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# SYNERGISTIC EFFECT OF *d*-LIMONENE AND ETHANOL ON THE TRANSDERMAL PERMEATION OF NB-818

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## ABSTRACT

The promoting effect of typical absorption enhancers and various terpenes on the transdermal permeation of NB-818, a new calcium entry blocker of dihydropyridine class, through a hairless mouse excised skin from an ethanolic suspension system was investigated in vitro. The permeability of NB-818 was markedly enhanced by incorporating *d*-limonene and *β*-pinene into the suspension. While the other enhancers tested only slightly affected the skin permeation of NB-818. The steady-state flux (*Jss*) of NB-818 in logarithmic scale linearly increased with increasing ethanol concentration up to 62.5%, then drastically enhanced by further increase of ethanol concentration. The trend in *Jss* differed from that in solubility suggesting that higher concentration of ethanol might have a strong direct effect on the stratum corneum. The *Jss* values of NB-818 at 25-50% ethanol concentration were significantly enhanced by the incorporation of 2% of *d*-limonene. Combined effect of *d*-limonene and ethanol on the transdermal permeation of NB-818 was investigated by comparing the *Jss* values of NB-818, *d*-limonene and ethanol. The both ingredients appeared to act synergistically on the skin permeation of NB-818.

## INTRODUCTION

NB-818, isopropyl methyl (±)-6-carbamoyloxymethyl 4-(2,3-dichlorophenyl)-1,4-dihydro-2-methyl 3,5-pyridinedicaboxylate, is a new calcium entry blocker in dihydropyridine class developed for the treatment of hypertension, the improvement of cerebral blood flow, etc. NB-818 has such

troublesome problems as extremely low solubility and poor absorbability in dosage form design. Moreover, NB-818 absorbed from the gastrointestinal tract undergoes hepatic first pass elimination. Although NB-818 could be rapidly absorbed after oral administration by means of a solid dispersion technique (1), its bioavailability was relatively low due to the deposition of NB-818 from its metastable state in the gastrointestinal tract and the hepatic first pass elimination of drug absorbed. Furthermore the biological half-life of NB-818 is too short to realize once a day dosing (2).

Transdermal drug delivery system offers many advantages over conventional routes of drug administration. Namely extended duration of activity, avoidance of hepatic first pass elimination, improvement of patient compliance, etc. (3). However, the absorption rate after transdermal application of most drugs is low and the plasma concentration can not reach the therapeutic level owing to the barrier property of the stratum corneum of skin (4,5). In order to overcome the low skin permeability of drug, many solvent systems (6-10) and many permeation enhancers (11-17) have been applied. There are two typical mechanisms of action for the improvement of drug permeation through the skin. One is an action of rising the partitioning of drugs between the stratum corneum and dosing vehicles. The other is an action of improving the permeability of drugs through the stratum corneum. However, both modes are considered to be closely related to the transdermal absorption of drugs in the case of the practical formulations and effects of enhancing system on the permeation usually depends upon physicochemical characteristics of drug.

In this study, we examined the efficacy of some typical enhancers and terpenes on the permeation of NB-818 through excised hairless mouse skin by a vertical cell method. The synergy of *d*-limonene which most effectively improved skin permeation of NB-818 under the coexistence of ethanol was investigated by comparing the *Jss* values of the drug, enhancer and ethanol.

## EXPERIMENTAL

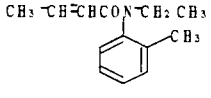
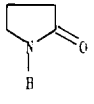
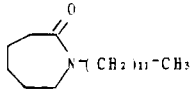
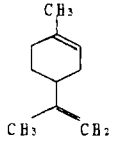
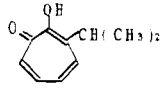
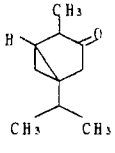
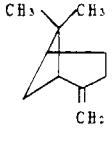
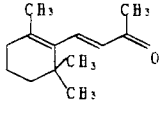
### *Materials*

Both NB-818 and its derivatives, NPK-127 (as an internal standard for NB-818), were synthesized in Banyu Co., Ltd. Sources of chemicals tested as absorption enhancers were listed in Table I. Bovine serum albumin Fraction V was purchased from Seikagaku Kogyo Co., Ltd. The other chemicals used were of reagent grade or HPLC grade.

