The Journal of Toxicological Sciences (J. Toxicol. Sci.)
Vol.39, No.3, 475-485, 2014

Original Article

Safety prediction of topically exposed biocides using permeability coefficients and the desquamation rate at the stratum corneum

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(Received January 8, 2014; Accepted April 24, 2014)

ABSTRACT — Advances in the synthesis and utilization of new chemical compounds have led to improvements in our daily lives. However, new chemicals may be both beneficial and toxic. Thus, exposure to these new compounds should be restricted in an attempt to limit their potential toxicities. We predicted the safety of three biocides (*p*-cresol, diazinon and resmethrin) by comparing their skin permeability coefficients and desquamation rate (the counter flux of permeability coefficient for chemical compounds induced by skin turnover) following skin exposure. *In vitro* skin permeation experiments revealed that the permeability coefficients of diazinon and resmethrin were smaller than the desquamation rate; therefore, these biocides could not permeate the skin, which resulted in very low skin concentrations of these compounds. On the other hand, the skin concentration of *p*-cresol was high because of its higher permeability coefficient than the desquamation rate. Furthermore, low *in vitro* cell viability was reported for skin exposed to *p*-cresol. These results in the present study indicate that the method described herein is useful for predicting the toxicities of chemicals following their topical exposure.

Key words: Biocide, Skin exposure, Safety prediction, Skin permeation, Permeability coefficient, Desquamation rate

INTRODUCTION

Recent advances in the synthesis and utilization of new chemical compounds have led to improvements in our daily lives. However, exposure to these chemicals should be limited because some are known to be toxic. Chemical exposure can be categorized into two types based on lifestyle and work environment; i.e., exposure of industrial workers and exposure of members of the general public. Safety evaluations of the exposure of industrial workers to chemicals should be undertaken by companies and industrial associations. In contrast, safety evaluations of chemical compounds for members of the general public (especially ordinary households) should be discussed by leading scientists, governments, and/or the World Health Organization (WHO).

Oral, respiratory (Mani *et al*., 2007), and dermal routes (Tomalik-Scharte *et al*., 2005) have been suggested as the possible exposure routes of humans to chemical compounds such as biocides. In the present study, we focused on the dermal exposure of members of the general public to biocides in the household. Insecticide spray contains insecticidal compounds as its main ingredient, and consumers can be exposed to these compounds through their daily use at home. However, oral and respiratory exposure can be avoided while spraying insecticides by shutting the mouth and closing the nose. Dermal exposure may still occur in spite of this caution because of insecticide residues on the flooring and walls, which can be absorbed through the skin.

Concentration-dependent toxicity of chemical compounds in blood and tissues can be generally determined by the Hill's equation (Sheiner *et al*., 1979). Skin concentration and blood concentration-time profiles

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can be predicted by skin permeation parameters, such as flux and permeability coefficient, which are obtained from *in vitro* skin permeation experiments. Thus, skin permeation rate would be a good index to predict or evaluate the safety of chemical compounds to the skin as well as the whole body. In the present study, systemic and local (dermal) toxicities of chemicals were predicted after their dermal exposure based on their permeation rates through the excised skin of hairless rats. Resmethrin and diazinon were chosen as model insecticides (biocides). *Para*-cresol (*p*-cresol) was also selected to predict whether the physicochemical properties of these biocides had any systemic and/or local toxic effects. Table 1 summarizes the chemical structures and physicochemical properties of the biocides used in this study (NIOSH, ICSC, 2008) (NPIC, Technical Fact Sheet: Diazinon, 2009) (NPIC, Technical Fact Sheet: Resmethrin, 2008).

Theoretical

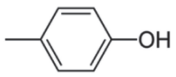
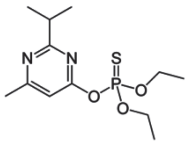
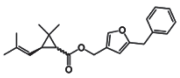
The toxicity of a chemical compound has been shown to depend on the concentration of the chemical compound exposed to viable cells (Eaton and Klaassen, 2008). Skin irritation is a typical example of the toxic effects caused by chemical compounds (Rice and Mauro, 2008). These toxic responses (or effective responses) can be classified into a direct reaction model, in which toxicity depends on the concentration of the chemical compound, and an indirect reaction model, in which toxicity is independent of the concentration of the chemical compound used (Eaton and Klaassen, 2008). Most cases of skin irritation caused by chemical compounds are regarded as a direct reaction (Rice and Mauro, 2008), in which the relationship between the toxicity (T) and concentration of the chemical compound in viable cells (C) can be explained using the Hill equation.

$$T = \frac{T_{max} C^{\gamma}}{TC_{50}{}^{\gamma} + C^{\gamma}} \qquad \text{(eq. 1)}$$

where T_{max}, TC_{50} and γ represent the maximum toxic effect, compound concentration showing 50% toxicity and Hill coefficient, respectively. This equation can be used to predict the severity of the toxic reaction based on the concentration of the chemical compound exposed to viable cells. Equation 1 can also be used to determine the relationship between the toxicity (T) and concentration (C) of the chemical compound in the skin as well as toxicity in the body (T) and systemic concentration (C) (i.e., blood or plasma) when chemical compounds are absorbed into the systemic circulation.

