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Influence of human nail etching for the assessment of topical onychomycosis therapies

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Abstract

The purpose of this investigation was to study the physico-chemical properties of hot-melt extruded films containing ketoconazole and to determine the influence of 'nail etching' on film bioadhesion and drug permeability for the assessment of topical onychomycosis therapies. Hot-melt extrusion (HME) was used to prepare films containing 20% w/w ketoconazole. Ketoconazole 0.125% gel was also prepared using Carbopol® 974P NF. Films were processed at a temperature range of 115–120 °C utilizing a Killion extruder (KLB-100), and were evaluated for post-extrusion drug content, content uniformity, bioadhesion, thermal behavior and nail drug permeation. The extruded films demonstrated excellent content uniformity and post-processing drug content. Tensile and peel tests were recorded to determine the bioadhesive profiles. In this study, work of adhesion and peak adhesive force determinations using the peel tests provided more sensitive results for evaluating the bioadhesivity of the HME films than the tensile tests. The in vitro permeability profiles have demonstrated, that nail samples treated with an 'etchant' demonstrated a significant increase in drug permeability compared to control. Differential scanning calorimetry (DSC) thermograms indicated that ketoconazole was in solid solution within the HME films. These findings are encouraging for the future design and formulation of novel drug delivery systems for the topical treatment of onychomycosis.

Keywords: Onychomycosis; Nail; Hot-melt extrusion; Bioadhesion; Permeability; Etching; Ketoconazole; Differential scanning calorimetry (DSC)

1. Introduction

Onychomycosis is a fungal infection of the fingernails or toenails. It causes the nails to thicken, discolor, disfigure, and split. It has been reported that at least 18% of the world population is afflicted with this disease (Scher, 1996; Gupta and Scher, 1998). These fungal infections are especially troublesome to people with compromised peripheral circulation such as the elderly and diabetic patients. Others afflicted with these infections include those in the medical field, ranchers, farmers, military personnel, and users of acrylic nail

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products (Repka et al., 2002b). Causative agents of onychomycosis are dermatophytes (90%), yeasts (7%) and nondermatophyte molds (3%) (Niewerth and Korting, 1999; Piraccini and Tosti, 1999; Tom and Kane, 1999; Tosti et al., 1999; Debruyne and Coquerel, 2001). The primary fungi that cause this disease are *Trichophyton rubrum* and *Trichophyton mentagrophytes*. Increased therapies with antineoplastic agents and a continually growing population of immunocompromised individuals, such as those infected with the human immunodeficiency virus (HIV) have shown an increased incidence of systemic morbidity from this persistent disease process.

1.1. Treatment modalities

Onychomycosis has received much attention recently because of the high incidence of nail infections and problems associated with its therapy (Myoung and Choi, 2003). Negative aspects associated with oral systemic antifungal therapy for onychomycosis include its limited success rate, toxicity, contraindications, drug interactions, high cost of medication and increased microbe resistance (Murdan, 2002). Though, topical therapy does not lead to systemic side effects or drug interactions, it is not yet effective and this failure may be due to poor penetration of drugs into the nail plate. Human nail is composed of multiple layers of horny cells joined in a tightly cemented continuous sheet. The nail cells are dead corneocytes without nuclei or organelles and filled with α-keratin, constituting almost the entire dry weight of the nail (Quintanar-Guerrero et al., 1998). These unique properties, particularly its thickness and relatively compact construction, make it a challengeable barrier to the entry of topically applied agents. The pathogen will penetrate into the nail to such a depth within the keratin that it is difficult for conventional topical drug therapy to achieve a corresponding in-depth fungicidal action capable of destroying the pathogens.

1.2. Etching

Dentistry was introduced to the acid-etch technique for tooth restoration in 1955 (Buonocore, 1955). This idea proposed and subsequently provided, an ideal surface for bonding restorative materials to enamel using 30–40% phosphoric acid. It was later demonstrated that the resulting ‘etch’ pattern was characterized by the formation of profuse microporosities which allowed the penetration of monomers to form resin tags that provided micro-mechanical retention of composite restorations. Fluoride containing materials have since been introduced to cause inter-diffusion of the ion within the enamel to stimulate remineralization.

It is hypothesized that this “principle”, may be applicable to another hard, durable structure in humans, that is, the nail plate. Although, structurally different from enamel and dentin, the development of microporosities within the dorsal nail surface may increase wettability with a resulting increase in surface area and a decrease in contact angle (Retief, 1973). This activity results in the interpenetration and bonding of a polymeric delivery system and the facilitation of interdiffusion of a therapeutic agent (e.g. an antimycotic). If consistent with the finding, that depth of polymer penetration into enamel decreases insignificantly with increased viscosity (Perdigao et al., 2000), a sustained-release hydrophilic polymer film drug delivery system (free-formed or in situ) may be applicable for the human nail plate that has been etched. Preliminary data indicate that this acid-etch technique may be applicable to the nail plate for effective antimycotic topical delivery for onychomycosis, other disease states and possible preventative therapy.

