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Evaluation and structure–activity relationship of synthesized cyclohexanol derivatives on percutaneous absorption of ketoprofen using artificial neural network

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Abstract

The effect of 35 newly synthesized *O*-ethylmenthol (MET) derivatives on percutaneous absorption of ketoprofen was investigated in rats. In order to understand the relationship between the structure of compounds and promoting activity (structure–activity relationship), an artificial neural network (ANN) was employed. In the in vivo percutaneous absorption study, male Wistar rats, weighing 160–180 g, were used. The apparent penetration rate (R_p) was estimated based on a pharmacokinetic model with a constant rate of penetration through the skin after a lag time. As an index of the promoting activity of each compound, an enhancement factor (Ef), defined as follows, was used: $Ef = R_p(\text{with enhancer})/R_p(\text{without enhancer})$. An irritation evoked on rat skin was microscopically judged at the end of the in vivo percutaneous absorption experiment and evaluated as a total irritation score (TIS). Ef and TIS were selected as output variables to determine the ANN structure. Calculated log *P*, molecular weight, steric energy (SE), van der Waals area, van der Waals volume, dipole moment, highest occupied molecular orbital (HOMO) and lowest unoccupied molecular orbital (LUMO) were used as factors to determine the structural nature of cyclohexanol derivatives. Among these parameters, log *P*, SE and LUMO significantly affected the prediction of Ef and TIS. The predicted values of Ef and TIS coincided well with in vivo percutaneous absorption experimental values. However, results observed with a linear regression method were poor compared with the ANN approach. The contribution index of log *P* was $\approx 50\%$ in the prediction of Ef, suggesting that lipophilicity among physicochemical properties contributes most of the promoting activity of these compounds.

Keywords: *O*-Ethylmenthol derivatives; Percutaneous absorption enhancer; Artificial neural networks (ANN); Structure–activity relationship

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1. Introduction

In transdermal drug delivery, co-administration with percutaneous absorption enhancer is considered an effective means of delivering a sufficient amount of drug systemically. Many compounds have been investigated for activity to promote skin permeation of drugs (Barry, 1987 Okamoto et al., 1988 Green et al., 1988 Takayama et al., 1991 Ghanem et al., 1992). Recently, we synthesized *O*-alkylmenthol and *O*-acylmenthol derivatives and evaluated their promoting activity for the percutaneous absorption of ketoprofen from hydrogel in rats in vivo and in vitro (Negishi et al., 1995 Nakamura et al., 1996). Among these compounds, *O*-ethylmenthol (MET) was the most effective and caused relatively little skin irritation. It was considered that MET distributed in the hydrophobic region of the stratum corneum and increased the permeability of the drugs. Okusa et al. reported that the urinary excretion was retained until 8 h after administration of oxybutynin hydrogel containing 0.5% MET (Okusa et al., 1997). It was also reported that MET was effective against, not only acidic drugs such as ketoprofen, but also basic drugs in skin permeation (Obata et al., 2000a). Furthermore, we synthesized thiomenthol derivatives and investigated their effect using ketoprofen (Takanashi et al., 1999). Unfortunately, in a series of thiomenthol derivatives, the compounds exhibiting strong enhancement action caused severe skin damage.

Not only promoting activity, but also the mode of action have been studied in detail using ESR, DSC and X-ray (Barry, 1987 Quan and Maibach, 1994 Hofland et al., 1995). These techniques are considered useful in clarifying the interaction between the promoting agents and stratum corneum intercellular lipids, considered to be dominated by the characteristics of the enhancers.

When discussing the characteristics of the compounds, the parameters representing lipophilicity (log *P* or solubility parameter) have been considered important (Okamoto et al., 1990). In this study, we tried to use other parameters to assess the structure of compounds and promoting activity (structure–activity relationship).

