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Evaluation of Skin Permeation and Accumulation Profiles of a Highly Lipophilic Fatty Ester

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The aim was to evaluate the skin permeation and accumulation profiles of a highly lipophilic fatty ester using the combination of various permeation enhancing techniques to study the potential of highly lipophilic fatty esters as local topical agents. Permeation and accumulation profiles of ketorolac stearate (C18:0) were studied using solubility improved formulation, supersaturated solution of permeant in enhancer vehicle, lipophilic receptor solution, enhancer pretreatment, and the removal of stratum corneum and delipidization of skins. Impermeability and minimal skin accumulation of ketorolac stearate could delineate a preliminary possibility for designing safer topical agents without systemic absorption.

Key words: Fatty esters, Ketorolac stearate, Skin permeation, Skin accumulation, Local topical agent

INTRODUCTION

Deep tissue accumulation and systemic absorption is not the aim of local topical medications. Unfortunately, most of the disorders requiring such medication are found in the area of high permeation flux and in most cases, stratum corneum (SC) barrier resistance is decreased allowing their easier permeation in order to attain plasma levels that could be sufficient to elicit adverse reactions (Trommer and Neubert., 2006). There have been many attempts to develop suitable approaches to minimize their systemic absorption and the resulting adverse effects. More recently, there have been interests in agents that may be used in topical formulations to prevent the permeation of active ingredients or excipients (Asbill and Michniak, 2000) into the systemic circulation. But, not all of these permeation retardants have yet been proven to be commercially viable and pharmaceutically suitable and in many cases, retardation has been achieved by compromising systemic and local toxicities of the retarders (Hadgraft, 1996). Therefore, it would be an advantage if the systemic absorption of topical agents could be minimized irrespective of the sites or altered skin barrier function without the co-application of other chemical retarders.

Although many fatty alcohols/acids/esters have been shown to act as penetration enhancers for a variety of drugs including ketorolac, their enhancing efficiency has been found to have a parabolic relationship with their lipophilicity (Kanikkannan *et al*., 2000; Aungst *et al*., 1986; Lee *et al*., 1993; Sloan, 1992; Andega *et al*., 2001; Kanikkannan and Singh, 2002; Cooper *et al*., 1985). In addition to their permeation retardant effect, self permeability of highly lipophilic molecules has been reported to be low due to their accumulation in SC because of their low aqueous solubility (Goodman and Barry, 1989; Williams and Barry, 1992). However, such compounds may sometime be ineffective retardants for other molecules or their self permeation could eventually increase because of their enzymatic degradation into parent drug and free enhancer molecule (fatty alcohols/acids/esters) that may increase the permeation of the liberated parent drug and/or the original

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ester. Hence, in case of fatty esters (that are enzymatically hydrolysed into free fatty alcohols or acids enhancers) in addition to their lipophilicity, their enzymatic stability in skin would be an important factor for their permeation retardant effect.