### *In Vitro Absorption Test*

The hairless mice (Hr-1, Hoshino test animals Co., Ltd.) weighing about 25 g were killed by cervical dislocation, and approximately 10 $cm^2$ area

## Table 1. Transdermal Absorption Enhancers tested in This Study

| Enhancers | Chemical structure | Molecular weight | State at RT | Source |
|---|---|---|---|---|
| Isopropyl myristate | $CH_3(CH_2)_{12}COOCH(CH_3)_2$ | 270.44 | liquid | TK[b] |
| Dimethylsulfoxide | $(CH_3)_2SO$ | 78.13 | liquid | KC[c] |
| Crotamiton (1) | (1)  | 203.27 | liquid | KK[d] |
| 2-Pyrroridone (2) | (2)  | 85.10 | liquid | TK |
| Azone™ (3) | (3)  | 281.48 | liquid | NR[e] |
| D-(+)-Limonene (4) | (4)  | 136.23 | liquid | TK |
| Hinokitiol (5) | (5)  | 164.20 | solid[a] | TK |
| Thujone (6) | (6)  | 152.23 | liquid | TK |
| β-Pinene (7) | (7)  | 136.23 | liquid | TK |
| β-Ionone (8) | (8)  | 192.29 | liquid | TK |
| Linalool | $(CH_3)_2C=CH(CH_2)_2C(OH)(CH_3)CH=CH_2$ | 154.24 | liquid | TK |
| Suqualane | $(CH_3[CH(CH_3)=CHCH_2CH_2]_2CH(CH_3)CH_2CH_2-)_2$ | 422.80 | liquid | TK |
| Farnesol | $(CH_3)_2C=CH(CH_2)_2C(CH_3)=CH(CH_2)_2C(CH_3)=CCH_2OH$ | 222.36 | liquid | TK |
| Citroneroll | $(CH_3)_2C=CH(CH_2)_2CH(CH_3)CH_2CH_2OH$ | 156.26 | liquid | TK |
| Geraniol | $(CH_3)_2C=CH(CH_2)_2C(CH_3)=CHCH_2OH$ | 154.24 | liquid | TK |

a) melting point: 37°C, b) Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan), c) Katayama Chemical Co.. Ltd. (Osaka, Japan), d) Kongo Kagaku Co., Ltd. (Toyama, Japan), e) Nelson Research & Development Co. (Carifolnia, USA).

of abdominal skin was excised. After removal of the subcutaneous fat, the skin was washed with saline and used within 1 hours. The skin was mounted and fixed between the cell body and cell head with epidermis side facing upward, as shown in Fig. 1. The surface area exposed to the donor phase was 4.9 $cm^2$. Ethanolic suspension of the donor phase contained 40 mg of NB-818 in 2 ml. The donor phase was sealed with a glass stopper and sealing film. The receiver phase, in contact with the underside of the skin, was 5% albumin/saline solution or 50% n-propanol solution. The cells were placed in a thermostatically controlled water bath at 37°C. Uniform mixing of the drug solution in the receiver phase was achieved by a small teflon stirring bar driven by an external magnetic stirrer at approximately 300 rpm. At an appropriate time interval, the receiver solutions (0.5 ml) were withdrawn and replaced by the same volume of fresh receiver solution to maintain a constant volume. The sample solutions were kept at -20°C until HPLC and GLC analysis.

*Solubility Measurementt*

An excess amount of NB-818 was added to the ethanolic solution with or without permeation enhancers, and the solutions were mixed well at 37°C for 3 days. After the aliquot of the mixture was passed through a membrane filter of 0.45 μm in pore size, the concentration of NB-818 in the filtrate was measured by HPLC.

*Measurement of Partition Coefficient*

Obtaining exact partition coefficient between stratum corneum and vehicle is difficult technically. Since isopropyl myristate (IPM) is supposed to have similar solvent characteristics as a stratum corneum (7), the partition coefficient was estimated by the ratio of NB-818 solubility in IPM to that in donor solvent at 37°C.

*Analytical Procedure of NB-818*

Concentration of NB-818 was determined by HPLC. Pretreatment of samples for HPLC determination of unchanged NB-818 was carried out according to the procedure of the BOND ELUT™ extraction technique shown in Fig. 2. More than 98% of NB-818 was recovered from the BOND ELUT absorbent at the methanol elution process of the step "(7)" in Fig. 2. A Shimadzu LC-6A solvent delivery system equipped with a system controller SLC-6A, an UV detector SPD-6AV set at 350 nm, a guard column, Nucleosil $_5C_{18}$ (4.6 mm i.d. x 50 mm), and a reverse phase column, Nucleosil $_5C_{18}$ (4.6 mm i.d. x 250 mm) was employed. The injection volume of the pretreated sample was 30 μl. Elution of NB-818 was carried out at the flow rate of 1 ml/min using the mixture of acetonitrile and water (65/35) at 40°C.