1.3. Ketoconazole

Ketoconazole was utilized as a model drug in this study. It is an imidazole antifungal agent with a five membered ring structure containing two nitrogen atoms. It is used to treat the fungal infections of the fingernails, toenails, mouth (thrush), lungs, vagina and blood. Ketoconazole works principally, by inhibition of cytochrome P450 14a-demethylase (P45014DM) (Lyman and Walsh, 1992). This enzyme is in the sterol biosynthesis pathway, that leads from lanosterol to ergosterol. The compound is insoluble in water, while it is soluble in methanol, acids and slightly in ethanol. The chemical structure is shown in Fig. 1.

1.4. Hot-melt techniques

Hot-melt extrusion (HME) is one of the most widely applied processing techniques in the plastic industry. For pharmaceutical systems, several research groups have recently demonstrated, that the HME technique

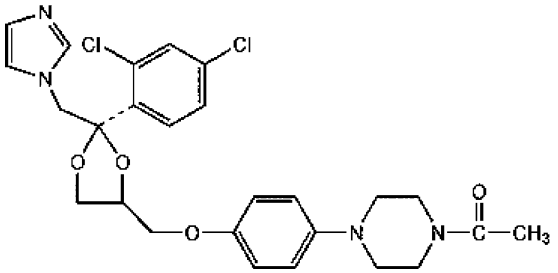


Fig. 1. Chemical structure of ketoconazole.

is a viable method to prepare numerous drug delivery systems. These systems include granules, sustained release tablets, transdermal and transmucosal drug delivery systems (Repka et al., 2002a; Follonier et al., 1994; Repka et al., 1999). Aitken-Nichol, et al. investigated the viability of HME technology for the production of thin, flexible acrylic films for topical drug delivery (Aitken-Nichol et al., 1996). Film casting (via organic or aqueous solvents) is the primary method used currently to produce transdermal and transmucosal drug delivery systems. However, Repka et al. discussed the numerous disadvantages accompanying this technique including long processing times, environmental concerns (organic solvent disposal), and excessive costs. Thus, for pharmaceutical applications, HME offers many advantages over traditional processing techniques which are as follows: (1) solvents are not used in this process; (2) fewer processing steps are needed; (3) there are no requirements for the compressibility of the active ingredients; (4) intense mixing and agitation during processing cause suspended drug particles to de-aggregate in the molten polymer, resulting in a more uniform dispersion of fine particles; and (5) bioavailability of the drug substance may be improved when it is solubilized or dispersed at the molecular level in HME dosage forms (Repka et al., 2002a).

In HME drug delivery systems, the active compound is embedded in a carrier formulation comprised of one or more meltable substances and other functional excipients. The meltable substances may be polymeric materials (Follonier et al., 1994; Cuff and Raouf, 1998; Aitken-Nichol et al., 1996; Repka et al., 1999; Grunhagen and Muller, 1995; Follonier et al., 1995; Zhang and McGinity, 1998) or low melting point waxes (Miyagawa et al., 1996; Sato and Miyagawa, 1997). The bioavailability of the drug substance could be improved, when it is dispersed at the molecular level in HME dosage forms. New chemical entities or natural product compounds that demonstrate a low bioavailability due to solubility issues are prime candidates for this technology.

1.5. Bioadhesion

Bioadhesion is a phenomenon related to the ability of biological or synthetic material to adhere to biological substrate (Wong et al., 1999). This results in the adhesion of the material to the tissue for a prolonged period of time (Noveon, 2002). Adhesive properties of transdermal systems can be explained on the belief that inter-atomic or inter-molecular forces are established at the interface of the adhesive and the substrate (adherent) or nail in these applications (Repka and McGinity, 2000). Numerous mechanisms of adhesion or mucoadhesion have been studied and proposed. However, one factor in common to achieve increased bioadhesion in any system is an increase in surface area. It was proposed that surface modification of the dorsal nail plate with a physical surface modifier, may increase microporosity (roughness) and thus increase surface area and wettability for a drug delivery system's bioadhesion and retention (Repka et al., 2002b). Increased bioadhesion is essential for the successful application of a bioadhesive drug delivery system for transnail delivery. In addition, it should release the drug in a controlled and predictable manner to elicit the required therapeutic response. Several techniques for in vitro determination of bioadhesion has been reported. They include tensile testing (Park and Robinson, 1987), shear stress testing (Smart et al., 1984), adhesion weight methods (Smart and Kellaway, 1982), fluorescent probe methods (Park and Robinson, 1984), flow channel techniques (Mikos and Peppas, 1986) and colloidal gold staining methods (Park, 1989). Texture Analyzer equipment, reported by Tobyn et al. (Tobyn et al., 1995) has been used to study bioadhesion in this project.