Not all of the previously synthesized ketorolac ester prodrugs were efficient to enhance the permeation of ketorolac e.g. faster enzymatic hydrolysis of ketorolac [(N, N-dimethylamino) carbonyl] methyl ester than that of ketorolac ethyl ester into parent ketorolac was the reason behind the increased permeation flux of ketorolac from methyl ester (Roy and Manoukian, 1994). In addition, in our earlier studies (Doh *et al*., 2003; Kim *et al*., 2005) on ketorolac alkyl ester prodrugs and amide prodrugs, it was found that their lipophilicities were proportional to their carbon chain length and good linear relationships between lipophilicity and capacity factor were observed (suggesting that the capacity factor could be the indication of lipophilicity index). Similarly, parabolic relationships were found between skin permeation rate and lipophilicity indicating a possibility for designing suitably lipophilic ketorolac esters without systemic washout. However, they were enzymatically unstable precluding their use as local topical agents. Since the lipophilicity and the stability have been reported to be improved by synthesizing fatty ester derivatives (Setoh *et al*., 1995), if suitably lipophilic and esterase stable fatty ester derivatives of a model drug (ketorolac) could be synthesized, their skin permeation might be low because of their lipophilicity and low aqueous solubility, and the application of exogenous esters might result in a more efficient barrier function (Man *et al*., 1993a, 1993b; Holleran *et al*., 1993; Feingold *et al*., 1990). Although a lot of earlier works have clearly shown as to how the skin permeation of drugs can be increased using free fatty alcohols/acids or fatty esters, less attentions have been paid to synthesize and evaluate lipophilic fatty ester soft prodrugs. Soft prodrugs which are readily degraded by plasma esterase after passing through the skin into systemic circulation and are metabolically stable in skin and are be especially useful for targeting skin with topical delivery and localized exposure (Ettmayer *et al*., 2004). Hence, the skin permeation and accumulation profiles of model lipophilic ketorolac fatty ester (esters) will be evaluated in this study.

MATERIALS AND METHODS

Materials

Ketorolac was purchased from Fluka (Sleeze, Germany) and ketorolac stearate (KS) (Fig. 1) was provided by Dongseo University, Busan, South Korea. It was received as the off-white powder. A specific purity study was not done because standard sample was not available. However, it was purified by column chromatography and no impurities were detected by Thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC). Moreover, the Nuclear magnetic resonance (NMR) spectra did not reveal any additional peaks indicating that no significant impurities were present (data not shown- to be published in the near future). All other reagents were of analytical grade and used without further purification.

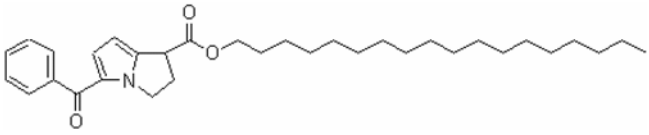

Fig. 1. Structure of ketorolac stearate

Determination of solubility and capacity factors

Solubility was determined as per our earlier method (Kim *et al*., 2005) in the mixtures of propylene glycol (PG): phosphate buffer (PB) pH 7.4 (0:100 to 100:0 v/v). Capacity factor was determined according to our earlier method (Doh *et al*., 2003; Kim *et al*., 2005) using methanol: distilled water (90:10 v/v) as the mobile phase. 90% methanol was used because of the relatively longer retention time of KS compared to pure ketorolac when the methanol concentration of mobile phase was less.

Enhanced solubility and dissolution of ketorolac stearate

To enhance its solubility and dissolution, phase solubility of KS was performed in various solutions of poloxamer 407 (P 407) at 25°C, and their solid dispersions (SDs) in 1:10 weight ratio was prepared by melting method in a locally designed jacketed vessel by circulating hot water (90-95°C) through a temperature controlled circulating water bath and magnetically stirring the resulting molten mixture at 700 rpm. After 10-15 minutes, the mixture was cooled by circulating cold water (<4°C) for about one hour and the solid mass was then ground. Surface morphology of KS, P 407, physical mixture and SDs were examined using a SEM (S-4100, Hitachi, Japan). The powders were fixed on a brass stub using double-sided adhesive tape and made electrically conductive by coating in a vacuum ($6P_a$) with platinum (6 nm/min) using Hitachi Ion Sputter (E-1030) for 240s at 15 mA. Solubility of KS from SDs was determined as per the method described in determination of solubility and capacity factors. Similarly, dissolution test was performed as per United States Pharmacopoeia paddle method in 500 ml distilled water (37°C) at 50 rpm in a standard tablet dissolution test apparatus.