In the pharmaceutical research, an artificial neural network (ANN) is being utilized to elucidate the structure–activity relationships including pattern recognitions. In this study, we focused on the structure–activity relationship of *O*-ethylmenthol derivatives (Obata et al., 2000b) in the percutaneous absorption of ketoprofen as well as skin damage. To clarify the mode of action of *O*-ethylmenthol derivatives, ANN was employed. Furthermore, a conventional statistical evaluation was performed comparing with the results obtained with ANN.

2. Materials and methods

2.1. Materials

Ketoprofen was purchased from Sigma (St. Louis, MO). Carboxyvinyl polymer (HIVISWAKO 105) was generously supplied by Wako Pure Chemical Industry Ltd. (Osaka, Japan). Other chemicals were of reagent grade.

2.2. Synthesis of cyclohexanol derivatives

The chemical structures of the synthesized cyclohexanol derivatives are shown in Fig. 1. These compounds were synthesized by the methods described previously (Obata et al., 2000b). The purity of each compound was characterized by nuclear magnetic resonance spectroscopy (JEOL PMX 270, Tokyo, Japan) and thin-layer chromatography (silica gel 60, with hexane/dichloromethan = 6/1 as the solvent system). The purity of each compound was $> 99\%$.

2.3. Preparation of hydrogel

The formulae of the ketoprofen hydrogels used in this study are listed in Table 1. The hydrogels were prepared as follows: carboxyvinyl polymer and triethanolamine were dissolved in distilled water. Separately, ketoprofen and each of the enhancers were dissolved in ethanol. Both solutions were mixed and the resulting hydrogel was stored at room temperature for 24 h under airtight conditions prior to use.

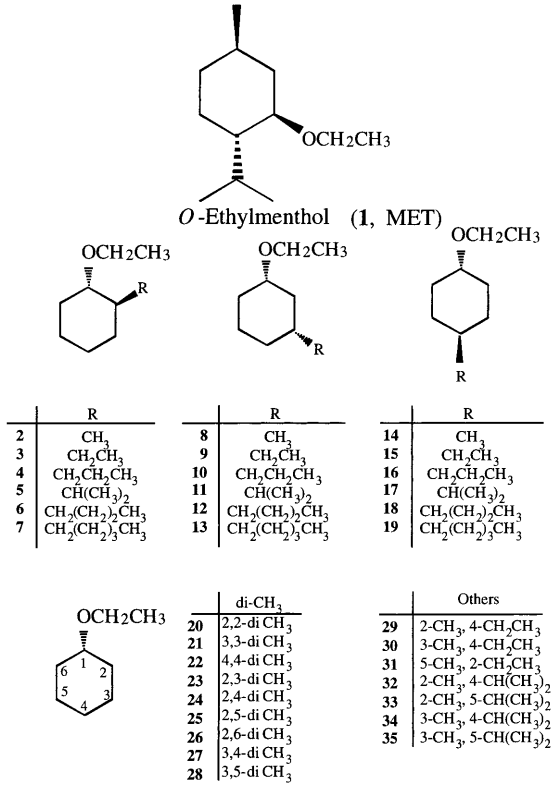

Fig. 1. Chemical structure of MET and MET derivatives.

2.4. In vivo percutaneous absorption study

Male Wistar rats, weighing 160–180 g, were used. After anesthetization with a urethane saline solution (25%; 3.0 ml/kg i.p.), the rats were secured on their back and the hair on the abdominal skin was removed with an electric animal clipper. Glass cells (16 mm inner diameter, 10 mm height) containing the hydrogel being tested (1.0 g) were attached to the skin with cyanoacrylate-type adhesives. Blood samples (0.5 ml) were taken via the jugular vein at 1, 2, 4, 6 and 8 h after the administration. Each was centrifuged and the plasma (0.1 ml) was thoroughly mixed with methanol (0.3 ml) containing an appropriate amount of *p*-hydroxybenzoic acid butyl ester as an internal standard. The mixture was again centrifuged for 1 min and the supernatant solution was filtered using a disposable filter unit (Gelman Science Japan Ltd., Ekikuro-Disk 3CR). The concentration of ketoprofen in each of the filtrates was then determined using the HPLC method subsequently described.

