.57 |
| C:M(2:1) | 0.271 ± 0.012 | 0.068 ± 0.037 | 74.77 | 0.158 ± 0.011 | 0.023 ± 0.010 | 85.58 |

[a] Percentage decrease = 100 − [(absorbance peak height due to treatment/absorbance peak height due to control) × 100].C:M(2:1) = chloroform:methanol (2:1).

Table 2
Changes in symmetric and asymmetric C–H stretching absorbance peak areas after treatment with alkyl acetates[a]

| Treatment | Peak area (mean ± S.D., $n = 3$) | | | | | |
|---|---|---|---|---|---|---|
| | Asymmetric | | | Symmetric | | |
| | Control | Treatment | Percentage decrease | Control | Treatment | Percentage decrease |
| Methyl acetate | 13.177 ± 1.019 | 10.833 ± 0.710 | 17.28 | 2.716 ± 0.101 | 2.283 ± 0.062 | 15.81 |
| Ethyl acetate | 12.774 ± 0.500 | 9.605 ± 0.564 | 24.63 | 2.914 ± 0.164 | 2.062 ± 0.233 | 28.79 |
| Propyl acetate | 15.915 ± 0.067 | 12.767 ± 0.453 | 19.78 | 3.183 ± 0.064 | 2.384 ± 0.167 | 25.14 |
| Butyl acetate | 13.839 ± 0.515 | 11.508 ± 0.091 | 16.78 | 2.944 ± 0.294 | 2.216 ± 0.341 | 25.02 |
| Pentyl acetate | 13.771 ± 2.139 | 12.120 ± 1.121 | 11.37 | 2.986 ± 0.334 | 2.195 ± 0.119 | 25.61 |
| Hexyl acetate | 13.590 ± 0.649 | 11.610 ± 0.236 | 14.40 | 3.128 ± 0.102 | 2.414 ± 0.199 | 22.64 |
| Octyl acetate | 14.052 ± 0.119 | 11.699 ± 0.387 | 16.76 | 3.194 ± 0.063 | 2.333 ± 0.303 | 26.98 |
| C:M(2:1) | 6.090 ± 0.212 | 1.117 ± 0.936 | 75.94 | 2.362 ± 0.620 | 0.226 ± 0.100 | 89.94 |

[a] Percentage decrease = 100 − [(absorbance peak area due to treatment/absorbance peak area due to control) × 100]. C:M(2:1) = chloroform:methanol (2:1).

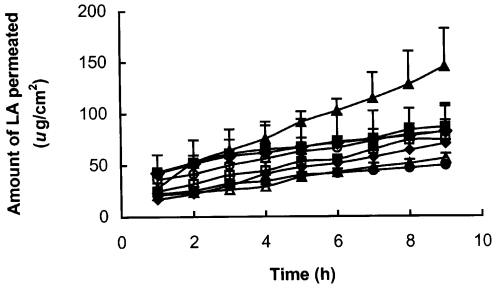


Fig. 2. Effect of alkyl acetate treatment on the in-vitro passive transport of leuprolide acetate through porcine epidermis. Each data point is the mean ± S.D. of three determinations. Key: (●) control; (○) methyl acetate; (▲) ethyl acetate; (△) propyl acetate; (■) butyl acetate; (□) pentyl acetate; (◆) hexyl acetate; (◇) octyl acetate.

where $C_v$ is the donor concentration of the solutes. Statistical comparisons were made using Student's $t$-test. The level of significance was taken as $P < 0.05$.

## 3. Results and discussion

Fig. 1 depicts the FT-IR spectra, and Tables 1 and 2 present the data derived from them. Decreases in peak heights and areas of the asymmetric and symmetric C–H stretching absorbances were observed showing an extent of lipid extraction. Lipid extraction caused by different alkyl acetates varied from one another. Ethyl acetate produced a greater percentage decrease in peak heights and areas of C–H stretching absorbances. After a 40 min treatment with ethyl acetate, the asymmetric C–H stretching peak height and area showed a percentage decrease of 29.8 and 24.6, respectively. Also, the symmetric C–H stretching peak height and area showed a percentage decrease of 25.2 and 28.8, respectively.

For peptide and protein drugs, the pH of the solution can control the charge of the peptide and protein molecule based on their p$K_a$ values. The pH

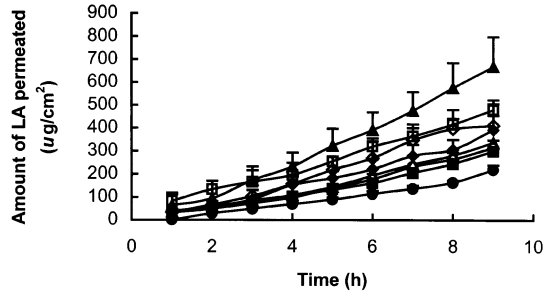


Fig. 3. Effect of alkyl acetate treatment on the in-vitro iontophoretic transport of leuprolide acetate through porcine epidermis. Each data point is the mean ± S.D. of three determinations. Key: (●) control; (○) methyl acetate; (▲) ethyl acetate; (△) propyl acetate; (■) butyl acetate; (□) pentyl acetate; (◆) hexyl acetate; (◇) octyl acetate.