Preparation of SC stripped and delipidized skins

Animals care and procedures were conducted accord-

ing to the guidelines for animal use in toxicology (Society of Toxicology USP 1989). SC free skins were obtained by adhesive tape stripping of intact skins for 30 times (Shah *et al*., 1992; Kuo *et al*., 1989). Delipidized skins were prepared according to the method described by Kuo *et al*., 1989 (Kuo *et al*., 1989). 0.01 %w/v gentamycin was added in the buffer solution, and the extracting solution was changed every 12 h and the extraction was performed for 48 h to achieve relatively more favorable lipid extraction.

Enhancer pretreatment and permeation studies

Skins were pretreated by placing 1 mL of PG in the donor compartment. The receptor compartment was filled with phosphate-buffered saline containing 0.01 %w/v gentamycin (33 mM phosphate buffer with 0.74% sodium chloride, pH 7.2) and thermostated at 37°C. After 12 h, the enhancer solution was removed and the remaining PG on the surface of skin was wiped off. Permeation studies were performed at 37°C (Doh *et al*., 2003; Kim *et al*., 2005) in PG pretreated intact, SC stripped and delipidized skins using a mixture of PG: PB (85:15 v/v- final pH 7.4) containing 0.01%w/v gentamycin as the receptor solution which was stirred magnetically at 600 rpm. Mounted skins were equilibrated with receptor solution for 2 h and 0.3 mL each of the saturated solution of ketorolac, KS and KS SDs in PG was placed on the donor compartment. Saturation was considered to be achieved by sonicating excess of materials in PG for 12 h at 37°C (Doh *et al*., 2003; Kim *et al*., 2005). Samples were collected at 2 h intervals for 48 h and analyzed by HPLC. The effect of improved solubility of KS on its permeation and accumulation profile was also evaluated using a saturated solution of SDs in PB (100%) and in a mixture of PB and PG (50/50 v/v) as the donor phase and a mixture of PG and PB (50:50 v/v) as the receptor solution.

Skin content analysis

Accumulation of KS at the end of permeation study was determined by cutting the effective permeation area of skins, which were then washed with 5% aqueous methanol solution, wiped off, dried at 40°C for 12 h, minced and homogenized in 3 mL mixture of THF (tetrahydrofurone): water (2:1 v/v), and centrifuged at 10000 × g for 10 min. 0.45 mL of the clear supernatant was mixed with 0.05 mL internal standard solution (flufenamic acid 1 mg/mL) and 0.5 mL THF:acetonitrile (ACN):dimethylsulphoxide (DMSO)-30: 65: 5 v/v (for maximum extraction and proteins precipitation). The clear supernatants obtained after immediate mixing and centrifugation for 2 minutes at 10000 × g were analyzed by HPLC.

Drug analysis and data interpretation

Ketorolac and KS were analyzed by Jasco P987 HPLC system equipped with UV-975 detector at 314 nm (column-C8, 5 μm, 4.6150 mm, Inertsil, GL Science; mobile phase flow rate-1.3 mL/min; injection volume-50 μL). Ratio of the mobile phase compositions (A-methanol:water:acetic acid -55:43:2 v/v, pH-3.0, B-methanol 100%) was controlled to accommodate their retention times. Lower limit of detection was 20 ng/mL, calibration curves were constructed at the concentration range of 0.02-50 μg/mL, and an excellent linearity was observed between the peak area ratios and drug concentrations over this range (r>0.993). Least-squared regression method was used to determine the regression coefficients and the equation for the best fitting line. The accuracy of the method was >90% and coefficient of variation (CV) did not exceed 10%. Data were compared for statistical significance by one way analysis of variance (ANOVA). The statistical significance of means was compared by multiple range method of least significant difference.