The skin permeability of chemical compounds can be expressed by their fluxes across the skin. The relationship between the steady-state flux ($Flux_{ss}$) and permeability coefficient (P) is represented by the following equation:

Table 1. Chemical structures, physicochemical properties and toxic parameters of the test biocides used in this study

CAS no.	Chemical structure	M.W.	m.p. (°C)	b.p. (°C)	log$K_{o/w}$*	Oral LD_{50} (mg/kg bw)**	Dermal LD_{50} (mg/kg bw)***
106-44-5	*p*-Cresol 	108.1	35	202	1.94	207	300
333-41-5	Diazinon 	304.4	120	306	3.11	1340	> 2020
10453-86-8	Resmethrin 	338.5	43-48	180	3.46	6091	> 2000

* National Institute of Occupational Safety and Health (NIOSH), ** in male rats *** in rabbits

$$P = \frac{Flux_{ss}}{C_v} \quad \text{(eq. 2)}$$

where C_v is the concentration of the chemical compound exposed to the skin surface. The permeability coefficient of low molecular compounds typically ranges from 10^{-6} to 10^{-10} cm/sec or low, and is dependent on the physicochemical properties of the compounds.

The steady-state blood (plasma) concentration (C_{ss}) of a chemical compound absorbed through the skin is represented by the following equation using $Flux_{ss}$ and total body clearance (CL_{tot}) in which the characteristics of the chemical compound can be explained by linear pharmacokinetics:

$$C_{ss} = \frac{Flux_{ss} \cdot A}{CL_{tot}} = \frac{C_v \cdot A}{CL_{tot}} \cdot P \quad \text{(eq. 3)}$$

where A is the application or exposure area (cm^2). According to equation 3, the blood (plasma) concentrations of chemical compounds absorbed through the skin can be predicted using P and CL_{tot}.

The epidermis is composed of keratinocytes, which undergo cell division once a day in the basal layer, and these cells continue to differentiate into the stratum corneum. Almost one month is needed for cells from the basal layer to reach the uppermost layer of the stratum corneum, which is sloughed off a day in a process called desquamation (Kimura *et al*., 2012). The desquamation rate of the stratum corneum has been calculated as about 1×10^{-9} cm/sec (Kimura *et al*., 2012) because the surface layer of stratum corneum (approximately 1 μm in thickness) is sloughed off in a day (24 hr × 60 min × 60 sec). Even if chemical compounds penetrate the first layer of the stratum corneum over 24 hr, this layer is desquamated by this turnover, which prevents these chemical compounds penetrating the deeper epidermis. Thus, chemical compounds with permeability coefficient much less than 1×10^{-9} cm/sec generally penetrate into deep stratum corneum layers and be only located in the uppermost layer in the stratum corneum.

Generally, chemical compound permeation through stripped skin, stratum corneum-removed skin, was much higher than that through intact skin. However, permeation of highly lipophilic compounds through stripped skin shows almost the same value to that through intact skin. This is because the main barrier in skin against the permeation would be changed from the stratum corneum for general compounds to the viable epidermis and dermis for highly lipophilic compounds. Therefore, lipophilic compounds might easily permeate the stratum corneum and thus higher concentration in the stratum corneum can be observed, even when the permeability coefficient is less than desquamation rate, ca. 1×10^{-9} cm/sec. A dermal toxicity or effect might be appeared, because chemical compounds could distribute from the stratum corneum and diffuse in the viable epidermis and dermis. Under these assumptions, desquamation rate, ca. 1×10^{-9} cm/sec, would be a good criterion to estimate the site of toxicity (either local [dermal] or systemic, or both) after skin exposure to chemicals.

Table 2 shows the permeability coefficients of topically applied drugs, which are referred from published data. These topically applied drugs can be classified into locally and systemically active drugs based on their therapeutic effects. Generally, local active drugs have slightly less permeability coefficients than 1×10^{-9} cm/sec and their molecular weight is higher than 500 Da except for pimecrolimus. On the other hand, systemically active drugs have markedly higher permeability coefficients than 1×10^{-9} cm/sec. These data suggest us that the permeability coefficient is greatly useful to predict whether the compounds pass through skin or distribute on the skin surface after skin application (or exposure). Thus, the percutaneous absorption (or skin permeation) rate is an important parameter that is used to predict or estimate the risk and safety of chemical compounds exposed to skin. This method is useful for chemical compounds that penetrate through the stratum corneum. Fig. 1 illustrates this concept.

Introduction continued

The aim of the present study is to calculate the permeability coefficients of three kinds of model compounds, *p*-cresol, diazinon and resmethrin, using *in vitro* skin permeation experiments to predict the possible site showing toxicity after dermal exposure to chemicals. Phosphate buffered saline (PBS, pH 7.4) and kerosene were used as solvents for the biocides. Kerosene has been utilized as a solvent for market spray pesticides. We also predict the safety of *p*-cresol, a model compound exposed to skin from the relationship between *p*-cresol concentration in skin and toxicokinetic parameters calculated from Hill equation. In addition, we also assess the toxicity level of *p*-cresol when it is absorbed into the systemic circulation.