Table 1
Formulae of ketoprofen hydrogel

Ketoprofen	0.30 g
Carboxyvinylpolymer	0.15 g
Triethanolamine	0.20 g
Ethanol	4.00 g
Enhancers	0.10 g
Water	ad. 10.00 g

2.5. Determination of plasma concentration of ketoprofen

The concentration of ketoprofen in the filtrate was analyzed with a HPLC system (Shimadzu, LC-10AS) equipped with a variable wavelength ultraviolet monitor (Shimadzu, SPD-6A). The column was a YMC-Pack A-302 S-5 120A ODS (4.6×150 mm; Yamamura Chemical Laboratories Co. Ltd.). The flow rate was 1.0 ml/min and elution was carried out at room temperature with a mobile phase consisting of 0.057% aqueous phosphoric acid–methanol (35:65). The column effluent was monitored at 254 nm.

2.6. Pathological study

The separated skin was fixed in 10% formalin for at least 24 h before routine processing, then cut vertical to the skin surface at the central region in 4-mm sections. Each section was dehydrated using a graded series of ethanol solutions and embedded in paraffin wax. Tissues were divided into small pieces (≈ 3 μm in thickness) and stained with hematoxylin and eosin. All sections were examined using optiphoto light microscopy.

2.7. Evaluation of promoting activity and skin irritation

From the results of the in vivo percutaneous absorption experiments using rats, the apparent penetration rate (R_p) of ketoprofen was estimated based on a pharmacokinetic model with a con-

stant rate of penetration through the skin after a lag time. The two-compartment model was applied to the data obtained from the i.v. administration.

$$C = \frac{R_p}{V_d k_{10}} \left\{ 1 + \frac{\alpha - k_{10}}{\alpha - \beta} e^{-\alpha(t - t_L)} + \frac{k_{10} - \alpha}{\alpha - \beta} e^{-\alpha(t - t_L)} \right\} \quad (1)$$

where C is the plasma concentration, R_p is the rate of penetration, t is time, t_L is the lag time, V_d is the distribution volume of the central compartment, k_{10} is the rate constant for elimination from the central compartment and α and β are the hybrid first-order rate constants. The mean values of V_d, k_{10}, α and β, estimated previously (Takayama and Nagai, 1991), were used in this study to determine R_p and t_L values. As an index to evaluate the promoting activity of each enhancer, an enhancement factor (Ef) was defined as follows:

$$\text{Ef} = R_p(\text{with enhancer})/R_p(\text{without enhancer}) \quad (2)$$

As well as the enhancement activities, skin irritation should be evaluated for the development of safer and more effective promoting agents. At 8 h after the application of hydrogel, the skin was excised and pathologically evaluated. As shown in Table 2, skin irritation was judged by these standards. A total irritation score (TIS) was obtained by summation of the scores of each part and used as an index for skin damage caused by the application of hydrogel.

2.8. Computer program

Physicochemical parameters of menthol derivatives were estimated using the computer programs 'CAChe' (Oxford Molecular Group Inc., Oxford, UK) with a Power Macintosh 8100/80 computer (Apple Japan Inc., Tokyo, Japan) and 'WinMOPAC' (Fujitsu Ltd., Chiba, Japan) for Windows '98. ANN and multiple regression analysis programs executable in Windows '98, written by us, were used for the study of structure–activity and structure–toxicity relationships. An extended Kalman filter algorithm was employed as a training method for ANN.