RESULTS AND DISCUSSION

Molecular weight and capacity factor of KS was 507. 37 and 14.17 (compared to 0.31 of ketorolac under the similar conditions) respectively. It was practically insoluble in PB-PG mixtures containing PG less than 80 %v/v, then its solubility increased with an increment in PG concentration and reached up to 1 mg/mL in 100% PG (Fig. 2). In our other unpublished studies (accepted in the J. Pharm. Pharm. Sci., and Bio. Pharm. Bull.) KS was like other ketorolac fatty esters was found to be highly stable toward chemical hydrolysis in PG-PB mixtures (80:15 %v/v, pH 3, 5, 7, 9, and 11), but it was readily hydrolyzed into parent ketorolac in PG-hairless mouse liver/skin homogenates or plasma mixtures (20: 80 %v/v at 37°C) in the order of

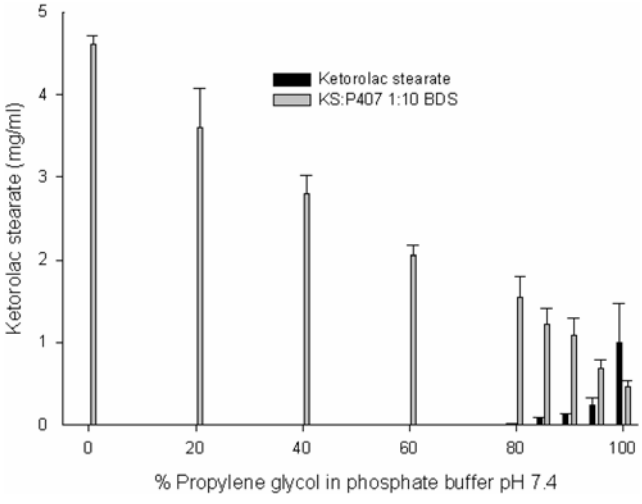

Fig. 2. Solubility of ketorolac stearate and its 1:10 w/w solid dispersion with poloxamer 407 in propyleneglycol and phosphate buffer pH 7.4 mixture at 25°C. Data are expressed as mean ± SD (n =3).

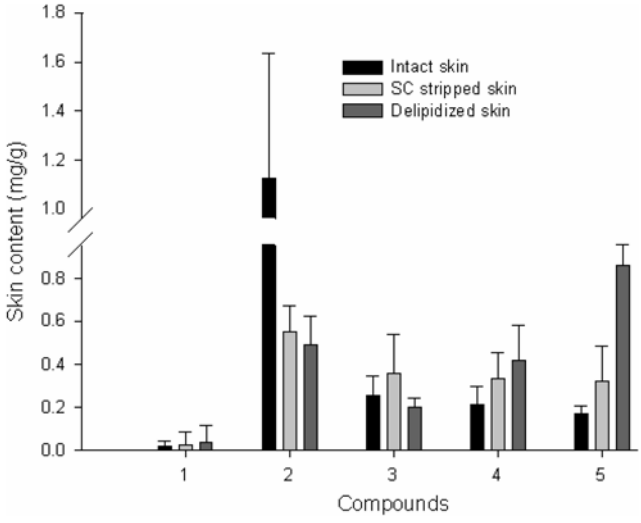

Fig. 3. Accumulation profiles in hairless mouse skins. 1. Ketorolac (control), 2. Ketorolac stearate, 3. SDs supersaturated in 100% propylene glycol in donor cell, 4. SDs supersaturated in a mixture of phosphate buffer pH 7.4: propylene glycol 50:50 %v/v in donor cell and phosphate buffer pH 7.4: propylene glycol 50:50 %v/v receptor solution as the receptor phase, and 5. SDs supersaturated in 100% phosphate buffer pH 7.4 in donor cell and phosphate buffer pH 7.4: propylene glycol 50: 50 v/v as the receptor phase. Data are expressed as mean ± SD (n =6).