Table 3
Flux and enhancement ratio of Leuprolide acetate (LA) due to alkyl acetates for passive and iontophoretic transport[a]

| Treatment | Flux (mg/cm$^2$/h) (mean ± S.D., $n = 3$) | | Enhancement ratio (ER) | |
|---|---|---|---|---|
| | P | I | ER-1 | ER-2 |
| Control | 2.82 ± 0.53 | 37.82 ± 3.64 | – | 13.41 |
| Methyl acetate | 5.07 ± 2.05 | 43.45 ± 3.49* | 1.80 | 15.41 |
| Ethyl acetate | 13.47 ± 5.85* | 89.79 ± 15.64* | 4.78 | 31.84 |
| Propyl acetate | 5.10 ± 1.08* | 45.79 ± 8.11 | 1.81 | 16.24 |
| Butyl acetate | 4.52 ± 2.37 | 39.23 ± 2.25 | 1.60 | 13.91 |
| Pentyl acetate | 6.30 ± 1.62* | 55.86 ± 0.82* | 2.23 | 19.81 |
| Hexyl acetate | 5.74 ± 0.02* | 46.59 ± 5.04* | 2.04 | 16.52 |
| Octyl acetate | 3.90 ± 0.04* | 53.89 ± 7.73* | 1.38 | 19.11 |
| C:M(2:1) | 205.17 ± 34.34* | 256.13 ± 54.05* | 72.76 | 90.83 |

[a] Control: no treatment; P: passive; I: iontophoresis; ER-1: passive flux of LA with treatment/passive flux of LA with control; ER-2: Iontophoretic flux of LA with treatment/passive flux of LA with control; C:M(2:1): chloroform:methanol (2:1). *Significantly greater than the control ($P < 0.05$).

is an important factor for drugs whose degree of ionization is pH-dependent. Divalent ions may migrate slowly as a result of interacting more strongly with charged sites in the skin than do monovalent ions (Burnette and Ongpipattanakui, 1987). Thus, there is a possibility that iontophoretic delivery of the leuprolide acetate would be more efficient with one charge at a pH as high as 7 than with two positive charge at pH 5 (Fu Lu et al., 1993). We chose normal saline (0.9% NaCl solution) to make a donor solution of leuprolide acetate for transport studies where the peptide would have a charge of +1. Also, because skin carries a net fixed negative charge, transdermal transport of positively charged ions is favored. Furthermore, transport of positively charged drugs across the skin is further enhanced by electroosmosis.

Figs. 2 and 3 show the transport profiles of Leuprolide acetate for passive and iontophoresis, respectively. Ethyl acetate, propyl acetate, pentyl acetate, hexyl acetate and octyl acetate showed a significant ($P < 0.05$) increase in passive flux and permeability coefficient when compared to the control (Table 3, Fig. 4). A multifold (seven to 14 times) increase in permeability coefficient was observed for each of the alkyl acetate when the iontophoretic permeability coefficient was compared with the passive permeability coefficient with the same alkyl acetate.

Table 3 compares the flux and enhancement ratios in flux for passive and iontophoretic transport of leuprolide acetate through the epidermis for different alkyl acetates and C:M(2:1). Ethyl acetate shows a remarkable increase in passive flux (13.47 μg/cm$^2$/h) as compared to its control (2.82 μg/cm$^2$/h). Also, the iontophoretic flux through ethyl acetate treated epidermis was significantly ($P < 0.05$) greater (89.79 μg/cm$^2$/h) than the iontophoretic flux through the control (37.82 μg/cm$^2$/h). Numerous studies suggest that the delipidization enhances the skin permeability by perturbing the barrier efficiency (Abrams et al., 1993; Levang et al., 1999).