Table 2
Judgement indices of skin irritation

Epidermis liquefaction	0–4
Subepidermis edema	0–4
Dermis	
Collagen fiber swelling	0–4
Inflammatory cell infiltration	0–4
Hypodermis	
Collagen fiber swelling	0–4
Inflammatory cell infiltration	0–4
Skin appendage degeneration	0–4
Total irritation score (TIS)	0–28

3. Results and discussion

3.1. Relationship between promoting activity and irritation

The 35 newly synthesized cyclohexanol derivatives were assessed for their activity to promote skin permeation of ketoprofen. Ef and TIS values of cyclohexanol derivatives showed almost a linear relationship (Fig. 2). The compounds that had good promoting activity also caused relatively

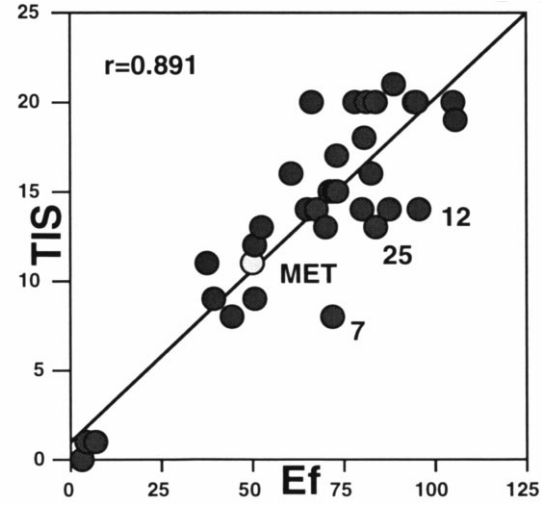

Fig. 2. Relationship between Ef and TIS of MET derivatives. Each point represents the mean of three determinations.

severe irritation. Among these derivatives, however, compounds 7, 12 and 25 showed relatively good promoting activity and low irritancy. These compounds were considered to be promising agents to enhance percutaneous absorption of ketoprofen. In addition, both compounds 7 and 12 had a hybrid structure of fatty acid and cyclic monoterpene. In the case of fatty acid esters, the esters that had fatty acid and alcohol moieties in their chemical structure promoted the permeation of indomethacin (Fujii et al., 1996). Although the mechanism was not clarified, it was speculated that the structures of these compounds enabled them to interact with the components of the stratum corneum and consequently, to enhance permeation of drugs through the skin. In the case of compounds 7 and 12, it was thought that such an interaction contributed to the promoting activity. In particular, compound 12 was twice as active as MET despite almost the same amount of irritation. Compound 12 has *O*-ethyl and 3-isobutyl moiety and it was thought that adequate lipophilicity and molecular size were needed to show the enhancing activity to skin permeation of ketoprofen compared with the other compounds. Thus, compound 12 was considered the most promising of these compounds.

3.2. Structure activity relationship as assessed using the conventional multiple regression method

When the characteristics of a compound are discussed in the study of percutaneous absorption enhancers, parameters representing lipophilicity (log *P* or solubility parameter) have been considered important (Okamoto et al., 1990). We tried to use other parameters to evaluate the structure–activity relationship. We tried to predict the value of Ef and TIS using structural parameters of the compounds. In this study, a series of compounds that had the same main structure but different functions were selected. It was considered that these compounds would help to elucidate the factors affecting the promoting activity systematically. Log *P*, molecular weight, steric energy (SE), van der Waals area, van der Waals volume, dipole moment, highest occupied molecular orbital (HOMO) and lowest unoccupied molecular orbital (LUMO) were selected as the factors to determine the structural parameters of the compounds. These were the typical parameters determined by molecular simulation. Log *P* was considered to be a parameter that related to the distribution of compound to the skin surface. To estimate log *P*, the computer program 'CAChe' was employed. Log *P* was calculated using the atom typing scheme (Ghose et al., 1988). The values calculated by 'CAChe' correspond to the partition coefficient between octanol and water. Molecular weight, SE, van der Waals area and van der Waals volume were used to determine the molecular size of compounds. Dipole moment, HOMO and LUMO were related to the electric properties. HOMO energy means the energy required to remove an electron from the highest occupied molecular orbital. LUMO energy refers to the energy when an electron is added to the lowest unoccupied molecular orbital. A minus translated value of LUMO means electron affinity. HOMO and LUMO energy were determined after optimizing the molecular geometry, first using augmented MM2 then using MOPAC with PM3 parameters.