*Analytical Procedure for d-Limonene and Ethanol*

Concentration of *d*-limonene and ethanol permeated were determined by gas liquid chromatography (GLC). To a 0.1 ml of sample solution, a 1 ml of 0.08% methanol solution was added as an internal standard and mixed well. A 1 μl of the solution was applied to the GLC. A Hewlett Packard 5840A gas chromatograph equipped with hydrogen flame ionization detector, an integrator and a PEG 20M bonded capillary column (0.53 mm i.d. x 25 m; Gasukuro Kogyo Inc.). Elution of ethanol and *d*-limonene was done at the flow rate of 10 ml/min using a helium gas as a mobile phase. The operating temperatures of injection port and column oven were 200 and 35°C, respectively.

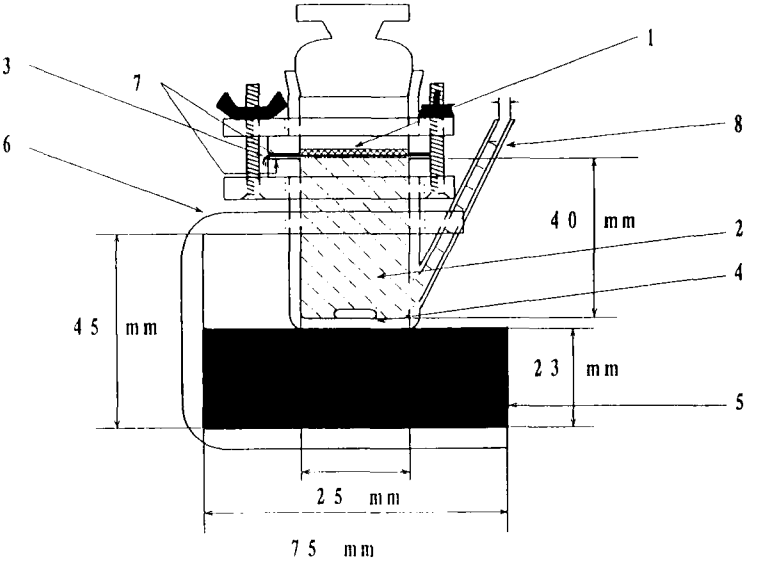



**FIGURE 1**

**Schematic Illustration of Vertical Cell for Evaluating Transdermal Permeation of Drug In Vitro**

**1; donor suspension (2 ml), 2; receiver solution (20 ml), 3; hairless mouse skin, 4; stirring bar, 5; stirrer, 6; stand, 7; O-ring, 8; sampling duct**

## RESULTS AND DISCUSSION

### *Effect of Enhancers on Skin Permeation of NB-818*

The influence of typical absorption enhancers and various terpenes on the permeation of NB-818 through a excised hairless mouse skin was evaluated in vitro diffusion method to find out effective enhancers. The saline solutions containing 5 % bovine serum albumin and neat ethanol were employed as a receiver solution and a donor one, respectively. The chemical structure of compounds tested in this study are summarized in Table 1. Effects of these compounds on the steady-state flux (*Jss*) and permeability constant (*Pss*) of NB-818 are summarized in Table 2 .

The *Jss* value is given by ;

$$Jss = Q/At = D\,PC\,Cp/L$$

where $Q$ is the amount of permeant that diffuses across the area, $A$, in time, $t$; $PC$, is the partition coefficient between the skin and donor solvent of the

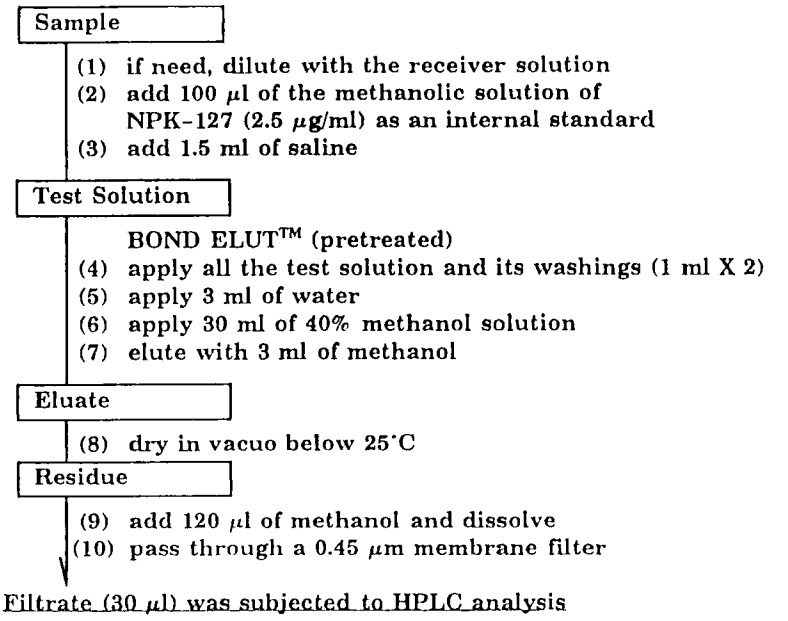



**FIGURE 2**

**Extraction Procedure of NB-818 for HPLC Analysis**



permeant ; *D* is the permeant diffusion constant in the skin of thickness, *L*, and *Cp* is the permeant concentration in the donor solvent. The steady state permeability constant, *Pss*, is given by *Jss*/*Cp*.

