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Skin Permeation of Testosterone and its Ester Derivatives in Rats

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Abstract

To establish the optimum conditions for improving the transdermal delivery of testosterone, we studied the relationship between the lipophilicity of testosterone ester derivatives and the rat skin permeation rate of testosterone. We performed a rat skin permeation study of testosterone and its commercially available ester derivatives, testosterone hemisuccinate, testosterone propionate and testosterone-17β-cypionate, using an ethanol/water co-solvent system.

The aqueous solubility and rat skin permeation rate of each drug, saturated in various compositions of an ethanol/water system, was determined at 37°C. The aqueous solubility of testosterone and its ester derivatives increased exponentially as the volume fraction of ethanol increased up to 100% (v/v). The stability of testosterone propionate in both the skin homogenate and the extract was investigated to observe the enzymatic degradation during the skin permeation process. Testosterone propionate was found to be stable in the isotonic buffer solution and in the epidermis-side extract for 10 h at 37°C. However, in the skin homogenate and the dermis-side extract testosterone propionate rapidly degraded producing testosterone, implying that testosterone propionate rapidly degraded to testosterone during the skin permeation process. The steady-state permeation rates of testosterone in the ethanol/water systems increased exponentially as the volume fraction of ethanol increased, reaching the maximum value ($2{\cdot}69 \pm 0{\cdot}69\ \mu g\ cm^{-2}\ h^{-1}$) at 70% (v/v) ethanol in water, and then decreasing with further increases in the ethanol volume fraction. However, in the skin permeation study with testosterone esters saturated in 70% (v/v) ethanol in water system, testosterone esters were hardly detected in the receptor solution, probably due to the rapid degradation to testosterone during the skin permeation process. Moreover, a parabolic relationship was observed between the permeation rate of testosterone and the log P values of ester derivatives. Maximum flux was achieved at a log P value of around 3 which corresponded to that of testosterone ($\log P = 3{\cdot}4$).

The results showed that the skin permeation rate of testosterone and its ester derivatives was maximized when these compounds were saturated in a 70% ethanolic solution. It was also found that a log P value of around 3 is suitable for the skin permeation of testosterone related compounds.

Testosterone, the primary androgenic hormone, is responsible for the normal growth and development of the male sex organs and for maintenance of secondary sex characteristics. Male hypogonadism (testosterone deficiency) results from a primary defect of the testes, or from a disorder of the hypothalamus or anterior pituitary resulting in inadequate gonadotropic stimulation of the testes. Symptoms associated with male hypogonadism include impotence and decreased sexual desire, fatigue and loss of energy, mood depression, and regression of secondary sexual characteristics. Bone mineralization, muscle strength, immune function, and carbohydrate metabolism may also be adversely affected by testosterone deficiency (Bizzarro et al 1987; Mooradian et al 1987). The usual treatment for testosterone deficiency is to replace the missing testosterone through injections, tablets or skin patches.

Recently, transdermal drug delivery systems have been introduced as suitable alternatives to conventional testosterone administration (Misra et al

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1996; Arver et al 1997). The potential advantages associated with transdermal drug delivery are well documented and include avoidance of hepatic first-pass elimination, decrease in side effects, and the relative ease of drug input termination in problematic cases (Williams & Barry 1992). Transdermal delivery of testosterone may also be helpful in maintaining suitable plasma concentration for a longer duration through a non-invasive zero-order delivery. Nevertheless, transdermal drug delivery has always been challenged by the formidable barrier property of the intercellular lipid bilayer in the stratum corneum. Testosterone, compared with other steroidal hormones, exhibits low skin permeability. To improve the permeation rate of testosterone, consideration of the lipophilicity/hydrophilicity balance may be helpful. There have been several reports on the relationship between the lipophilicity of steroidal compounds and skin permeability. Chien (1992) introduced hydroxy groups into progesterone to reduce the lipophilicity, and observed a linear relationship between the skin permeability of these compounds and their partition coefficient up to a log P value of 2·5. Chien (1992) also reported that the ester-type prodrugs of oestradiol are extensively metabolized during the course of skin permeation by the esterase in skin tissue to regenerate oestradiol. Moreover, the rate of regeneration of oestradiol from the prodrug-type esters was dependent on the alkyl chain length. The purpose of this study was to investigate the relationship between the lipophilicity and the rat skin permeation rate of testosterone to establish optimum conditions for improving the transdermal delivery of testosterone. Three commercially available ester derivatives of testosterone were used, testosterone hemisuccinate as a model hydrophilic derivative, and testosterone propionate and testosterone-17β-cypionate as model lipophilic derivatives.