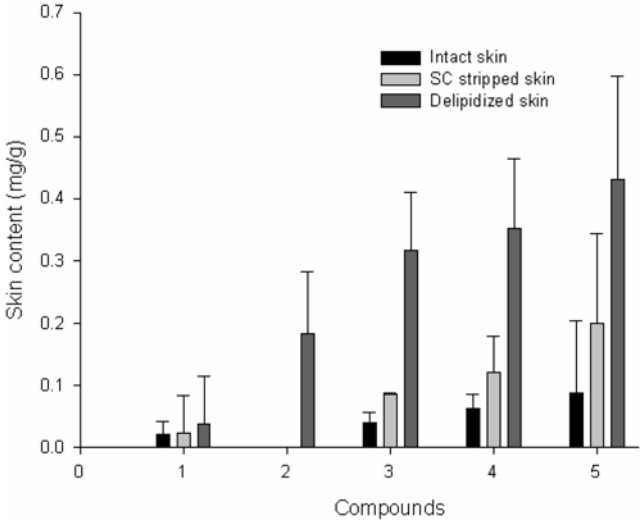

Fig. 4 Accumulation profiles of parent ketorolac released from enzymatic hydrolysis of ketorolac stearate in hairless mouse skin during skin permeation. 1. Ketorolac (control), 2. Ketorolac stearate, 3. SDs supersaturated in 100% propylene glycol in donor cell, 4. SDs supersaturated in a mixture of phosphate buffer pH 7.4: propylene glycol 50:50 %v/v in donor cell and phosphate buffer pH 7.4: propylene glycol 50:50 %v/v receptor solution as the receptor phase, and 5. SDs supersaturated in 100% phosphate buffer pH7.4 in donor cell and phosphate buffer pH 7.4: propylene glycol 50: 50 v/v as the receptor phase. Data are expressed as mean ± SD (n =6).

plasma > liver homogenate > skin homogenate. Moreover, KS was the most lipophilic and stable among the ketorolac esters tested. Thus in this study, its solubility and dissolution was enhanced and its permeation and accumulation tests were performed using various strategies. In skin permeation study, unlike parent ketorolac (control), KS did not appear in the receptor solution from all skins (even from the solubility and dissolution improved SDs). Permeation parameters of parent ketorolac were not calculated and a graph was not drawn because KS (test) did not permeate. Accumulation of KS in all skins was significantly higher compared with parent ketorolac ($P<0.05$) (Fig. 3). But, its accumulation was not significantly different in different skins ($P>0.11$). Its skin accumulation was 1.125, 0.55 and 0.494 mg/g in intact, SC stripped and delipidized skins respectively. The amounts of parent ketorolac released during skin permeation test was found to be 0.18 mg/g in delipidized skin but it did not degrade in intact and SC stripped skins (Fig. 4). Conversion of KS into parent ketorolac indicated that it was a prodrug. However, its stability during permeation study implied that it was susceptible to esterase at high enzyme concentration, and was a soft prodrug because its order of hydrolysis was highest in plasma followed by liver and the skin (Ettmayer *et al*., 2004). This justifies the objective of this study which was to synthesize a highly lipophilic ketorolac fatty ester soft prodrug to evaluate its accumulation and permeation profiles.

Stability of KS in intact and SC stripped skins during permeation study could be due to the relatively lower enzyme concentration per substrate (KS) molecule compared to the skin homogenate. Esterases are soluble in aqueous phase, and for hydrolysis to occur, enzyme and substrate must be in the same phase in a close contact. KS was stable in intact and SC stripped skins because of its low aqueous solubility and improving its solubility enhanced its degradation from SD. However, hydrolysis in delipidized skin might be because of the fact that it was present in aqueous phase where its interaction with esterase was relatively easier than in lipid phase (intact and stripped skins). This was in accordance to the finding of Setoh *et al*. (1995), who reported that the modification of tetragastrin into fatty esters increased its lipophilicity and reduced its degradation in the viable skin. As the hairless mouse skin expresses greater esterase activity than human skin (Sloan, 1998), KS would be relatively more stable in human skin.

Since the degradation of KS results into free stearyl alcohol enhancer having synergistic enhancing effect with PG for the simultaneously released ketorolac (Gwak and Chun, 2002; Young and Gwak, 2004), absence of ketorolac in receptor solution indicated the permeation retarding effect of KS on the parent drug because of its lipophilicity.