Ethyl acetate and methyl acetate have been reported as effective penetration enhancers for

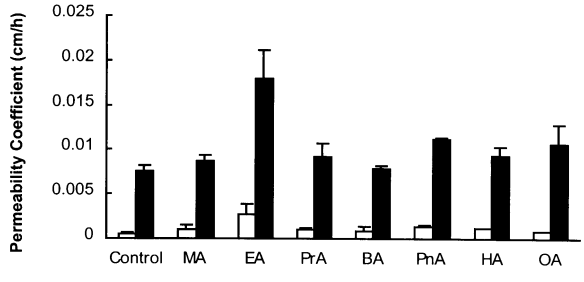


Fig. 4. Permeability coefficient of leuprolide acetate through porcine epidermis after alkyl acetate treatment. Key: (□) passive; (■) iontophoresis; MA, methyl acetate; EA, ethyl acetate; PrA, propyl acetate; BA, butyl acetate; PnA, pentyl acetate; HA, hexyl acetate; OA, octyl acetate.

indomethacin (Friend and Heller, 1993). Ethyl acetate has also been tested for percutaneous absorption enhancement of estradiol, hydrocortisone, 5-fluorouracil and nifedipine (Friend et al., 1989). Enhancement ratios of 200, 650, 74 and 115 with ethyl acetate in comparison to water were observed for estradiol, hydrocortisone, 5-fluorouracil and nifedipine, respectively. Ethyl acetate extracts lipids from the SC, which would lower the diffusional resistance of the skin. The DSC and FT-IR experiments on hairless mouse SC indicated a greater lipid extraction with longer exposures of ethyl acetate (Catz and Friend, 1989). In fact, ethyl acetate has been used as a delipidizing agent to remove sebum in clinical studies (Millns and Maibach, 1982).

Ethyl acetate has been examined in an occlusive patch test (10% petrolatum) in humans over 24 h, during which it was found to be non-irritating and non-sensitizing (Opdyke, 1974). Ethyl acetate is generally recognised as safe by the Food and Drug Administration. It has a relatively low toxicity, and the joint FAO/WHO Expert Committee on Food Additives has given ethyl acetate an unconditional acceptable daily intake of 0–25 mg/kg. Therefore, it is reasonable to consider ethyl acetate as an additive in the commercial transdermal delivery systems in order to enhance the percutaneous absorption of drugs.

We also studied the effect of C:M(2:1) treatment of SC/epidermis on the extent of lipid extraction and leuprolide transport. C:M(2:1) is a standard solvent used for lipid extraction. C:M(2:1) treatment of epidermis leads to almost complete ( $>80$–90%) removal of lipids (Raykar et al., 1988). A 40 min treatment with C:M(2:1) showed a percentage decrease of 75–90% in peak heights and areas of asymmetric and symmetric C–H stretching absorbances (Tables 1 and 2). A 40 min treatment of epidermis with C:M(2:1) showed an enhancement ratio of 72.76 and 90.83 for the passive and iontophoretic flux when compared with the passive flux through the control epidermis (Table 3). Thus, these findings suggest that SC lipid extraction in vitro leads to an enhancement in the passive and iontophoretic flux of a peptide such as leuprolide acetate. Such delipidization of the SC in vivo can also be realized by putting a cotton swab saturated with the solvent/enhancer on the skin for desired period of time before application of passive or iontophoretic patches. Topical methods for the in-vivo extraction of the SC lipids can be found in several studies (Menczel and Touitou, 1989; Bronaugh and Maibach, 1999).

The extracellular lipids that form the only continuous structure from the exterior to interior of the SC have been shown to represent the primary pathway (and barrier, as a consequence of the inherently high diffusional resistance) for the penetration of solutes (Elias et al., 1981; Grubauer et al., 1989). Ogiso et al. (1995) found a linear relationship between flux and removal of ceramides from hairless rat skin, indicating that the removal of intercellular lipids would cause dramatic dilations between adherent cornified cells. This would facilitate the iontophoretic and passive transport of hydrophilic solutes due to the resultant increase in free volume. Our findings provide evidence that alkyl acetates and C:M(2:1) cause lipid extraction, which creates a free volume in order to facilitate the passive and iontophoretic permeability of leuprolide acetate.

## 4. Conclusions

The present studies identify the synergy between alkyl acetates and iontophoresis as a technique to enhance and control the transdermal delivery of leuprolide acetate. Several simple alkyl acetates (i.e. ethyl acetate, propyl acetate, pentyl acetate, hexyl acetate and octyl acetate) significantly ($P < 0.05$) increased the passive flux of leuprolide acetate in comparison with the control (epidermis not treated with alkyl acetate). Iontophoresis further increased ($P < 0.05$) the flux of leuprolide acetate through alkyl acetate-treated epidermis. Ethyl acetate caused a greater lipid extaction and passive and iontophoretic permeability among all the alkyl acetates studied. FT-IR results showed that the SC treated with alkyl acetates decreased the percentage peak heights and areas for both asymmetric and symmetric stretching absorbances in comparison to untreated SC. Thus, alkyl acetates increased the leuprolide acetate permeability by enhancing the SC lipid extraction. Finally, our findings indicate

that iontophoresis supplemented with lipid extraction shows real promise as a tool for delivering biologically active macromolecules like peptides and proteins across the skin.

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