Materials and Methods

Materials

Testosterone, testosterone hemisuccinate and testosterone-17β-cypionate were purchased from Sigma Chemical Co. (St Louis, MO). Testosterone propionate was obtained from Tokyo Kasei Organic Chemicals (Tokyo, Japan). HPLC grade acetonitrile and ethanol were purchased from Fisher Scientific (Fair Lawn, NJ). All other chemicals were reagent grade or higher and were used as received.

Preparation of rat skin

Male Sprague–Dawley rats (220–250 g) were obtained from Dae-Han Laboratory Animal Research Center Co. (Dae-Jeon, Korea). The rats had free access to food and water until they were used for experiments. The rats were killed in a CO_2 chamber immediately before the experiments. The dorsal hairs were removed with a clipper and full-thickness skin (approximately 16 cm^2) was surgically removed from each rat. The skin specimen was cut into appropriate sizes after carefully removing subcutaneous fat and washing with normal saline.

HPLC analysis of testosterone and its ester derivatives

Concentrations of testosterone and its ester derivatives were determined using a high-performance liquid chromatography (HPLC) system equipped with a binary pump system (Gilson Model 305 and 306) and an automatic injector (Gilson Model 234). A Merck C_{18} LiChroCAT 125-4 column (5 μm, 125 × 4 mm, Merck, Darmstadt, Germany) was used as an analytical column at ambient temperature. The mobile phase was an acetonitrile–acetate buffer (50 mM, pH 4·0) combination (60% acetonitrile for testosterone, testosterone hemisuccinate, and testosterone propionate, and 90% for testosterone-17β-cypionate) at a flow rate of 1·0 mL min^{-1}. The variable wavelength UV detector (Gilson Model 118) was set at 242 nm. All solutions to be analysed were injected at a volume of 20 μL. Retention times of testosterone, testosterone hemisuccinate, testosterone propionate, and testosterone-17β-cypionate were 3·3, 3·2, 14·2, and 9·1 min, respectively.

Determination of lipophilicity

Partition coefficient (P). The partition coefficient was determined according to the method of Dearden & Bresnen (1988). An n-octanol/water mutual saturation was carried out for 12 h with gentle mechanical stirring, and then each phase was separated. Ethanolic solutions of testosterone, testosterone hemisuccinate, testosterone propionate, and testosterone-17β-cypionate (10 mg mL^{-1}) were placed in glass tubes (300 μL each), and 1·0 mL of each saturated solvent was added to the tubes after completely evaporating the ethanol. The stoppered tubes were shaken for 30 min at 20 inversions min^{-1}, and then the phases were separated by centrifugation at 3000 g for 20 min. Concentrations of each compound in each phase were

determined by HPLC after appropriate dilution with methanol.

Capacity factor (k′). The k′ values of testosterone, testosterone hemisuccinate, testosterone propionate, and testosterone-17β-cypionate were determined isocratically (60% acetonitrile in acetate buffer solution, 1·0 mL min^{-1}) using HPLC, as described above. Retention times of each compound were measured, and the k′ values were calculated from the following equation:

$$k' = (t_i - t_o)t_o^{-1} \quad (1)$$

where t_o is the retention time of methanol and t_i is the retention time of each compound.

Solubility study
The solubility of testosterone and its ester derivatives in ethanol/water co-solvent systems of varying volume fraction were measured at 37°C. An excess amount of each compound was added to the co-solvent system and mixed by vortexing. The solution was immersed in a shaking water bath at 37°C and allowed to equilibrate for 48 h. The saturated solutions were then filtered through Minisart RC 4 filters (0·45 μm, Sartorius, Germany). Concentrations of compound were analysed by HPLC after appropriate dilution with methanol.