The *Jss* value of NB-818 from the system with 2% *d*-limonene (code F) or 2% β-pinene (code L) was about 30 times greater than that from control (code A), and the both compounds were superior to a typical strong enhancer, 2% AZONE™ (code G), in transdermal permeation promoting effect for NB-818. The other compounds tested gave only a weak promoting effect on the skin permeation of NB-818. In addition, effects of combined use of AZONE with terpenes on transdermal permeation were also evaluated. In spite of the low activity of AZONE or β-ionone alone, permeation enhancing ability of the combined system tested (code R) was rather great. The effects of these compounds on the thermodynamic properties, solubility and partition coefficients, of NB-818 were studied and the results are summarized in Table III. No relationship was seen between the *Jss* value and thermodynamic properties of NB-818 in donor suggesting that the enhancement of skin

**Table 2. Effect of Enhancers on Transdermal Absorption of NB-818 through excised Hairless Mouse Skins In Vitro Vertical Cell Method at 37°C**

| Donor Solutions | | Steady state flux | Permeability constant |
|---|---|---|---|
| Enhancers | Concentration (%) | (Jss; $\mu g/h \cdot cm^2$) | (Pss; $10^{-5}$ cm/h) |
| None (Control) | 0 | 0.660 ± 0.066[a)] | 5.596 ± 0.560[a)] |
| Isopropyl myristate | 2 | 5.356 ± 0.794* | 44.900 ± 6.655* |
| Dimethylsulfoxide | 2 | 0.620 ± 0.016 | 4.068 ± 0.104 |
| Crotamiton | 2 | 0.473 ± 0.014* | 4.127 ± 0.122 |
| 2-Pyrroridone | 2 | 0.567 ± 0.033 | 3.664 ± 0.213 |
| Azone™ | 2 | 1.726 ± 0.407 | 15.274 ± 3.602 |
| D-(+)-Limonene | 2 | 19.491 ± 1.612** | 153.755 ± 12.72** |
| Hinokitiol | 2 | 0.940 ± 0.134 | 7.947 ± 1.006 |
| Thujone | 2 | 2.018 ± 0.910 | 23.667 ± 7.692 |
| β-Pinene | 2 | 17.681 ± 0.665** | 145.167 ± 5.460** |
| β-Ionone | 2 | 1.063 ± 0.180* | 7.197 ± 1.459 |
| Linalool | 2 | 0.628 ± 0.193 | 7.080 ± 1.668 |
| Suqualane | 2 | 1.464 ± 0.480 | 19.477 ± 4.252 |
| Farnesol | 2 | 1.772 ± 0.547 | 19.504 ± 4.880 |
| Citroneroll | 2 | 1.084 ± 0.094* | 10.509 ± 0.839* |
| Geraniol | 2 | 1.257 ± 0.574 | 14.957 ± 4.996 |
| Azone™/D-(+)-Limonene | 2(1/1) | 16.521 ± 1.998** | 128.468 ± 15.54** |
| Azone™/β-Ionone | 2(1/1) | 9.664 ± 2.603* | 77.873 ± 20.98* |

a) Each value represents the mean ± S.E. of 3–6 determinations.
b) Significant difference from control (*; $p<0.05$, **; $p<0.001$, t-test).

**Table 3. Solubility of NB-818 in Donor Solutions and Partition Coefficient of NB-818 between Isopropyl Myristate and Donor Ethanol Solutions**