1.6. Nail permeation

The human nail forms a resistant barrier to the topical penetration of actives (Gupchup and Zatz, 1999). Thus, treatment of nail disorders, such as fungal infections, remains a challenge because of the difficulty encountered in achieving therapeutic

concentrations of drugs at the site of infection, which is often under the nail (Malhotra and Zatz, 2002). Investigation of the scientific literature suggests, that a key to successful treatment of onychomycosis by a topical antifungal product lies in it, effectively, overcoming the nail barrier. Current topical treatments have limited effectiveness, possibly because they cannot sufficiently penetrate the nail plate to transport a therapeutically sufficient quantity of antifungal drug to the target sites to eradicate the infection (Hui et al., 2002).

The purpose of this investigation was to study the physical and chemical properties of HME films containing ketoconazole and to determine the influence of 'etching' on film bioadhesion and drug permeability for the assessment of topical onychomycosis therapies. These bioadhesion and drug permeability findings coupled with ongoing studies may be useful for the design and formulation of novel drug delivery systems for the topical treatment of onychomycosis.

2. Materials and methods

2.1. Materials

Tip nail pieces were obtained from the fingers of healthy volunteers (University of Mississippi, IRB # 03-045), using nail clippers. The samples were collected and immediately sealed in four mil polyethylene bags. Klucel® (Hydroxypropylcellulose, HPC), was kindly gifted by Aqualon, Division of Hercules, Inc., Wilmington DE. Carbopol® 974P NF, was provided by BF Goodrich Speciality Chemicals (Cleveland, OH 44141). Polyethylene oxide (PEO) was purchased from Aldrich Chemical Company, Milwaukee, WI. Potassium dibasic phosphate and ketoconazole were obtained from Spectrum Chemical, Inc., Gardena, CA. Phosphoric acid (PA) gel was obtained from Henry Schein, Inc., Melville, NY.

2.2. Methods

2.2.1. Preparation of HME films containing Ketoconazole

Five 250 g batches (I-V) of HPC and/or PEO films containing ketoconazole (20%) were extruded using a Killion extruder (Model KLB-100) (Fig. 2). The extruder was pre-heated to 115 °C melt temperature. For purging purposes, polyethylene pellets were added to the hopper and passed through the extruder for 5 min (this procedure was repeated for each individual batch). All additives were blended thoroughly and dried at 50 °C for 24 h before extrusion. The dry blend of the drug and polymer was fed into the hopper and trans-

Fig. 2. Killion extruder (Model KLB–100).

ferred inside the heated barrel by a rotating extruder screw. The extrusion temperatures ranged from 115 °C to 120 °C. During HME, polymeric materials soften and become flexible primarily due to the shearing effect of the rotating screw, however, heat from the thermal devices attached to the barrel also aid in the softening of the polymers (Repka et al., 2002a). The molten polymeric mass is continuously pressurized as it moves forward in the barrel due to the rotation of the screw. In this study, a 6 in. flexible lip die was utilized. Homogeneous films were obtained with a thickness range of 9–13 mm or 0.23–0.33 mm (1 mm = 25.4 μ or 0.001 in.). The width of the films produced was, approximately, 5 in. (±0.25). The extrudate was collected in rolls, labeled, and sealed in 5 mm, opaque, polyethylene bags.

2.2.2. Instrumentation and chromatographic conditions

The chromatographic system consisted of a Waters 600 pump and a dual wavelength Waters 2487 UV detector. A 3.9 × 300 mm, μBondapak column, 10 mm particle size (Waters, Milford, MA, U.S.A) was used for the analysis of drug. The mobile phase used was 75% methanol and 25% of pH 7.5 phophate buffer. The flow rate was 2 ml/min. The injection volume for the standard and the sample preparations was maintained at 20 μl, and the column effluent was monitored by UV absorption at 225 nm for the active drug.

2.2.3. Determination of drug content by high performance liquid chromatography (HPLC)

Stock solutions of ketoconazole were prepared using the mobile phase. Nine calibration standards were prepared by diluting the stock with the mobile phase in appropriate quantities and were then injected into the high performance liquid chromatography (HPLC) system. Regression analysis was performed on the data points generating the calibration curve. Random samples ($n = 4$) were taken from all the batches. The samples were weighed and dissolved in 10 ml of the mobile phase, sonicated for 10 min or until the entire film was dissolved. These samples were then centrifuged for 18 min at 4000 rpm. The supernatant was removed and filtered using a 0.45 μl nylon filter and injected into the chromatographic system. The content of ketoconazole was calculated from the equation obtained from the regression analysis. Drug content in the physical mixtures (prior to extrusion) was also determined.