Stability study
Enzymatic degradation of testosterone propionate during the skin permeation process was observed in both the rat skin extract and the homogenate. Extraction of rat skin was conducted using Valia-Chien permeation cells at 37°C, as described by Kim & Chien (1995). Briefly, freshly excised skin specimen was mounted between the two half-cells, with the stratum corneum facing the donor half-cell and the dermis facing the receptor half-cell. Both donor and receptor half-cells were filled with isotonic phosphate buffer solution (IPB, pH 7·4), and extraction of enzymes from the skin was carried out for 24 h. After 24 h, the donor and receptor solutions from each pair of half-cell were separately combined and stored in the freezer until used. Skin homogenate was prepared from the freshly excised rat skin added to tenfold IPB (pH 7·4) and homogenized for 15 min in an ice bath. The supernatants were obtained after centrifugation for 20 min at 9000 *g*. Gentamicin (0·01% (w/v)) was added to all solutions used for stability studies to inhibit the bacterial degradation of testosterone propionate. Testosterone propionate solutions in IPB, skin extract, and homogenate (1·0 μg mL^{-1}) were placed in a shaking water bath (50 rev min^{-1}) at 37°C. At predetermined time intervals, the concentration of testosterone propionate in each solution was measured by HPLC for 10 h. Considering the initial molar concentration of testosterone propionate as 100%, the remaining molar percentage of testosterone propionate and testosterone in each solution was determined as a function of time.

Skin permeation study
In-vitro skin permeation across rat skin was conducted with Keshary-Chien diffusion cells at 37°C. Freshly excised skin was mounted between the donor and receptor cell (stratum corneum side facing the donor). The area of diffusion for all in-vitro experiments was 2·01 cm^2. The donor cells, faced with the stratum corneum surface, contained a saturated solution of testosterone or its ester derivatives in various compositions of an ethanol/water co-solvent system (3·0 mL), and was occluded with Parafilm. The receptor cells, which faced the dermis side, were filled with normal saline solution containing 40% (v/v) polyethylene glycol 400 to maintain sink conditions (12·0 mL). At predetermined time intervals, 0·5 mL receptor solution was withdrawn and refilled with the same volume of fresh receptor solution. Samples were kept in a freezer (−20°C) until analysed by HPLC.

Results and Discussion

Determination of lipophilicity
Lipophilicity of testosterone and its ester derivatives was determined by the n-octanol/water partition coefficient and capacity factors. Testosterone was a relatively lipophilic molecule with a log P value of 3·42, which is close to the value of 3·32 previously reported by Misra et al (1996). As expected, testosterone hemisuccinate was more hydrophilic than testosterone, while testosterone propionate and testosterone-17β-cypionate were more lipophilic than testosterone. Since the plot of log P vs log k′ yielded a straight line with a correlation coefficient (r^2) of 0·71 (Figure 1), the capacity factors determined by HPLC seem to be an easy and convenient method to estimate the lipophilicity of compounds.

Solubility study
The effect of the volume fraction of ethanol on the solubility of testosterone, testosterone hemisuccinate, testosterone propionate, and testoster-

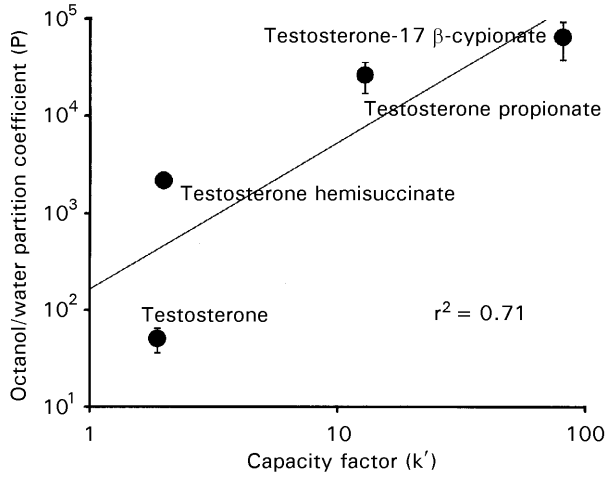

Figure 1. Plot of capacity factor (k′) vs octanol/water partition coefficient (P). Octanol/water partition coefficients of testosterone and ester derivatives were determined in mutual saturation of octanol and water as described in the literature (Dearden & Bresnen 1988). The capacity factor of each drug was determined using an isocratic HPLC system (60% acetonitrile in acetate buffer solution as a mobile phase at 1·0 mL min^{-1} flow rate).