| Ethanolic Donor Solution | | Solubility of NB-818 | Partition Coefficient[a)] |
|---|---|---|---|
| Enhancers | Concentration (%) | (mg/ml) | |
| None (Control) | 2 | 11.79 | 0.0450 |
| Isopropyl Myristate | 2 | 11.93 | 0.0444 |
| Dimethylsulfoxide | 2 | 15.24 | 0.0348 |
| Crotamiton | 2 | 11.47 | 0.0462 |
| 2-Pyrroridone | 2 | 15.48 | 0.0342 |
| Azone™ | 2 | 11.30 | 0.0469 |
| D-(+)-Limonene | 2 | 12.67 | 0.0418 |
| Hinokitiol | 2 | 13.32 | 0.0398 |
| Thujone | 2 | 12.83 | 0.0413 |
| β-Pinene | 2 | 12.18 | 0.0435 |
| β-Ionone | 2 | 12.34 | 0.0429 |
| Linalool | 2 | 11.57 | 0.0458 |
| Suqualane | 2 | 11.29 | 0.0469 |
| Farnesol | 2 | 11.21 | 0.0473 |
| Citroneroll | 2 | 11.21 | 0.0473 |
| Geraniol | 2 | 11.49 | 0.0461 |
| Azone™/D-(+)-Limonene | 2(1/1) | 12.86 | 0.0412 |
| Azone™/β-Ionone | 2(1/1) | 12.41 | 0.0427 |

a) estimated by the ratio of drug solubility in isopropyl myristate to that in donor solution

permeability of NB-818 obtained by *d*-limonene and β-pinene might be due to a direct effect on the stratum corneum.

In relation to the skin permeation promoting effect of cyclic terpenes, Obata et al reported that absorption of hydrophilic sodium dicrofenac was markedly enhanced in the presence of *l*-menthol or *dl*-menthone, while was not enhanced in the presence of *d*-limonene (18). In contrast to this result, *d*-limonene significantly promote the absorption of indomethacin and ketoprofen which have a similar nature to NB-818 in its high lipophilicity (12). This agrees with the result of NB-818 observed in this study. Several investigators also reported the permeant-specificity of promoting effect of terpenes (11,17,19, 20). Neat ethanol, which was well known to increase the diffusibility of permeant in both pore and lipid pathways, significantly rose the *Jss* value of NB-818 shown below. The promoting activity of enhancers except *d*-limonene and β-pinene was poor. It was also reported that the spectrum of skin permeation enhancing activity by promotor varied greatly with changing concentration of ethanol, and that the combined effect of promoters and ethanol on the permeation of permeant was synergistic (21). Thus, transdermal permeation has been revealed to be influenced not only by the intrinsic promoting activity of promotor but also by the physicochemical congeniality between permeant and promotor, the thermodynamic activity of the both in a formulation, and the direct effect of promotor and solvent on a skin. Thus, the effect of concentration of donor solvent ethanol and combined effect of *d*-limonene and ethanol on the skin permeation of NB-818 was investigated.

*Transdermal Absorption of NB-818 from Binary Solvent System*

Figure 3 shows the effect of ethanol on the solubility in logarithmic scale of NB-818 in binary solvent system (open circle). In subsequent experiments 50% n-propanol/water was used as a receiver solution which can be directly applied to the GLC. The effect of ethanol concentration on the *Jss* value in logarithmic scale of NB-818 is also shown in Figure 3 (closed circle). Both of *Jss* value and solubility of NB-818 increased as a function of concentration of ethanol in the donor solution, however, the trend of both curves was not similar. With increasing the concentration of ethanol up to 62.5%, *Jss* values in a logarithmic scale of NB-818 increased linearly, and this tendency coincided well with increasing the solubility in the logarithmic scale of NB-818 in donor solution. Inflection point on the concentration-permeation curve of NB-818 was observed at 62.5% of ethanol and an impressive elevation in the *Jss* value of NB-818 was brought about at the