In addition, degradation of KS during permeation study was low; hence the available amount of released enhancers might have been inefficient to exert any enhancing effect for lipophilic KS or ketorolac. This was in accordance to Goodman and Barry (1989) and Williams and Barry (1992), who reported that the compounds with high affinity to the skin lipids have low self permeation rate and they also retard the penetration of other drugs. Thus its impermeability into receptor solution could be due to its accumulation in and strong affinity for skin lipids.

In order to corroborate the assumption of systemic impermeability, permeation studies were performed using the combination of proven permeation enhancing techniques for lipophilic molecules. Lipophilic receptor solution was used to achieve more favorable skin/receptor solution partition of KS (Chris *et al*., 2003). PG was preferred because it back diffuses into the dermis to drag KS and also acts as a permeation enhancer. Skins were pretreated with PG for its permeation enhancing effect (Bendas *et al*., 1995). In addition, PG readily permeates the skin and in doing so, might have carried the drug molecules across (Squillante *et al*., 1998). Moreover, the use of this cosolvent in combination with enhancers like fatty alcohols (in this case, the enzymatically released free stearyl alcohols) or fatty esters (KS itself) might offer synergistic enhancing effect for ketorolac (Cooper *et al*., 1985; Arellano *et al*., 1999; Gwak and Chun, 2002; Young and Gwak, 2004). During the study, KS and ketorolac were saturated in PG in order to keep a constant driving force with maximum thermodynamic activity. In addition to its relatively higher lipophilicity, the exogenous bulk lipid provided by the stearate side chains might have contributed to an increment in the total lipid proportion (in skin) there by a more effective barrier (Man *et al*., 1993a, 1993b; Holleran *et al*., 1993; Feingold *et al*., 1990). However, the exact mechanism for its inability to partition from lipid layer into the relatively lipophilic receptor medium cannot be explained without further works. Its accumulation and impermeability was in accordance to Goodman and Barry (1989) and Williams and Barry (1992), who reported that the permeability of highly lipophilic molecules was low because of their accumulation in SC due to their low aqueous solubility. Thus its solubility was improved by solid dispersion technique to study the effect of solubility in its permeation and accumulation profiles.

Phase solubility study showed that the solubility of KS linearly increased as the concentration of P 407 increased (R^2 = 0.98) (Fig. 5). In scanning electron micrographs, KS appeared as irregular crystals with rough surface (A) and P 407 as smooth surfaced spherical particles (B). Physical mixture had attached KS particles on their surface (C) and the SDs looked smooth surfaced, uniform, and homogeneously mixed mass (D) (Fig. 6). Compared to pure KS,

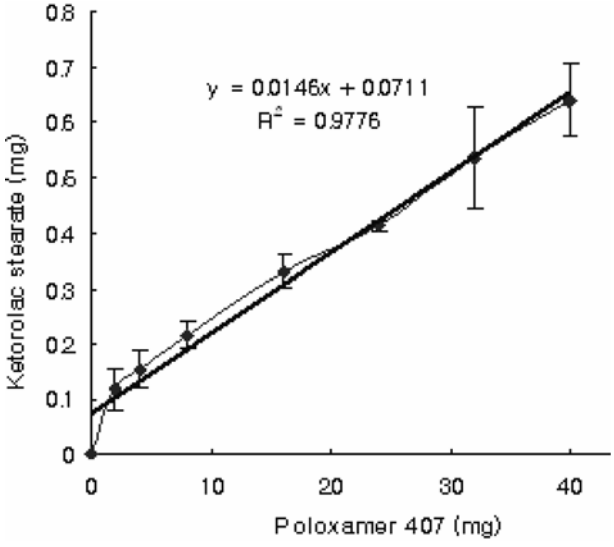

Fig. 5. Phase solubility behavior of ketorolac stearate in poloxamer 407 at 25°C. Data are expressed as mean ± SD (n =3).