one-17β-cypionate in the ethanol/water system is shown in Figure 2. The aqueous solubility of these compounds increased exponentially as the volume fraction of ethanol increased up to 100% (v/v). Similar results were reported in an earlier study with the lipophilic contraceptive drug levonorgestrel (Chen et al 1995). It was not possible to determine the aqueous solubility of testosterone-17β-cypionate, since it degraded in the co-solvent system with lower than 40% ethanol in water. It is interesting to note, however, that the water solubility of these compounds was in the order of testosterone hemisuccinate > testosterone > testosterone propionate > testosterone-17β-cypionate, which coincided with the hydrophilicity of these compounds.

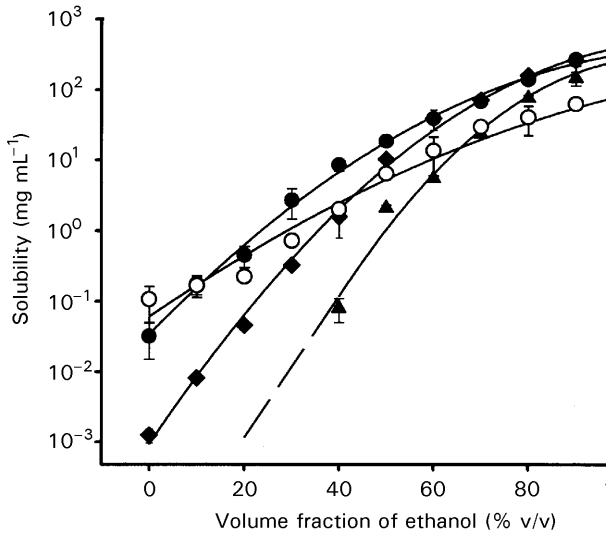

Figure 2. The solubility of testosterone and its ester derivatives in various compositions of the ethanol/water co-solvent system saturated at 37°C for 48 h (n = 3). ● Testosterone; ○ testosterone hemisuccinate; ◆ testosterone propionate; ▲ testosterone-17β-cypionate.

Stability study

Difficulties were encountered in the HPLC analyses of testosterone hemisuccinate and testosterone-17β-cypionate. This was because the retention time of testosterone hemisuccinate was too close to testosterone while that of testosterone-17β-cypionate was too far away. Thus, only testosterone propionate was chosen to study the enzymatic degradation of ester derivatives to testosterone during the skin permeation process. The stability of testosterone propionate in skin homogenate and skin extract was compared using IPB as a control (Figure 3). No significant degradation of testosterone propionate in IPB and the extract of the epidermis side were noted during 10 h of experiments. However, in the skin homogenate and the extract of the dermis side, testosterone propionate quickly degraded producing testosterone. It could therefore be confirmed that the degradation of testosterone propionate was due to the contact with skin (especially dermis side), and not to the physicochemical stability of testosterone propionate in the solution.

Skin permeation studies

Figure 4 shows the permeation profile of testosterone saturated in various volume fractions of the ethanol/water system across the rat skin at 37°C. In ethanol/water co-solvent systems, steady-state permeation rates of testosterone increased as the volume fraction of ethanol increased, reaching a maximum at 70% (v/v) ethanol in water, and then decreasing with further increases in the volume fraction of ethanol. Table 1 summarizes the skin permeation parameters of testosterone in various volume fractions of the ethanolic solution. The maximum flux of 2·69 $\mu g\,cm^{-2}\,h^{-1}$ was achieved with the saturated solution of testosterone in 70% (v/v) ethanol.