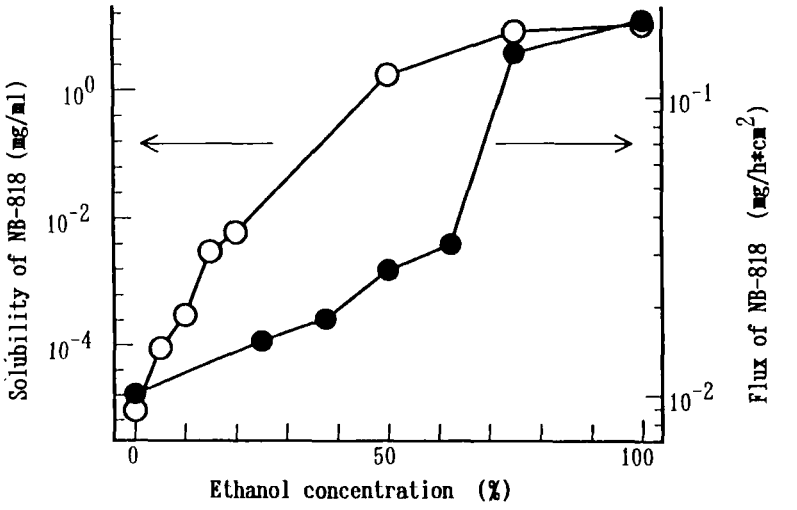



**FIGURE 3**

**Solubility Profile (○) and Steady State Flux (●) of NB-818 as a Function of Concentration of Ethanol**

**Each point represents the mean of 2-3 determinations.**

higher concentration of ethanol. While, the gradual increase of the solubility of NB-818 in donor solution was observed with further increase of ethanol concentration. The trend of the permeation can not be explained satisfactorily by the change of solubility of NB-818 in the donor solution. The permeation-enhancing effect by ethanol has been reported by many investigators. Seki et al. reported that the *Jss* value of nicardipine hydrochloride as a function of ethanol concentration correlated well to the solubility of the drug in donor solution (10). This result is significantly different from our one. Our result is in rough accord with the results in highly lipophilic ibuprofen and flurubiprofen (22). Hatanaka et al reported that a skin penetration-enhancing effect by ethanol depended upon its concentration in donor and that the phenomena might be induced by the direct effect on both lipid and pore pathway (22). In this way NB-818 is considered to be mainly permeated through a lipid pathway similarly to ibuprofen, flurubiprofen, etc., and the permeation was drastically enhanced by the presence of higher concentration of ethanol than 75%. However, such high concentration of ethanol leads to a serious damage on the skin. Thus

the combined effect of a prominent enhancer, *d*-limonene, and ethanol was investigated to design suitable formulation.

The Synergy of *d-Limonene with Ethanol on Permeation*

Permeation profiles of NB-818 (permeant), *d*-limonene (promoter) and ethanol (solvent) from the donor systems were investigated to reveal the role of enhancer and solvent on the transdermal permeation of NB-818. Figure 4 shows the effect of ethanol concentration on the absorption profiles of NB-818, *d*-limonene and ethanol itself through the skin from the donor system with 2% *d*-limonene. Ethanol dramatically enhanced the permeation of not only NB-818 but also *d*-limonene, and ethanol itself was most rapidly permeated (Fig. 4c). Apparent equilibrium concentrations in permeation of ethanol were observed to be dependent on the initial concentration of ethanol in donor solvent, and the time until achieving equiriblium concentrations were very short, while it takes 8-24 hours in the case of *d*-limonene.

Figure 5a shows the effect of ethanol concentration on the *Jss* values of NB-818 from the donor system with or without 2% *d*-limonene. In the case of donor system without *d*-limonene, the *Jss* values of NB-818 were very small at the lower concentration range of ethanol (0-62.5%) and then increased markedly at the higher concentration of ethanol (more than 75%). On the other hand, the *Jss* value from the donor system with 2% *d*-limonene significantly increased almost linearly up to 50% ethanol concentration. The lag time remarkably decreased as shown in Figure 5b suggesting that the observed increase in the *Jss* value mainly depend upon the increase of diffusivity of NB-818 in the skin. Increasing concentration of ethanol to 75% did not lead to further enhancement in the *Jss* value, and the value was almost the same as that in the case of 50% ethanol. In the donor system, *d*-limonene was excess up to 85% ethanol, and rapidly passes into further concentrated ethanol. Accordingly the decrease of the *Jss* value of NB-818 in neat ethanol can be explained by the decrease of thermodynamic activity of *d*-limonene. These observations clearly indicate the synergistic effect of *d*-limonene with ethanol on the *Jss* value of NB-818 over 25-50% of ethanol concentration. Figure 6a shows the *Jss* value of *d*-limonene increased almost linearly with increasing the concentration of ethanol over from 0-75% and decreased with further increasing the ethanol concentration. Figure 6b shows the *Jss* value of ethanol observed with the same conditions as that of *d*-limonene. When neat ethanol was used, the *Jss* value of NB-818 without

