Feasibility of Use of Several Cardiovascular Agents in Transdermal Therapeutic Systems with *l*-Menthol–Ethanol System on Hairless Rat and Human Skin

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Effect of the simultaneous use of *l*-menthol and ethanol on the skin permeation of six potent cardiovascular agents: nicardipine hydrochloride, atenolol, captopril, nifedipine, vinpocetine and nilvadipine (in hydrophilic order) was investigated to evaluate the feasibility of their use in a transdermal therapeutic system (TTS). *In vitro* diffusion experiments were carried out using excised hairless rat and human skin, and the application area of TTS required for the minimum therapeutic effect was estimated by a simple pharmacokinetic calculation. Marked enhancing effect by the *l*-menthol–ethanol system was found independent of drug lipophilicity, but the mode of action was dependent on the lipophilicity of the drug. The action of the system on lipophilic drugs (nifedipine, vinpocetine and nilvadipine) was mainly due to their increase in solubility in the system, while that on hydrophilic (or water soluble) drugs (nicardipine hydrochloride, atenolol and captopril) was the result of increase in their skin permeability coefficient. This enhancing effect was adequate to assure their minimum effective concentration (MEC) in human. The area of application of a drug to maintain the MEC was calculated to be 0.15 cm^2 for hydrophilic or water soluble drugs and 3.7—13 cm^2 for lipophilic drugs.

Keywords enhancer; *l*-menthol; ethanol; skin permeation; cardiovascular agent; feasibility study

Since the transdermal therapeutic system (TTS) provides a non-invasive and parenteral route of administration, it is appropriate as a dosage form for patients who cannot take medicines by themselves. The skin permeability of most drugs, however, is not sufficient to exert its therapeutic effect, making it necessary to enhance skin permeation to develop the actual potential of TTS. Use of a chemical enhancer is one method,[1-3] but few enhancers have been found which are both safe and effective. The effect of enhancer is dependent on the physicochemical property of a drug.[4-6] Therefore, in developing TTS with the desired pharmacological effect, two major factors must be known: enhancement characteristics of the selected enhancer, and the candidate drug which can best exert its therapeutic effect in combination with existing enhancers.

We have already reported a simultaneous use of *l*-menthol and ethanol (M-E) to increase skin permeation of morphine hydrochloride.[7] The effect was more marked than that obtained with Azone®, Sefsol®-318, isopropyl myristate or other enhancers. As a subsequent experiment, cardiovascular agents were combined with M-E to identify the enhancement characteristics of M-E, and to determine the optimal candidate drug for use with M-E. Six cardiovascular agents: nicardipine hydrochloride (NC), atenolol (ATL), captopril (CPT), nifedipine (NF), vinpocetine (VIN) and nilvadipine (NL) (in hydrophilic order) were selected as model drugs. These drugs are potent and beneficial in keeping blood level constant for the control of cardiovascular diseases.[8,9]

An *in vitro* diffusion experiment was done using hairless rat and human skin. Hairless rat skin was used in the study on enhancement characteristics of M-E because of the restricted availability of human skin in Japan, whereas the feasibility study on combination of an individual drug and M-E was carried out using human skin. To evaluate the applicability of TTS to human patients, the least TTS diffusion area to maintain a minimum effective concentration was calculated from the *in vitro* permeation data using human skin.[2] Some information on species difference in the enhancing effect of M-E is also reported.

Experimental

All procedures for NC, NF and NL were carried out under subdued lighting conditions due to their high sensitivity to light.

Materials CPT and NL were generously supplied by Sankyo Company Ltd. (Tokyo, Japan) and Fujisawa Pharmaceutical Co., Ltd. (Osaka, Japan), respectively. NC, ATL and VIN were gifts from Nissan Chemical Industries (Tokyo), Kyukyu Pharmaceutical Co., Ltd. (Tokyo) and Kowa Pharmaceutical Industries, Ltd. (Saitama, Japan), respectively. NF was purchased from Wako Pure Chemical Industries, Ltd. (Osaka). *l*-Menthol (JP grade) and ethanol (EtOH, Wako Pure Chemical Industries) were used as an enhancer and a coenhancer, respectively. *N*-(1-Pyrene)maleimide (NPM, Wako Pure Chemical Industries) was used as a derivatizing reagent for CPT assay. Nitrendipine (NT) as an internal standard to assay for dihydropyridines was generously supplied by Yoshitomi Pharmaceutical Industries, Ltd. (Osaka). Other reagents were of reagent grade or HPLC grade. The composition of M-E, the enahncer–coenhancer system, was 5% *l*-menthol and 40% EtOH in water.[7]