2.2.4. Ketoconazole gel

Ketoconazole, 0.125% gel was prepared using Carbopol® 974P NF. The model antifungal drug and methyl paraben were dissolved in ethanol. Carbopol® was blended in a mortar and pestle into a hydroalcoholic dispersion. The active solution was then added, followed by addition of an appropriate quantity of triethanolamine.

2.2.5. Etching

Tip nail pieces were obtained from the fingers of healthy volunteers using nail clippers. Dorsal nail surfaces were subjected to PA gel, 10% for 60 s. The excess gel was removed by washing with 100 ml of distilled water for 2 min. Non-treated nail samples were used as controls.

2.2.6. Bioadhesive studies

Bioadhesion tests were performed on HME films containing ketoconazole using a Texture Analyzer (TA.XT2i, Texture Technologies Corp., Scarsdale, NY/Stable Micro Systems, Godalming, Surrey, UK) (Fig. 3) equipped with Texture Expert™ software. The extruded films used in this work were wetted with 300 μl of nanopure water for, approximately, 60 s to allow the polymer chains to fully hydrate prior to testing. The films were then applied to human nail samples, in vitro (non-treated and etched). Each nail sample was placed and secured on a slotted die-cut fixture (a modified TA Indexable Adhesive Test Ring) on the base of the Texture Analyzer. Tensile and peel tests were recorded depending upon the position angle of the nail. The Texture Expert software was programmed, such that, the probe was lowered at a speed of 1 mm/s, until contact was made with the nail at a force of 1.5 N for a pre-determined duration of contact time. At the end of contact time, the probe was then withdrawn at a speed of 0.5 mm/s (parameters based on preliminary studies). This rate is the slowest speed for the apparatus and has been chosen, since lower rates of deformation produce more even distribution of stress at the film-substrate interface (Rowe, 1980). During the withdrawal phase of the probe, the Texture Expert software records the force deflection profiles. The maximum force required to detach the film on the upper probe from the secured nail sample, known as peak adhesive force (PAF) and the area under curve (AUC), representing the work of

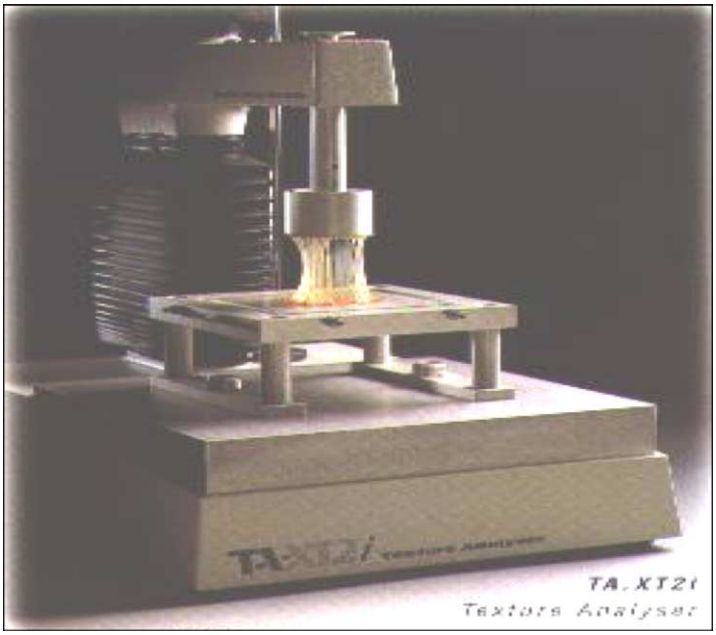


Fig. 3. Photo of Texture Analyzer (TA.XT2i).

adhesion, was determined by the generated profiles. Each measurement was performed in triplicate.

2.2.7. Differential scanning calorimetry (DSC)

The differential scanning calorimetry (DSC) thermograms was recorded on a DSC (Perkin-Elmer Pyris I DSC). Approximately, 3–4 mg of each sample of pure drug, physical mixture, and extruded film were hermetically sealed in a flat-bottomed aluminum pan and heated over a temperature range of 20–200 °C at a linear heating rate of 10 °C.