Ethanol is one of the most commonly used skin permeation enhancers (Williams & Barry 1992). Ethanol used as part of a co-solvent system with water has been observed to increase permeation of a wide range of drugs through human and animal skins (Ghaneum et al 1987; Berner et al 1989; Pershing et al 1990; Obata et al 1993; Megrab et al 1995). Berner et al (1989) reported the linear

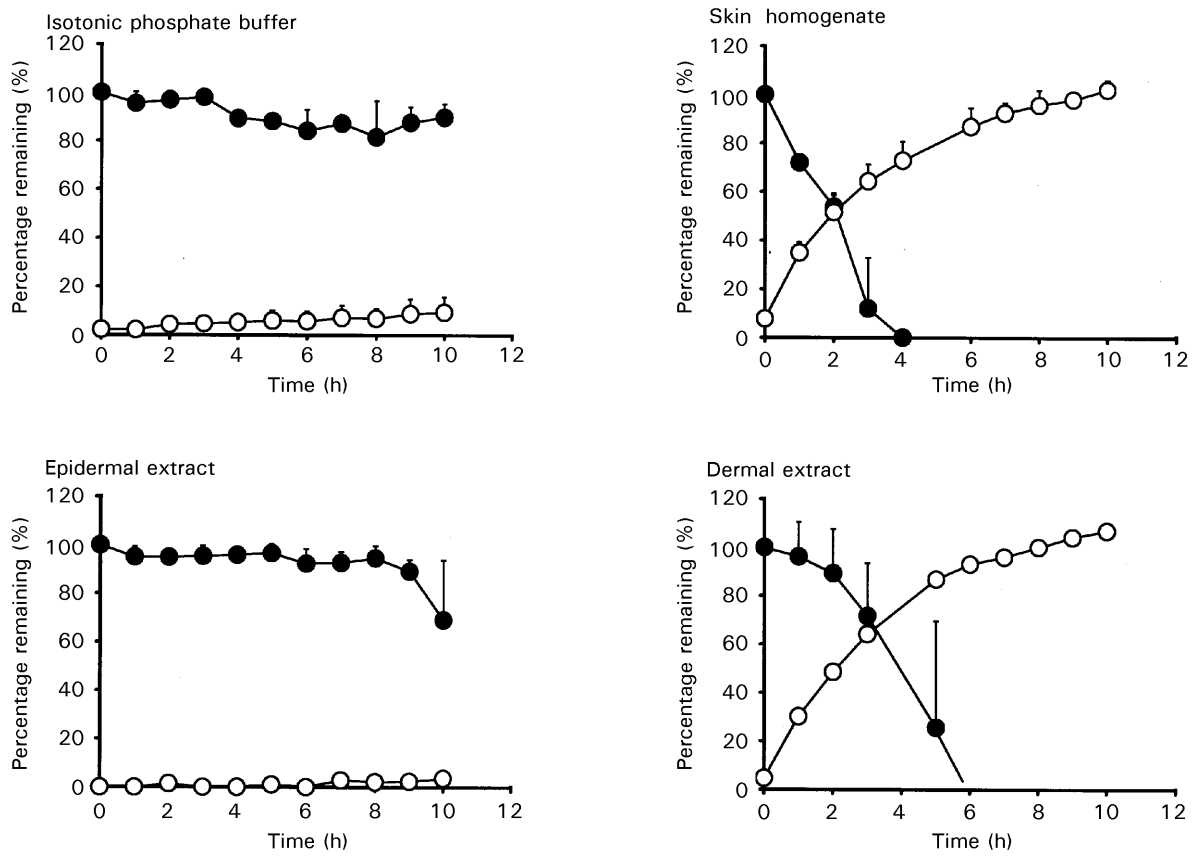

Figure 3. The stability of testosterone propionate in the isotonic phosphate buffer, skin homogenate, epidermal extract, and dermal extract of rat skin at 37°C. Testosterone propionate (●) was rapidly degraded producing testosterone (○) in skin homogenate and dermal extract.