Skin Membrane Preparations Hairless rat skin was excised from the abdomen of male WBN/ILA-Ht rats (average weight 160 g, Life Science Research Center, Josai University, Saitama, Japan) immediately before the permeation experiment. Human skin was obtained following unrelated surgical operation (Department of Surgery, Saitama Medical Center, Saitama Medical School); sources were the chests of 43 to 74 years old female patients. The human skin samples were stored at −20 °C until used. The thickness was adjusted to about 750 μm by whittling the dermis side.[10] Prior to use in the permeation experiment, the dermis side was washed with distilled water. It was confirmed that the treatment would not affect the skin permeability of the drugs to be tested. Human skin pieces from the same site of a patient were used to compare the M-E with a water vehicle.

Skin Permeation Procedure Skin permeation experiments were done according to the method of Okumura *et al.*[11] A diffusion cell consisting of two half-cells with a water jacket connected to a water bath at 37 °C was used. Each half cell had a volume of 2.5 ml and an effective area of 0.95 cm^2. The dermis side of the skin was in contact with the receiver

compartment and the stratum corneum with the donor compartment. Donor compartment was filled with the drug solution or suspension and the receiver compartment with distilled water or 40% polyethylene glycol 400/water (40% PEG). The PEG solution was used for hydrophobic drugs to prevent dissolution limiting into the receiver medium. It was reported that the skin barrier function was not influenced by 40% PEG.[12] For NC, ATL and CPT, 1% drug solution was used in the donor compartment and the receiver medium was distilled water, while 3%, 1% and 2% drug suspensions were used in the donor compartment for NF, VIN and NL, respectively, and the receiver medium was 40% PEG for these drugs. Distilled water was previously bubbled with nitrogen gas, and edetate disodium (1 mM) and ascorbic acid (1 mM) were added to the distilled water to prevent oxidation of CPT.[13] Both donor and receiver compartments were stirred with a starhead bar driven by a constant-speed synchronous motor (MC-301, Scinics, Tokyo) at 1200 rpm. Each sample was withdrawn from the receiver compartment at predetermined times for assay. A sink condition was always maintained in the receiver compartment. Each experiment using hairless rat skin was triplicated. Because of the restricted availability of human skin, only a single measurement was made for each human skin experiment.

Determination of Solubility and Partition Coefficient Excess drug was added to 1 ml of water, octanol or M-E and equilibrated at 37 °C for 24 h. The saturated drug concentration was determined by HPLC. The octanol/water partition coefficient of drugs (K_{OW}) was determined as a solubility ratio in octanol/water at 37 °C.

Assays The HPLC system for analyzing drug concentrations consisted of a pump (LC-6A, Shimadzu, Kyoto, Japan), either an ultraviolet (SPD-6A, Shimadzu) or a fluorescent detector (RF-535, Shimadzu), a 4.6 mm × 250 mm stainless column packed with Nucleosil $5C_{18}$ (Macherey Nargel, Germany), and an integrator (C-R6A, Shimadzu). The HPLC conditions are listed in Table I.

Sample Preparation for Assay CPT was derivatized for assay. After centrifugation of sample containing CPT withdrawn from receiver, 100 μl of supernatant and 50 μl of NPM (2 mM acetone solution) were dropwisely added to 2 ml of pH 7 phosphate buffer to derivatize CPT to the fluorescent adduct.[13] After stirring for exactly 15 min at room temperature, an aliquot was quickly injected to HPLC.

Two ml of sample containing NL withdrawn from the receiver compartment and 100 μl of NT methanol solution (200 μg/ml) as an internal standard were added to 2.0 ml of pH 9.0 phosphate buffer, and then both NL and NT were extracted with 2.0 ml of benzene–hexane (1 : 1) by shaking for 15 min. Following centrifugation, the upper layer was transferred to a clean test tube and was evaporated to dryness under nitrogen gas. The residue was reconstituted by the mobile phase and was subjected to HPLC.

For the other drugs, samples from the receiver were added to the same volume of methanol (with or without internal standard), and shaken by a vortex mixer. Following centrifugation, supernatant was subjected to HPLC.