2.2.8. Permeability studies

Satisfactory release of active ingredients from dosage forms is a pre-requisite for therapeutic efficacy (Chattaraj et al., 1995). Key to successful treatment of onychomycosis lies in it, effectively, overcoming the nail barrier. In this study, a physical surface modifier, PA gel 10% was used to enhance the nail plate permeation. A system employing 9 modified Franz diffusion cells (Fig. 4) was used for the permeability studies. Six sets of full thickness nail plates (non-treated 'control' and 'etched') were individually sandwiched between polypropylene adapters and were mounted on the cells with the dorsal nail plate facing the donor compartment (receptor volume 5.2 ml, donor surface area 0.7 cm^2). The receptor fluid (isotonic phosphate buffer solution with 0.5%, Brij 58) was maintained at 37 ± 0.5 °C, and was continually stirred with a magnetic bar. The ketoconazole-containing HME film (Batch V) and gel were applied to the dorsal nail surfaces (in separate experiments). Samples of the receptor phase were withdrawn at pre-determined time intervals up to 48 h,

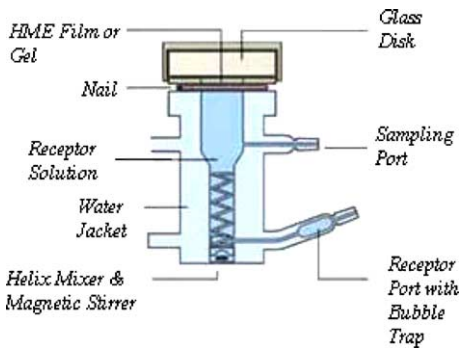


Fig. 4. Vertical diffusion cell used for permeability studies (from Hanson Research, Inc.,).

and immediately replaced with fresh receptor solution. Analysis of the samples was corrected for the previous drug removed. Drug content in the receptor media was determined using HPLC.

2.2.9. Data analysis

Statistical analysis was carried out using Microsoft Excel® and the results are reported as mean ± S.D. A $P < 0.05$ was considered statistically significant for drug content, bioadhesive uniformity and nail drug permeation for the extruded films.

3. Results and discussion

3.1. Drug content

The HME films demonstrated excellent chemical content uniformity and theoretical post-extrusion content. The theoretical post-extrusion content of ketoconazole found in the random samples collected from different areas of the extruded film ranged from 97.8% (±1.4) to 106.9% (±2.1). These results are shown in Table 1. Recent studies found similar results with HME clotrimazole films (Repka et al., 2003). The data presented in the table also suggests that the films had good drug content uniformity. This could be due to ketoconazole existing in solid solution within the extrudate or due to its solubilization in PEO, which will melt well below the melting point of ketoconazole. These results are supported by the DSC thermograms in Fig. 5. Ketoconazole in its crystalline form produced, an endotherm representing a melting point of 148 °C. However, after the HME process, films of the polymeric extrudate containing 20% ketoconazole did not exhibit a melting peak representing ketoconazole crystals. This indicates that ketoconazole is in amorphous form within the film; thus, a solid solution has most likely been formed (Repka et al., 2003). The solid-state dissolution of the drug may also attribute to the good content uniformity of the HME system.

Table 1
Drug content remaining in HME films after processing

Batch	Total % (theoretical)
I. 082703-001	97.8 (1.4)
II. 082703-002	103.1 (0.9)
III. 082703-003	99.4 (3.3)
IV. 082703-004	103.7 (1.9)
V. 082703-005	106.9 (2.1)

Films contain HPC, Noveon AA1, PEO, and Ketoconazole. Note: S.D. is denoted in parenthesis.

3.2. Bioadhesion

The peel test parameters, PAF and AUC, were determined to be greater for the etched human nail samples compared to that of the control for each of the instrument variables studied (Fig. 6a and b). Utilizing the peel test, for a contact time of 10 s, the PAF was, approximately, 2.5-fold higher for the treated sample compared to that of the control. Indeed, PAF was statistically higher in the etched samples at all contact intervals tested than in those nails that were not etched.

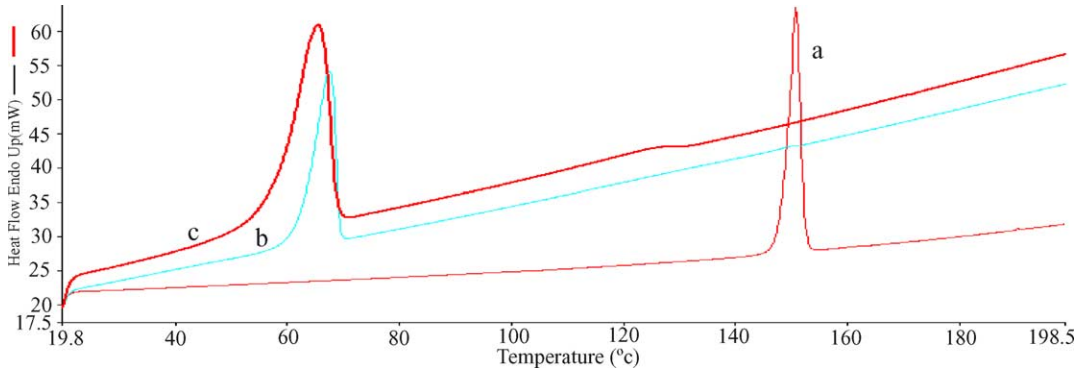


Fig. 5. Differential scanning calorimetry (DSC) thermograms (overlay) of, (a) pure ketoconazole, (b) physical mix (before extrusion), (c) HME ketoconazole incorporated film.