relationship between the skin permeation of nitroglycerin and the transdermal flux of ethanol, which implies that ethanol penetrates through the skin and changes the permeation properties of the skin. They suggested that when less than 70% ethanol is used, permeation of both ethanol and drug is enhanced by hydrating the entire stratum corneum. However, when more than 80% ethanol is used, the outer layer of the stratum corneum is substantially dehydrated, and this dehydrated portion increases barrier properties to the permeation of ethanol and drug. Recently, the influence of ethanol in aqueous solutions upon the transport behaviour of several lipophilic and hydrophilic permeants in hairless rat skin was systematically investigated (Kim et al 1996). Some researchers have demonstrated an ethanol concentration-dependent enhancement mechanism in which ethanol altered the lipid component at low fractions whereas new pores formed in the stratum corneum at higher ethanol fractions (Ghaneum et al 1987). In other studies, it was suggested that at high ethanol concentrations, the pore pathway formed as a result of a combination of changes in protein conformation, reorganization within the lipid polar head regions, and extractions of lipids by ethanol (Kai et al 1990; Kurihara-Bergstrom et al 1990).

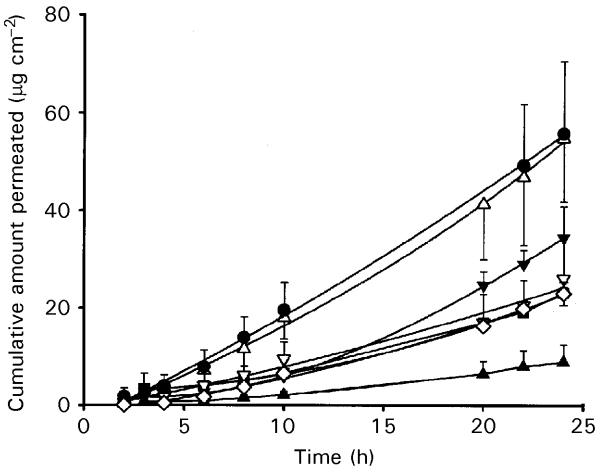

Figure 4. Permeation profiles of testosterone across the rat skin at 37°C. Testosterone was saturated in various compositions of ethanol/water co-solvent systems at 37°C for 48 h (n = 3). ▲ 20; ■ 40; ◇ 50;▼ 60; ● 70; △ 80, and ▽ 100% (v/v) ethanol.

In this study, testosterone ester derivatives were selected as prodrugs for enhanced skin permeation.

Table 1. Skin permeation parameters of testosterone saturated in various volume fractions of ethanol/water co-solvent systems at 37°C.

Ethanol (v/v)%	Flux $\mu g\,cm^{-2}\,h^{-1}$	Solubility ($mg\,mL^{-1}$	Permeability coefficient ($cm\,h^{-1}$) × 1000	Lag time (h
20	0·49 ± 0·19	0·45 ± 0·15	1·08 ± 0·43	5·83 ± 2·30
40	1·13 ± 0·19	8·53 ± 1·60	0·13 ± 0·02	4·44 ± 0·81
50	1·24 ± 0·21	18·52 ± 1·17	0·07 ± 0·01	5·41 ± 1·05
60	1·99 ± 0·47	38·95 ± 3·70	0·05 ± 0·01	7·16 ± 2·04
70	2·69 ± 0·69	68·32 ± 2·92	0·04 ± 0·01	2·89 ± 0·84
80	2·56 ± 0·67	140·01 ± 11·91	0·02 ± 0·00	3·36 ± 0·08
100	1·08 ± 0·29	334·03 ± 23·49	0·00 ± 0·00	2·09 ± 0·67

Each value represents the mean ± s.d. of triplicate experiments

A prodrug, by definition, is a pharmacologically inactive derivative of a parent drug molecule that requires spontaneous or enzymatic transformation within the body to release the active drug, and that has improved delivery properties over the parent drug molecule (Bungaard et al 1985). Figure 5 shows the permeation profiles of testosterone when various testosterone esters are saturated in a 70% ethanol/water co-solvent system. The amount of ester derivatives in the receptor solution was negligible, probably due to the rapid degradation to testosterone during the skin permeation process. At the start of this investigation, we expected that more testosterone would penetrate through the skin with more lipophilic ester derivatives. However, although the permeation rate of testosterone was higher than testosterone hemisuccinate as expected, it was also higher than testosterone propionate and testosterone-17β-cypionate, which are more lipophilic than testosterone.