TABLE I. HPLC Conditions for the Analysis of Drugs Used in This Experiment

Drug	Mobile phase	Detection (nm)	Internal standard
NC	Acetonitrile : 0.02 M potassium phosphate, monobasic (60 : 40)	UV 240	NT
ATL	Methanol : 1% phosphoric acid (60 : 40) + 2.5 mM sodium dodecylsulfate	Ex. 280 Em. 333	—[a]
CPT	Acetonitrile : 0.1% phosphoric acid (47 : 53)	Ex. 340 Em. 390	—[a]
NF	Acetonitrile : water (40 : 60)	UV 235	NT
VIN	Acetonitrile : 0.1% phosphoric acid (60 : 40) + 5 mM sodium dodecylsulfate	UV 229	*p*-Hydroxybenzoic acid hexyl ester
NL	Acetonitrile : water (60 : 40) + 1% phosphoric acid 5.25 ml + ammonium phosphate 2.5 g + 10% tetra-*n*-butylammonium hydroxide 10 ml	UV 254	NT

a) Absolute calibration method was used.

Data Analysis The maximum flux (J_{max}) is generally expressed as

$$J_{max} = P \times S \quad (1)$$

where P is the permeability coefficient and S is the solubility of the drugs.[14] Enhancing ratio (ER) is given by the following equation[15]

$$ER = J_{M-E}/J_w \quad (2)$$

where J_{M-E} and J_w are J_{max} of the drug with and without M-E as a vehicle, respectively. With zero-order delivery, the desired steady state drug input rate may then be obtained from the following equation:[16]

$$\text{drug input rate} = \text{target plasma level} \times \text{total clearance} \quad (3)$$

By substituting MEC (minimum effective concentration) for the target plasma level, Eq. 3 gives the lowest drug input rate to maintain a therapeutic effect. This input rate is defined as J_{req}. Using the parameter, A_{req}, the minimal diffusion area to maintain MEC is given as

$$A_{req} = J_{req}/J^H_{M-E} \quad (4)$$

where J^H_{M-E} is the J_{M-E} in human skin.

The resulting A_{req} was used to evaluate the applicability of TTS to human patients. Most of the values of MEC and CL_{tot} used to calculate A_{req} were cited from previous reports.[17–20] When MEC was unknown, the mean plasma concentration after ordinary dosing was substituted. In a case where CL_{tot} was unclear, the values were estimated from previous pharmacokinetical data.[21–25]

Results and Discussion

Time course of the cumulative amount of each drug to permeate through the excised hairless rat and human skin is shown in Fig. 1.

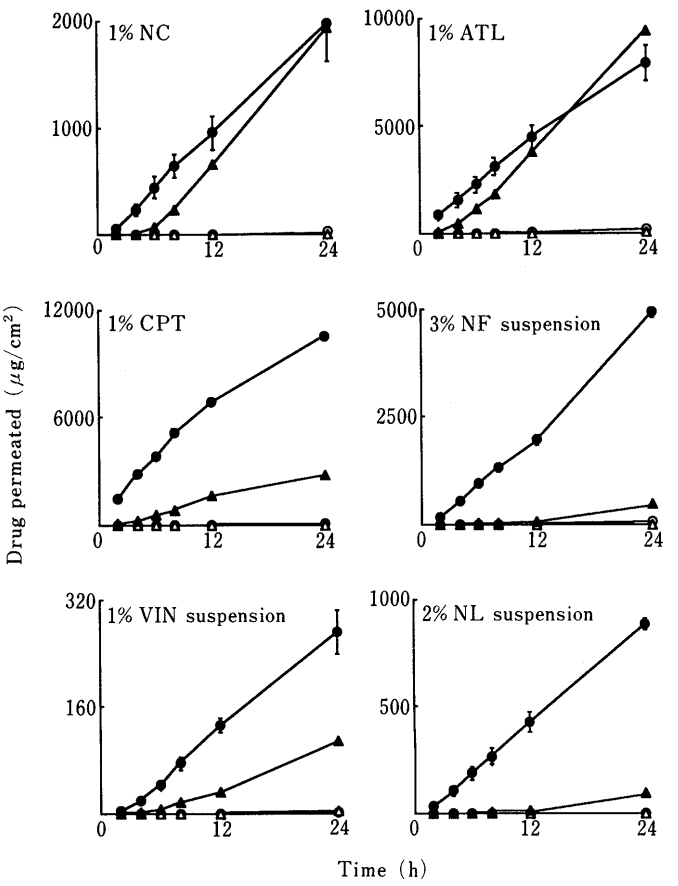

Fig. 1. Permeation Profiles of Drugs through Hairless Rat Skin (Circles) and Human Skin (Triangles) with Two Vehicles: Water (○, △) and M-E (●, ▲)

In the experiment with hairless rat skin, each data point represents the mean and S.E.M. of 3 experiments.