the aqueous solubility of KS from SDs was remarkably higher but decreased from 4.61 mg/mL to 0.46 mg/mL as the concentration of PG in PG: PB mixture increased from 0: 100 to 100: 0 %v/v (Fig 2). Similarly, its dissolution was significantly improved (Fig. 7). Enhanced solubility and dissolution of KS could be related to the combination of factors such as the surface activity, wetting effect which may lead to reduced agglomeration and hence increased surface area, and solubilizing effect of P 407. Permeability of KS was not achieved by increasing its solubility but its skin accumulations were significantly decreased ($P<0.05$) to 0.25, 0.36 and 0.19 mg/g in intact, SC stripped and delipidized skins, respectively (Fig. 3). Interestingly, the amounts of parent ketorolac released from the hydrolysis of KS from SDs during skin permeation test increased to 0.04, 0.085 and 0.32 mg/g in intact, SC stripped and delipidized skins, respectively (Fig. 4). Increasing the amount of PB in the donor and receptor solutions decreased the accumulation of KS in intact and SC stripped skin but its accumulation in delipidized skin and the amount of parent ketorolac generated during the permeation test were increased.

There was no statistically significant difference ($P>0.05$) in KS accumulation in SC-stripped and delipidized skins, suggesting that the predominant route for its passive penetration of SC-layer might be the intercellular pathway (Lee *et al*., 1997). Lower accumulation in delipidized skin compared to normal skin or stripped skin suggested that it had relatively strong affinity for lipids compared to other components in the skin. However, higher accumulation in SC (intact skin>SC stripped skin) suggested that it had high affinity for SC lipids than the dermal lipids. KS was not highly taken up in both lipid and proteinaceous phases.