The skin is composed of a comparatively lipophilic stratum and hydrophilic viable skin (epidermis and dermis). Therefore, partitioning into the stratum corneum becomes the rate-determining step of skin permeation for hydrophilic drugs, whereas partitioning out of the stratum corneum into the viable epidermis becomes important for drugs with high lipophilicity. Testosterone propionate and testosterone-17β-cypionate have relatively high lipophilicity (log P = 4·39 and 4·79, respectively), and so they can easily partition into the stratum corneum but may not be able to escape out of the stratum corneum into the viable epidermis. Yano et al (1986) reported parabolic relationships between skin permeability and log P values, with peak permeability at log P of 2·24 and 2·43 for homo-

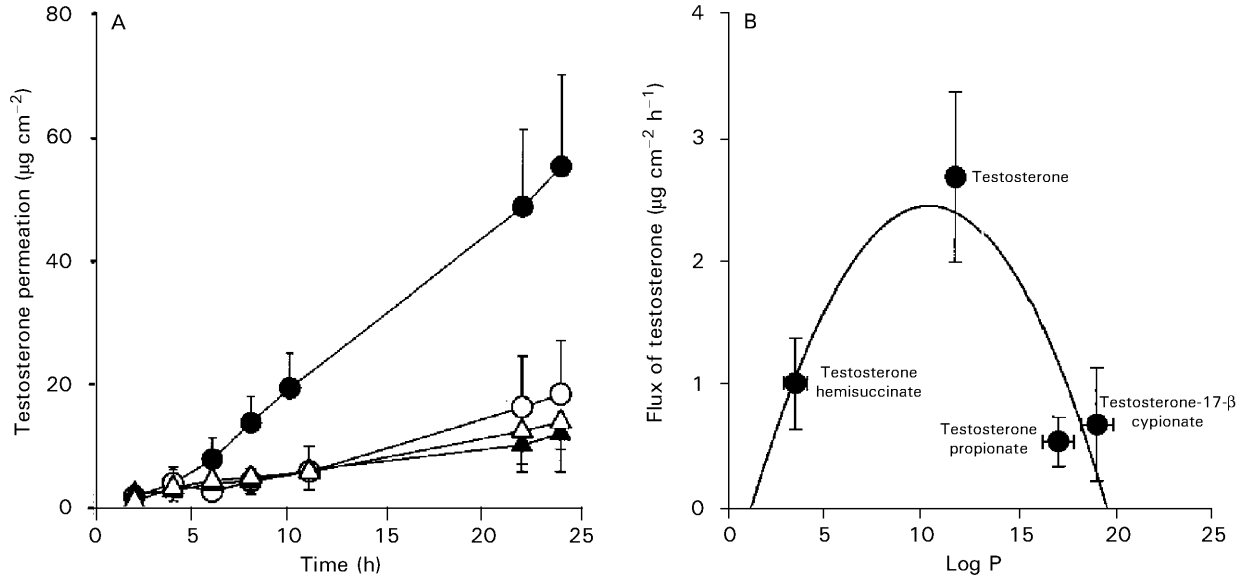

Figure 5. A. Skin permeation profiles of testosterone (●), testosterone hemisuccinate (○), testosterone propionate (▲), and testosterone-17β-cypionate (△), saturated in 70% (v/v) ethanol in water solution at 37°C for 48 h. The cumulative amount of testosterone was plotted as a function of time since the amount of testosterone derivatives in the receptor solution was negligible. B. Plot of octanol/water partition coefficient vs flux of testosterone when each drug was saturated in 70% (v/v) ethanol in water solution.

logous salicylate and non-steroidal anti-inflammatory drug series, respectively. In other studies, characteristic parabolic shapes were reported for the relationship between skin permeability and lipophilicity, with a maximum permeability at log P of approximately 2–3 (Kai et al 1990; Dies et al 1991; Lee et al 1994). In this study, we observed also a parabolic relationship between the skin permeation rate and log P value, and the maximum flux with testosterone which has the log P value of 3·4 (Figure 5). From the results of this study, it was found that compounds with log P values of around 3 are suitable for skin permeation. Also, skin permeation rate was maximized when these compounds were saturated in a 70% ethanol/water co-solvent system.

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