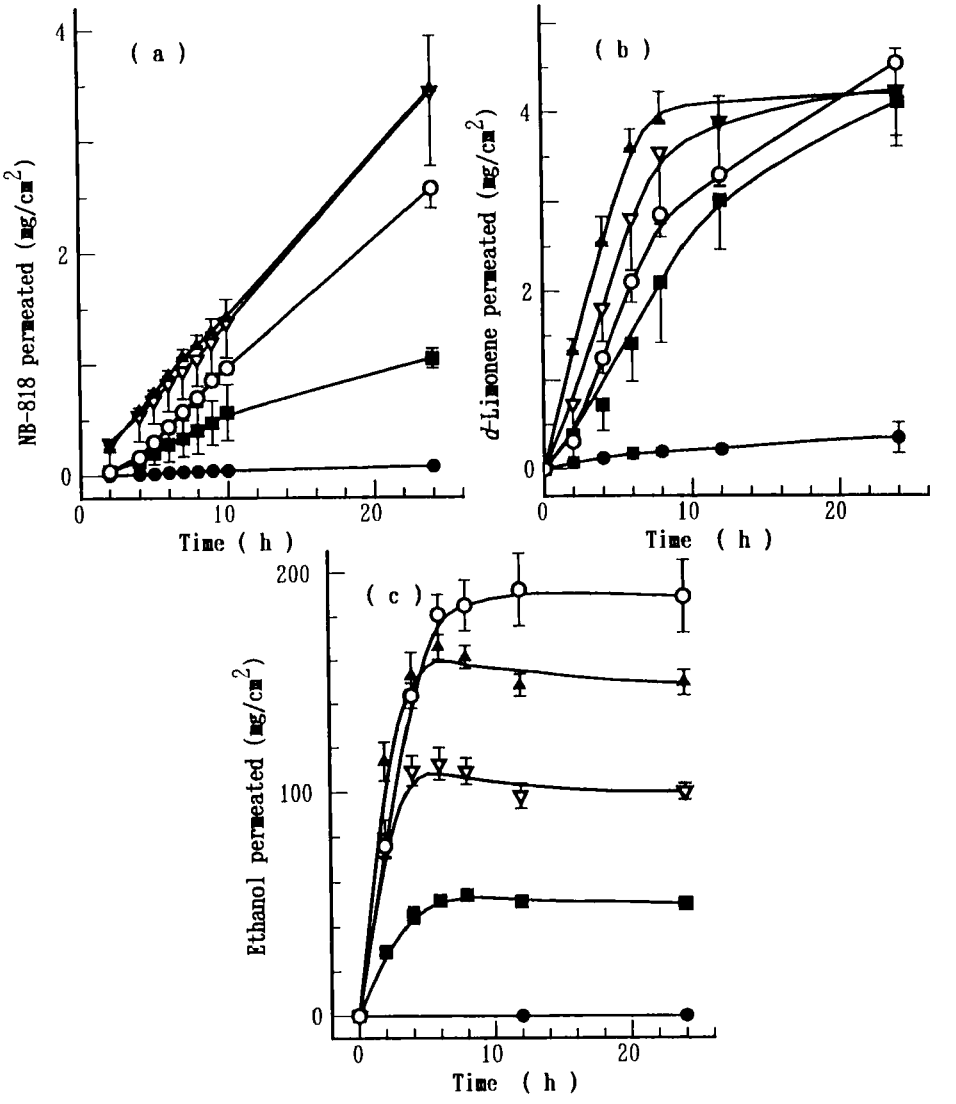



**FIGURE 4**

**Transdermal Permeation Profiles of NB-818 (a), *d*-Limonene (b) and Ethanol (c) through Hairless Mouse Skin from NB-818 Ethanolic Suspension with d-Limonene**

**Each points represents the mean ± S.E. of 3-5 determinations.**
**● ; 0% ethanol, ■ ; 25% ethanol, ▽ ; 50% ethanol,**
**▲ ; 75% ethanol, ○ ; 100% ethanol,**

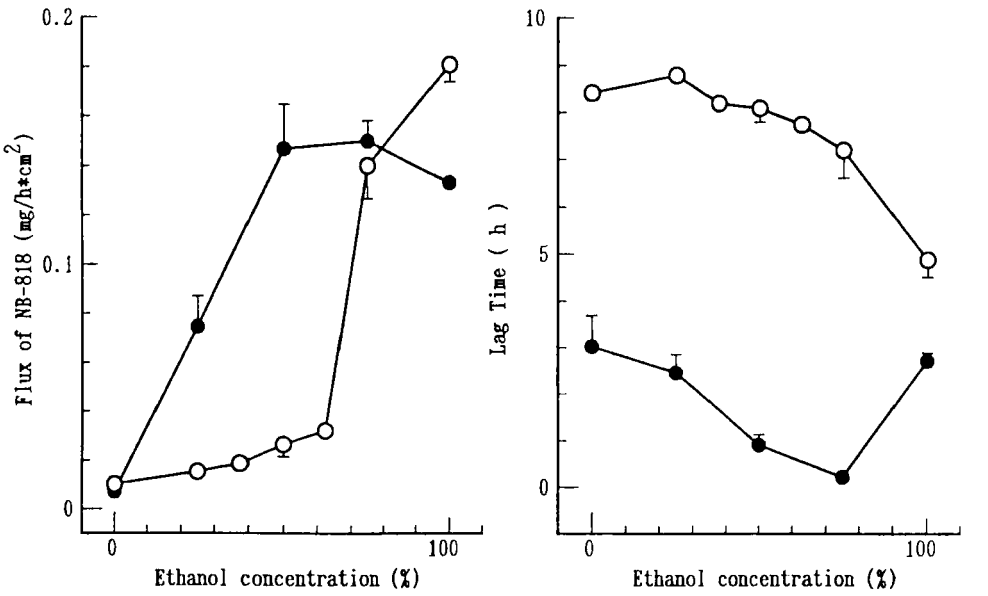



**FIGURE 5**

**Steady State Flux (Jss) and Lag Time of NB-818 through Hairless Mouse Skin from Suspension with (closed) or without (open) 2% *d*-Limonene in Donor Solution**