TABLE II. Estimated Parameters and *in Vitro* Permeation Data

		NC	ATL	CPT	NF	VIN	NL
MEC (ng/ml)		20	200	31[a]	15	0.8[a]	0.5
CL_{tot} (l/h)		23	11	48	27	43[b]	50[b]
J_{req} (μg/h)		460	2200	1488	405	34	25
Literature		17)	18, 19)	21)	20)	22,23)	24, 25)
Solubility	Water	12.7 mg	25.8 mg	138 mg	6.50 μg	5.00 μg	1.33 μg
(mg/ml or μg/ml)	Octanol	1.31 mg	12.9 mg	209 mg	14.4 mg	18.3 mg	17.9 mg
at 37 °C	M-E	319 mg	267 mg	992 mg	10.4 mg	1.39 mg	5.23 mg
$\log K_{ow}$		−0.99	−0.30	0.18	3.35	3.56	4.15
J_{max} (μg/h/cm^2)							
Rat	: Water	1.24	26.0	49.1	3.4	0.16	0.03
	: M-E	2690	7522	30683	249	11.7	38.3
Human	: Water	0.12	0.49	13.8	0.13	0.20	0.006
	: M-E	3400	12440	10049	31.2	6.45	6.84
ER	Rat	2169	289	625	73	73	1277
	Human	28333	25388	728	240	32	1086
$\log P$ (cm/s)							
Rat	: Water	−7.6	−6.6	−7.0	−3.8	−5.1	−5.2
	: M-E	−5.6	−5.1	−5.1	−5.2	−5.6	−5.7
A_{req} (cm^2)		0.14	0.18	0.15	13.0	5.3	3.7

a) Mean plasma concentration with ordinary dosing. *b*) Estimated value.

Definition of Flux to Evaluate Feasibility of Drugs and Characterization of M-E In the previous study,[7] we found that the enhancing effect of M-E on the skin permeation of morphine hydrochloride was partly attributable to the skin permeation of vehicle components, which suggested continuous change in donor condition (percent permeated: EtOH; 13.3%/2 h, 26.4%/4 h, 45.2%/8 h: *l*-menthol; 0.46%/2 h, 0.95%/4 h, 1.85%/8 h). The change in condition might also be influenced by the permeation of PEG400 from receiver to donor. The concentration of PEG400 in the donor compartment was therefore checked at 8 and 24 h after beginning of the permeation experiment by the determination method of Calzolari *et al.*[26] The concentration was 2.8±0.6% at 8 h and 14.1±4.1% at 24 h when M-E was used, whereas it was not detected with water. Therefore, it is difficult to define the most appropriate period of time for calculating mean permeation rate and permeability coefficient by these solvent migrations across the skin. Since no lag time was found or was very short when M-E was applied to hairless rat skin (Fig. 1), however, a mean permeation rate of from 2 to 4 h and drug solubility or initial concentration in M-E of lipophilic or hydrophilic drug, respectively, was utilized to evaluate the characterization of M-E. Utilization of a mean permeation rate of from 12 to 24 h is advisable, however, to avoid overestimating the feasibility of a drug. Fortunately, the mean permeation rates of 2 to 4 h were not greatly different from those of 12 to 24 h in all drugs examined (the maximum value as a ratio of 2—4 h/12—24 h was about double in captopril). Furthermore, the characterization of M-E was evaluated by plotting log *P versus* $\log K_{ov}$, and then the mean permeation rate from 12 to 24 h was used to calculate permeability coefficient.

Permeation data and estimated parameters are summarized in Table II.

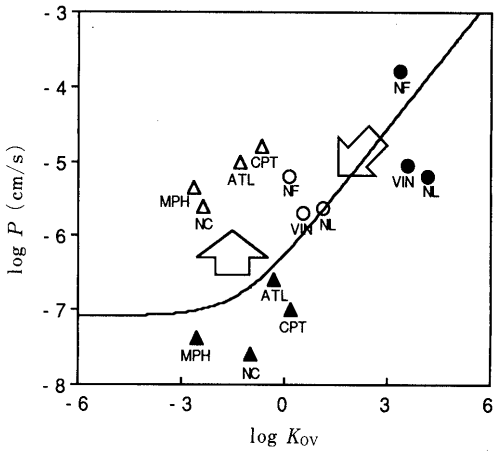

Fig. 2. Change in Relation between Partition Coefficient and Permeability Coefficient by Alteration of Vehicle from Water (Closed Symbols) to M-E (Open Symbols)

Circles, lipophilic drug; triangles, hydrophilic drugs; solid line, regression curve reported by Hatanaka *et al.*[27]

Enhancement Characteristics of M-E M-E is a skin permeation-enhancing system which was originally found to increase the permeation of morphine hydrochloride (MPH, $\log K_{ow} = -2.53$), a water soluble drug.[7] In the present study, the M-E showed a marked enhancement effect not only for water soluble drugs but also for lipophilic drugs (Fig. 1). When comparing ER in rat, the values for water soluble drugs were several times greater than those for lipophilic drugs (except NL, because of its very low permeability by water). Since the difference in ER between the two groups of drugs was not large, however, the M-E was considered to have an enhancing effect for both the

hydrophilic and lipophilic drugs. Skin permeation-enhancement of the drugs by M-E was due to an increase in permeability coefficient in the case of water soluble drugs, and a marked increase in solubility and decrease in permeability coefficient in lipophilic drugs (Table II).