The prediction made using the optimal regression equation is shown in Fig. 3. The quantitative relationship was investigated using the equation. Ef or TIS values were plotted according to predicted values. An equation for the Ef values was obtained as follows:

$$\begin{aligned} \mathrm{Ef} &= 562(\pm 153)\log P - 87.3(\pm 24.7)(\log P)^2 \\ &\quad - 817(\pm 233) \\ r &= 0.822,\ n = 35,\ s = 15.2,\ F(2,32) \\ &= 33.3\ (P < 0.01) \end{aligned} \tag{3}$$

where r is the multiple regression coefficient, s is the S.D. of the residual and F is the ratio of mean square regression to mean square residual (observed F value). An equation for the TIS values was obtained as follows:

$$\begin{aligned} \mathrm{TIS} &= 139(\pm 26)\log P - 22.0(\pm 4.1)(\log P)^2 \\ &\quad - 65.2\ \mathrm{LUMO} - 22.3 \\ r &= 0.902,\ n = 35,\ s = 2.55,\ F(3,31) \\ &= 45.0\ (P < 0.01) \end{aligned} \tag{4}$$

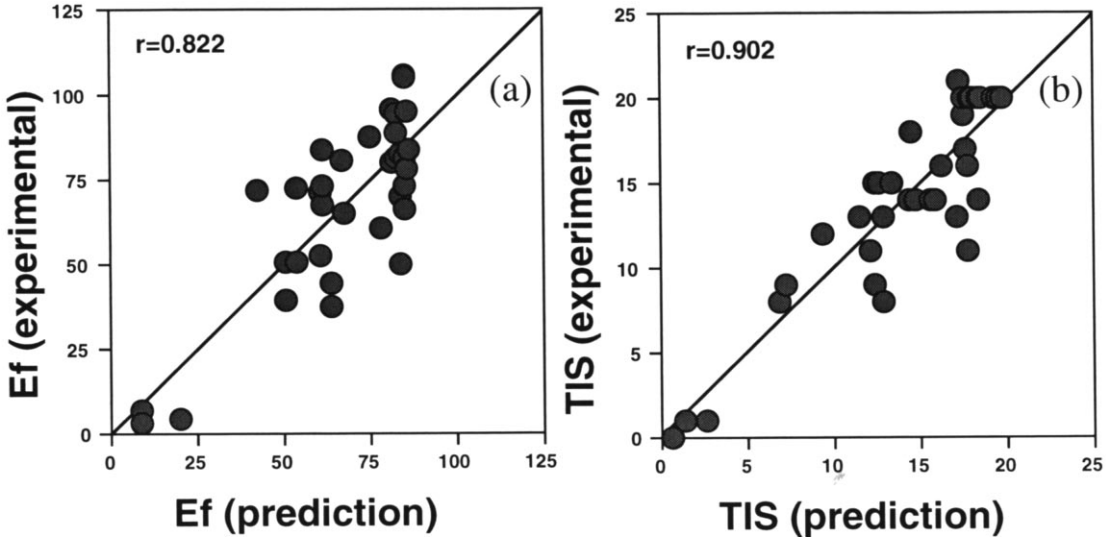

Fig. 3. Relationship between predicted and experimental values of Ef (a) and TIS (b) based on the optimal regression equation.

A combination of parameters was investigated for prediction of Ef and TIS. Only log P significantly affected the prediction of Ef, whereas log P and LUMO significantly affected the prediction of TIS. In Eqs. (3) and (4), correlation coefficients ($r = 0.882$ for Ef and $r = 0.902$ for TIS) were not sufficient for the prediction, suggesting a limitation of the predictions based on a linear combination of parameters.