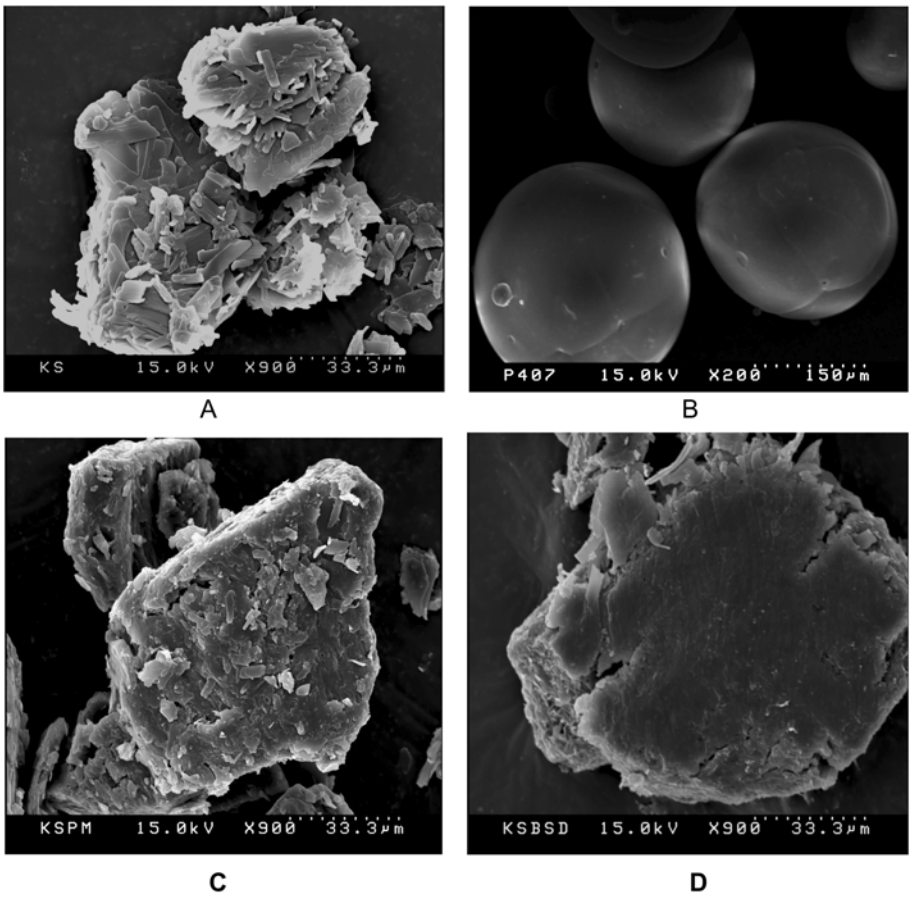

Fig. 6. Scanning electron micrographs A. Ketorolac stearate, B. Poloxamer 407, C. Ketorolac stearate: Poloxamer 1:10 w/w physical mixture, D. Ketorolac stearate: Poloxamer 1:10 w/w solid dispersions.

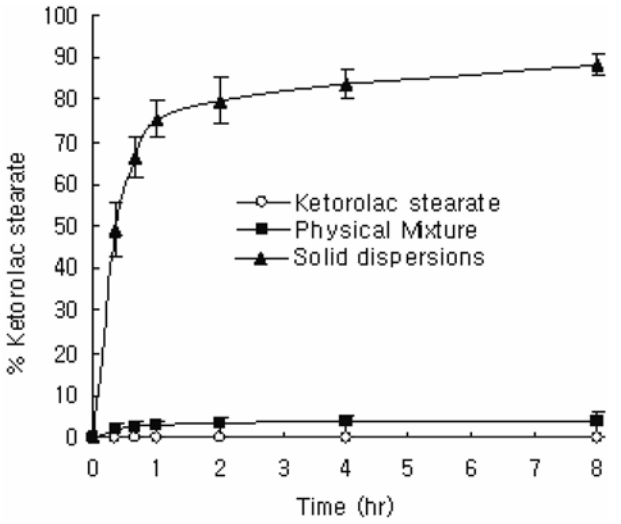

Fig. 7. Dissolution of ketorolac stearate and its 1:10 w/w solid dispersion with poloxamer 407 in propyleneglycol and phosphate buffer pH 7.4 mixture at 25°C. Data are expressed as mean ± SD (n =3).

Its smaller uptake in the lipid phase had no positive effects in its skin permeation because of its strong affinity for skin lipid and other components. Similarly, its uptake in the proteinaceous phase seems meaningless in this case because of its impermeability (ketorolac is prescribed for its systemic effect), and the lower accumulation (not suitable as depot for dermal or transdermal delivery). Since the KS was impermeable because of its affinity for dermal lipids and low aqueous solubility, its solubility was improved by formulating SD. However, the permeability of KS was not achieved and the accumulation was further decreased possibly because of lower solubility of SDs in the PG vehicle. Similarly, even less accumulation in intact and SC stripped skin, when PG concentration of donor solution was decreased might be due to the absence of inherent permeation enhancing effect of PG. Removal of lipids might have resulted in favorable partition of KS from SD into aqueous proteinaceous phase leading to its higher accumulation in delipidized skins. Increased hydrolysis KS (from SD) in delipidized skin might be because of the possibility that the KS was present in aqueous phase. Thus, considering the accumulation and permeation pattern of KS, it could be possible to suitably modify the physicochemical properties of some drugs by synthesizing suitably

lipophilic fatty esters soft prodrugs for topical application with minimal potential for systemic absorption provided that their pharmacological activity is not lost on esterification.

CONCLUSION

Lower accumulation and absence of permeation using the combination of common permeation enhancing techniques including enhanced aqueous solubility, and enzymatic stability in all type of skins during permeation studies of KS could delineate a preliminary possibility for designing safer topical agents with minimum potential for systemic absorption (without the co-application of permeation retarders).

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