We reported earlier the relationship between lipophilicity and permeability coefficient of drugs.[27] This relationship makes it possible to determine the enhancement characteristics of M-E.

Each data point in Fig. 2 shows the relationship between permeability coefficient and K_{OV} (solubility ratio of octanol/vehicle) of drugs learned in the present study. The solid line in the figure is a regression curve ($P = 4.78 \times K_{OW}^{0.589} + 8.33 \times 10^{-8}$) obtained from a previous permeation experiment[27] using hairless rat skin as a membrane and water as a vehicle. Closed symbols are the data from the experiment using water as a vehicle in the present study, while open symbols are the data using M-E as a vehicle. To clarify the character, the data of MPH (left side plot on the graph) reported earlier[7] was added to the figure. Water soluble drugs (triangles) and the lipophilic drugs (circles) shifted to the upper portion of the graph and the lower left, respectively, when the vehicle was changed from water to M-E. As a result, all the symbols gathered in the center of the graph. From these results it was considered that M-E might act to give similar permeability coefficient to the drugs regardless of their lipophilicity. The reason may be the almost constant barrier function in the viable epidermis and dermis independent of lipophilicity of drug. Therefore, J_{max} of the drug mainly depends on the solubility of the drug in M-E, as suggested by Eq. 1. Drugs which dissolve well in M-E, then, are candidates.

Feasibility of Drugs Since TTS containing cardiovascular agents have an important role in clinical stages, there have been many studies on percutaneous absorption and TTS.[8,28-33] Calcium channel antagonist is one of the series of cardiovascular agents, and a comparative study on its percutaneous absorption was reported recently.[34] NC, NF and NL are frequently used calcium channel antagonists. In the present study, the feasibility of fabricating their TTS with M-E was found to be on the order of $NC \gg NL > NF$ in a comparison of A_{req}. The reason the smallest A_{req} of NC was found is due to its high solubility in M-E, and the reason the A_{req} in NL was smaller than that of NF is the smaller MEC of NL. Among these calcium channel antagonists, therefore, NC would be the best candidate for TTS fabrication using M-E. This was consistent with a study by Diez *et al.* using 50% ethanol and hairless rat skin.[34] The cumulative amount of NF in the present experiment over the first 24 h, however, was about ten times greater than their data.

Since ATL and CPT show high solubility in M-E, the A_{req} of both drugs was small (0.18 and 0.15 cm^2, respectively), suggesting that these drugs can also be candidates. ATL is a β-blocking agent and CPT is an angiotensin converting enzyme (ACE) inhibitor. Because pharmacotherapy for hypertension is generally done using a combination of drugs with different pharmacological mechanisms of action, the development of TTS for both drugs might contribute to the clinical therapy. VIN is one of the drugs used in treatment of cerebral disease originating from a vascular or cerebral metabolic disturbance. A_{req} of VIN is small enough (5.3 cm^2) to develop TTS.

Species Difference of Enhancement Effect of M-E Investigation of species difference was not the main purpose of this study, but some information on the species difference in the enhancing effect of M-E can be discussed. Although permeability of hydrophilic drugs through human skin from water was lower than that through hairless rat skin, J_{max} of M-E was similar in hairless rat and human. As a result, ER of hydrophilic drugs for human skin was greater than that for hairless rat skin. The stratum corneum, the main barrier to drug permeation, was assumed to have lipid and pore permeation pathways.[27] It was also suggested that contribution of the pore pathway to the complete permeation of a drug through hairless rat skin was greater than through human skin.[35] In the present study, however, contribution of the pore pathway in human became similar to that in hairless rat when using M-E.

Further investigation is needed to clearly evaluate species difference of the enhancement.

Conclusion

We investigated the enhancement characteristics of M-E and determined the drugs suitable for M-E to develop TTS as a new dosage form. The results indicated that the M-E system could become a suitable vehicle for drugs that were soluble in the system. NC, ATL and CPT, which had high solubility in M-E, seemed to be good candidates for TTS.

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