3.3. Employment of ANN to predict promoting activity of and irritation by MET derivatives

In order to attain a more precise prediction of promoting activity (Ef) and irritation (TIS), ANN was employed. The structure of ANN was optimized on the basis of the simulated annealing technique with the help of Akaike's information criteria (AIC) as a judging standard (Kirkpatrick et al., 1983). Furthermore, combinations of the parameters and the number of hidden layer units were investigated. At first, all parameters were challenged to organize the partitioned ANN and then, the parameters, which did not contribute to the prediction of Ef or TIS values, were sequentially deleted. Finally, it was confirmed that log P, SE and LUMO are causal factors to predict Ef and TIS values. To achieve a more accurate prediction, a partitioned ANN was employed in this study (Takayama et al., 1999). The optimized structure of ANN used in this study is depicted in Fig. 4. In the prediction of Ef, the optimized structure of ANN had 4 units in a hidden layer. On the other hand, in the prediction of TIS, ANN was organized using 3 units in a hidden layer.

The predicted values of Ef and TIS are shown in Fig. 5. A fairly good linear relationship was observed between predicted and experimentally

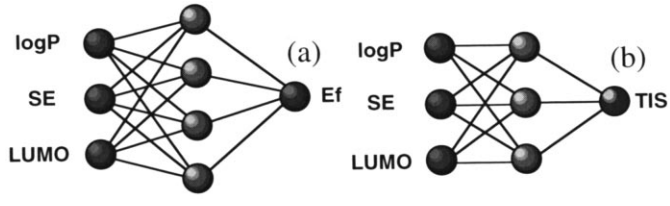

Fig. 4. Optimized structure of the partitioned ANN.

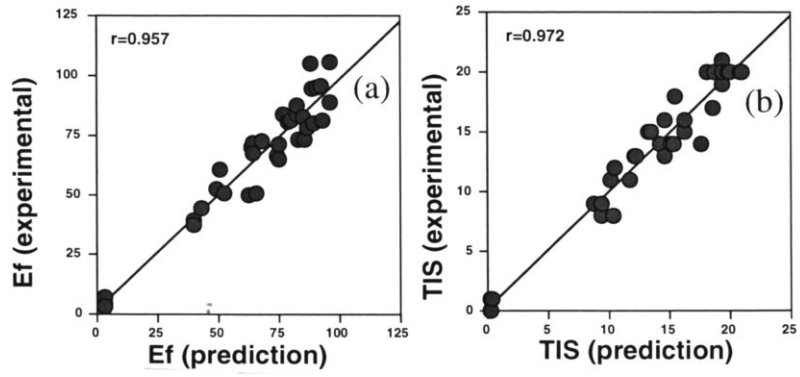

Fig. 5. Relationship between predicted and experimental values of Ef (a) and TIS (b) based on the optimized ANN.

obtained values for both Ef and TIS ($r = 0.957$ in Ef and $r = 0.972$ in TIS). The predictive ability of ANN was superior to that of multiple regression analysis. In addition to the log P value, a more precise prediction can be attained by taking other parameters, such as SE and LUMO, into consideration.

3.4. Significance of parameters for the prediction of Ef and TIS in ANN

In general, the internal structure of ANN is unknown, therefore it is rather difficult to estimate the level or degree of contribution of each parameter (input variables) to the prediction of responses (output variables). To solve this problem, we have introduced a novel method based on a Monte Carlo approach. First, a large number of parameter sets (X_n) except the parameter under test (X_{test}) are generated by means of an arithmetic random number within the range of experimental region. However, X_{test} is generated sequentially within the range of experimental region for at least 100 steps. Namely, a large number of data sets for X_n (at least $100 \times n$ sets) are generated randomly at every step of X_{test} values. The values of each response are estimated at every step of X_{test}, and then, the S.D. is calculated. Finally, the mean of S.D., the response values calculated at all steps of X_{test} is defined as a contribution index (CI). CI values are affected only by the change of X_{test} because the effect of X_n variation on the response variables is thought to be equivalent in every step of X_{test} values. It would be possible to compare the CI values directly for understanding the degrees of contribution of each parameter on the prediction of Ef and TIS.