**Each point represents the mean ± S.E. of 3–5 determinations.**

***d*-limonene became greater than that with *d*-limonene (Fig. 5a). In that case the *Jss* value of ethanol was also decreased by *d*-limonene (Fig. 6b), and the thermodynamic activity of ethanol with *d*-limonene was naturally smaller than that without *d*-limonene because of mutual dilution.**

**From the results mentioned above, apparent improvement in the *Jss* value of NB-818, which was brought about by the coexistence of *d*-limonene with ethanol, could be explained as follows. Transdermal permeation of ethanol was very fast compared to that of *d*-limonene and NB-818, as shown in Figure 4. Accordingly, ethanol at first penetrates into the stratum corneum of the skin. Permeation promoting mechanism by ethanol has been reported to relate the increase of lipid fluidity in stratum corneum. As the *Jss* value of *d*-limonene was greater than that of NB-818, *d*-limonene transports into the stratum corneum following disposition of ethanol. At this time, it is also obvious from Figure 4 that the coexistence with ethanol in the donor phase**

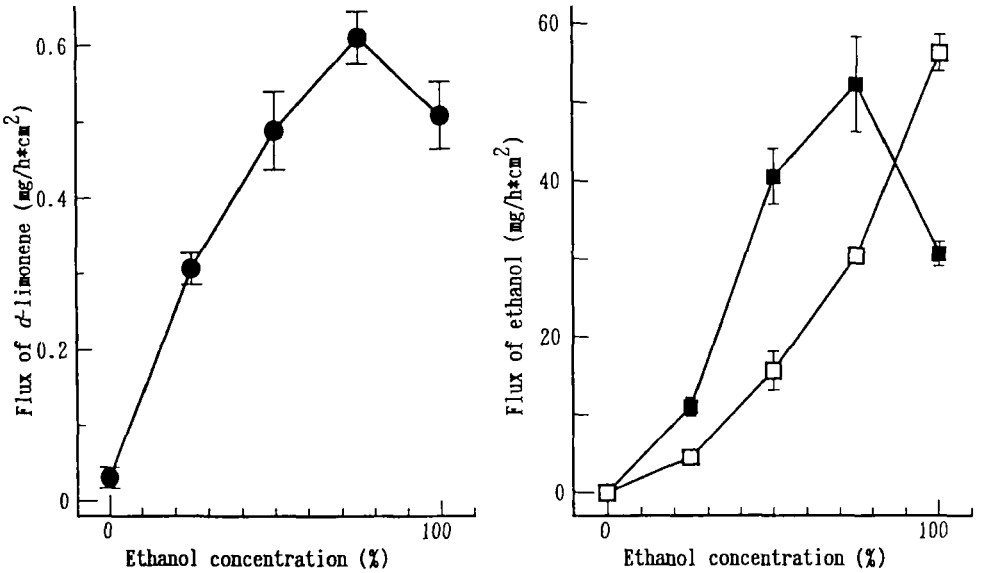



**FIGURE 6**

**Steady State Fluxes (Jss) of *d*-Limonene (a) and Ethanol (b) in In Vitro Absorption Test using Hairless Mouse excised Skin**

**Each point represents the mean ± S.E. of 3-5 determinations. Samples containing 2% NB-818 and 0% (open) 2% (closed) *d*-limonene in donor solution.**

**is essential. *d*-Limonene transported into the stratum corneum under the coexistence with ethanol may decrease the barrier function of stratum corneum on the permeation of NB-818 through the skin, and thereby results in the marked increase of the *Jss* value. Enhanced solubility of NB-818 would help the disposition of NB-818 to stratum corneum. In conclusion, it was found that *d*-limonene enhanced synergistically the transdermal absorption of NB-818 under the coexistence with ethanol at lower concentrations (25-50%). Moreover, the possibility of absorption enhancement by combined use of enhancers was also observed in such a system as β-ionone/AZONE. Clarifying the permeation behavior of all permeants and synergy among permeants is important to understand the mode of action of many candidate compounds as absorption enhancers.**

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