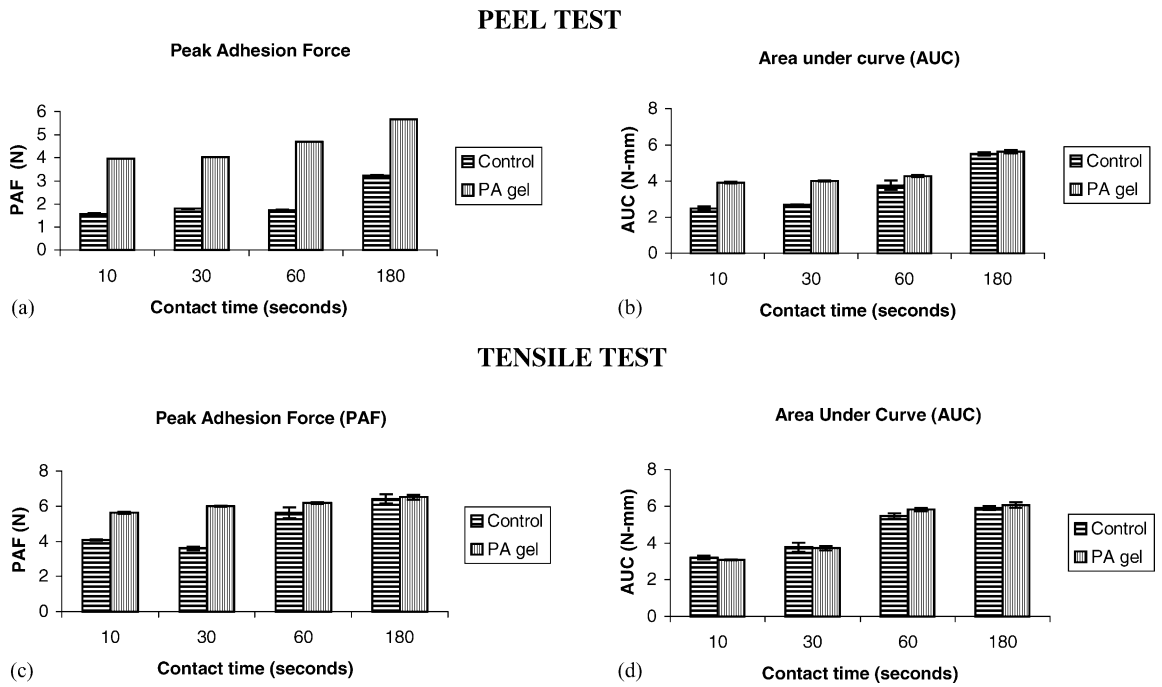
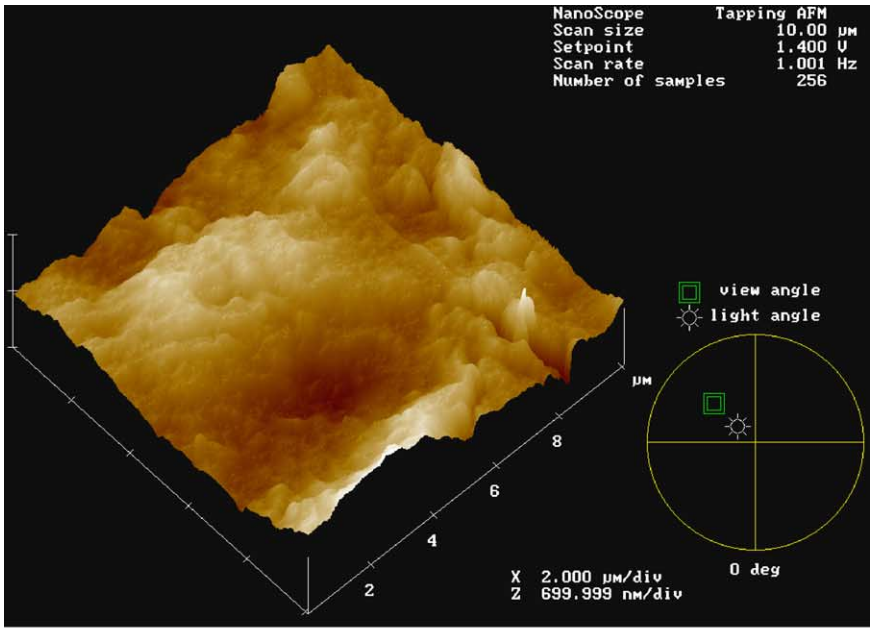


Fig. 6. Bioadhesive measurements of HME films containing ketoconazole on human nail, (a) peak adhesion force (peel test), (b) area under curve (peel test), (c) peak adhesion force (tensile test), (d) area under curve (tensile test).