Fig. 6 shows the CI values of each parameter for the prediction of Ef and TIS. The contribution of log P was estimated to be $\approx 50\%$ more than twice that of either SE or LUMO. This means that the lipophilicity of a compound is the most important factor in the prediction. Degrees of contribution of SE and LUMO were almost equal to the prediction of Ef. In the case of TIS, the CI value of log P was nearly 60%. The effect of log P on the skin damage was serious compared with the case of Ef. Furthermore, the contribution of SE was greater than that of LUMO.

Fig. 7 shows the effect of log P on the estimated values of Ef and TIS under constant values of SE and LUMO. Namely, SE and LUMO were taken

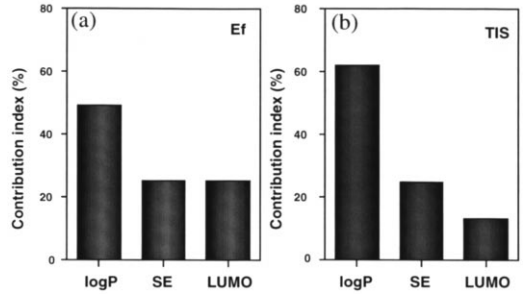

Fig. 6. Contribution index of parameters for prediction of Ef (a) and TIS (b).

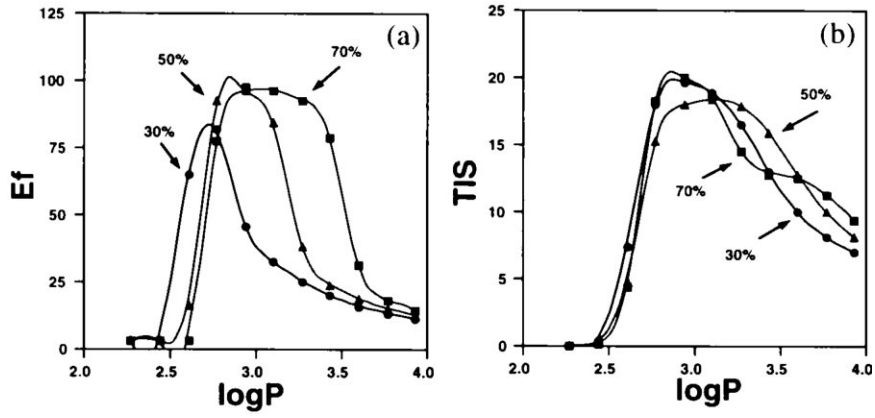

Fig. 7. ANN prediction of Ef (a) or TIS (b) as a function of log P at 30–70% of background factors (SE and LUMO). ●, 30%; ▲, 50%; ■, 70%.

as background factors for these plots. In the case of Ef, the predicted value varied depending on the change of background factors (SE and LUMO) from 30 to 70%. On the other hand, the predicted value of TIS scarcely changed at all. It was suggested that the skin damage was mainly due to the lipophilicity of the chemical enhancer (log P), whereas the size of molecules (SE) and the electric nature of chemicals (LUMO) were important to the enhancing activity as well as the lipophilicity (log P).

4. Conclusion

ANN composed of several structural parameters as input variables accurately estimated the values of Ef and TIS. The prediction of Ef and TIS was significantly affected by the parameters log P, SE and LUMO as an index of lipophilicity, molecular size and the electric nature of enhancers, respectively. The prediction made using a multiple regression method was poorer than the result observed with ANN, suggesting that the ANN was useful for clarifying the mechanism of action of the compounds using the structural parameters.

Acknowledgements

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The animal experiments were conducted in accordance with the Guide for Care and Use of Laboratory Animals adopted by the Committee on Care and Use of Laboratory Animals of Hoshi University, which is accredited by the Ministry of Education, Science, Sports and Culture, Japan.

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