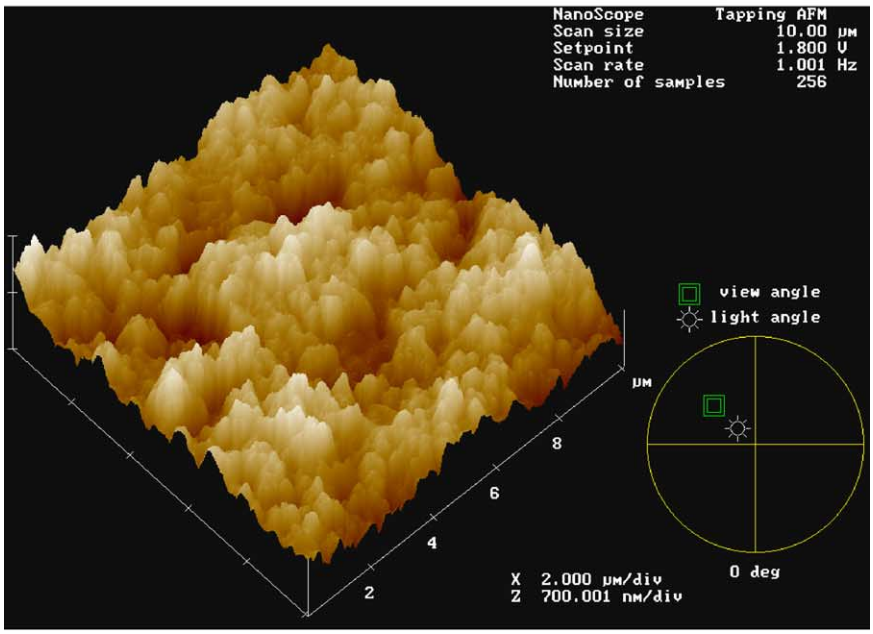
The tensile tests at contact time intervals of 10 and 30 s recorded a statistically higher PAF for the treated nail surface versus the control (Fig. 6c). This increase in bioadhesion for the treated samples can be explained by the fact, that increase in surface area provides, a greater opportunity for polymer chains to inter-diffuse and bond with the nail plate, improving bioadhesion and retention of a drug delivery system (Fig. 7) (Repka et al., 2002b). These results confirm the significance of previous work demonstrating the change in topography from the non-treated dorsal surface resulting in increased roughness and a consequent increase in surface area of the treated specimen. Another supporting explanation for the different bioadhesion profiles between the controlled 'unetched' and 'etched' nail samples is that, the polycarbophils contain a large number of carboxylic acid groups, that provide the ability to form a greater number of hydrogen bonds with the increased surface area of the etched human nail (Repka and McGinity, 2000). In this study, work of adhesion and PAF determinations using the peel tests provided more sensitive results for evaluating the bioadhesivity of the HME films to the dorsal nail surface than the tensile tests.

3.3. Differential scanning calorimetry

Fig. 5 depicts an overlay of DSC thermograms of pure ketoconazole, the physical mixture and HME films containing ketoconazole. The endotherm exhibited by ketoconazole (Fig. 5) at 148 °C indicates that the drug is in crystalline form. It was also observed that, there was no ketoconazole peak present in either the film or the physical mixture. The observation from the thermogram of the film could be due to ketoconazole existing in solid solution within the extrudate or due to its solubilization in PEO, which will melt well below the melting point of ketoconazole. Therefore, it is highly probable that ketoconazole is present in solid solution within the HME films. These findings are in agreement with previous research on hot-melt extrudates containing clotrimazole (Repka et al., 2003). Studies utilizing X-ray diffraction techniques are ongoing to confirm the crystalline prop-



(a) **Dorsal Surface - Control**



(b) **Dorsal Surface - Etched**

Fig. 7. AFM photomicrographs of the dorsal surfaces of the human nail; (a) control vs. (b) etched (Reprinted with permission from (Repka et al., 2002b).

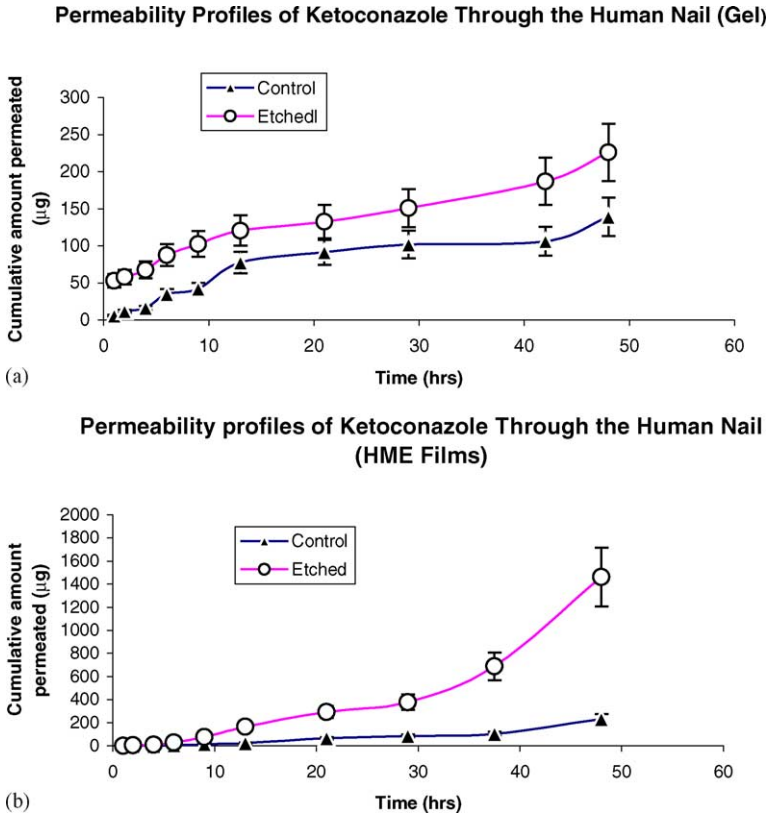


Fig. 8. Permeability profiles of ketoconazole through the human nail, (a) Gel, (b) HME Film.

erties of these and other hot-melt extruded dosage forms.

3.4. Permeation profiles

For the gel dosage form, ketoconazole that permeated the 'etched' nail plates was, approximately, 60% higher than that diffusing the controlled unetched nails (Fig. 8a). It should be noted that this significant difference was attained with a drug level of only 0.125%. The HME films containing ketoconazole exhibited six-fold higher total drug permeation (Fig. 8b) for the etched nail plates versus the control (non-etched). The increase in drug permeation for the etched nail plates can be explained by the controlled disruption of the dorsal surface, thereby, decreasing the 'effective membrane thickness' for drug permeation. There is also an increase in surface area for the etched nail due to the development of microporosities. This activity results in the interpenetration and bonding of a polymeric delivery system and the facilitation of inter-diffusion of the therapeutic agent. These data are in agreement with the work of Buonocore and co-workers, who demonstrated that the resulting etch pattern on enamel caused by 30–40% phosphoric acid was characterized by the formation of profuse microporosities which allowed the penetration of monomers to form resin tags that provided micro-mechanical retention of composite restorations (Buonocore, 1955). In Fig. 7 one can observe the change in topography from the non-treated dorsal surface to that of the etched nail surface. These researchers reported increased roughness scores and a consequent increase in surface area of the treated specimen (Repka et al., 2002b). The in vitro permeability profiles of ketoconazole-incorporated HME films and gels have demonstrated that nail samples treated with an 'etchant' exhibited, a significant increase in drug permeability compared to that of the control. Though,

an appreciable amount of the drug is releasing from the gel, there are several disadvantages which are as follows: (1) dissipation of the dosage form in relatively short periods of time (Repka et al., 2002b) and (2) cross-linking may increase the hydrophobicity of a gel and diminish the nail permeation of the drug (Martin, 2001). HME films, on the other hand, have several advantages. The in vitro permeability data of the extruded films when coupled with excellent post-extrusion drug content findings and in vitro bioadhesion testing data, the formulation and the delivery system prepared by HME may be a promising prototype for extended release of ketoconazole and other antifungal candidates for the treatment of onychomycosis.

4. Conclusions

Data from this study have indicated that nail samples treated with an 'etchant' demonstrated, a significant increase in both film bioadhesion and drug permeability compared to that of the control. While, atomic force microscopy (AFM) in combination with scanning electron microscopy (SEM) and polarized light microscopy (PLM) provided both qualitative and semi-quantitative information for the evaluation of nail morphology, the impact of these morphological findings on antimycotic nail permeation via two dosage forms has been demonstrated in this work. These bioadhesion and drug permeability findings coupled with ongoing studies may be useful in the design and formulation of novel drug delivery systems for the topical treatment of onychomycosis.

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