MATERIALS AND METHODS

Materials

p-Cresol, diazinon, kerosene and 2,6-di-*t*-butyl-4-methylphenol (BHT) were obtained from Wako Pure Chemi-

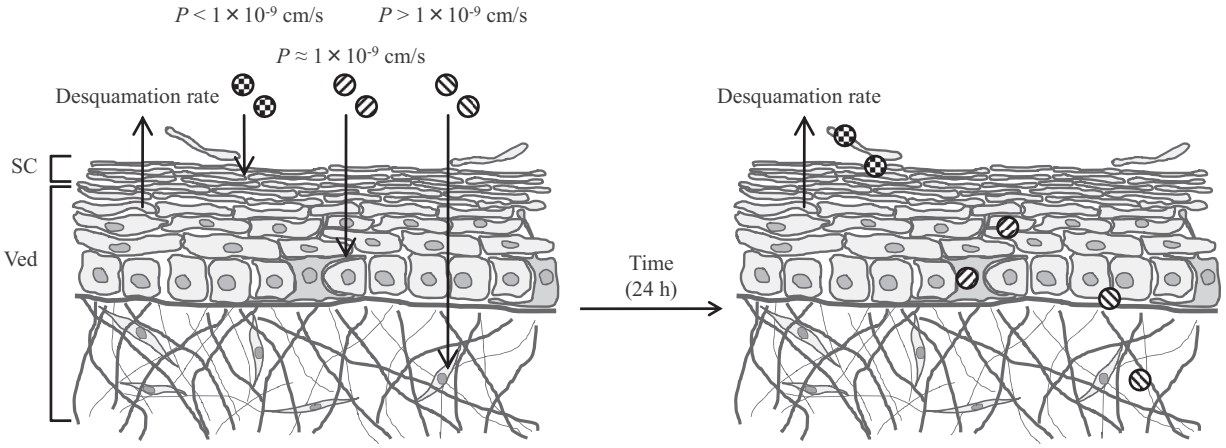


Fig. 1. Schematic representation of skin permeation images expected by comparing the permeability coefficient, *P*, with the desquamation rate (≈ 1 × 10^{-9}cm/sec). SC; stratum corneum, Ved; viable epidermis and dermis.

Table 2. Permeability coefficients of topically applied drugs

Expected site of effect	Compound name	Molecular weight	Permeability coefficient (cm/sec)	References
Local	Acyclovir	225.2	4.7×10^{-7}	Hasler-Nguyen *et al*., 2009
	Clobetasol propionate	410.9	2.2×10^{-8}	Fang *et al*., 1999
	Clotrimazole	344.8	1.4×10^{-10}	Schmook *et al*., 2001
	Diclofenac	269.1	1.8×10^{-7}	Ansari *et al*., 2006
	Hydrocortisone	362.5	8.3×10^{-10}	Johnson *et al*., 1995
	Metronidazole	171.2	7.5×10^{-8}	Ansari *et al*., 2006
	Tacrolimus	804.0	2.6×10^{-9}	Meingassner *et al*., 2005
	Terbinafine	291.4	2.8×10^{-10}	Schmook *et al*., 2001
	Penciclovir	253.3	2.8×10^{-8}	Hasler-Nguyen *et al*., 2009
	Pimecrolimus	810.5	4.2×10^{-11}	Meingassner *et al*., 2005
Systemic	Caffeine	194.2	2.9×10^{-8}	Mitragotri, 2003
	Estradiol	272.4	8.3×10^{-8}	Schmook *et al*., 2001
	Fentanyl	336.5	9.7×10^{-6}	Roy and Flynn, 1990
	Isosorbide dinitrate	236.1	1.3×10^{-6}	Singh *et al*., 1999
	Methylphenidate	233.3	7.1×10^{-7}	Farahmand and Maibach, 2009
		233.3	1.8×10^{-8}	Singh *et al*., 1999
	Nicotine	162.2	7.5×10^{-7}	Zorin *et al*., 1999
	Nitroglycerine	227.1	9.9×10^{-5}	Minghetti *et al*., 1999
	Norelgestromin	327.5	4.1×10^{-6}	Farahmand and Maibach, 2009
	Oxybutynin	357.5	2.8×10^{-8}	Farahmand and Maibach, 2009
	Rivastigmine	250.3	1.5×10^{-6}	Farahmand and Maibach, 2009
	Rotigotine	315.5	3.6×10^{-8}	Honey-Nguyen *et al*., 2003
	Salicylic acid	138.1	6.1×10^{-7}	Schmook *et al*., 2001
		138.1	4.1×10^{-7}	Farahmand and Maibach, 2009
	Scopolamine	303.4	1.4×10^{-8}	Mitragotri, 2003
		303.4	2.2×10^{-7}	Farahmand and Maibach, 2009
	Sumatriptan	295.4	6.9×10^{-9}	Femenía-Font *et al*., 2006
	Testosterone	288.4	1.1×10^{-7}	Schmook *et al*., 2001

cal Industries, Ltd. (Osaka, Japan). Resmethrin was provided by Sumitomo Chemical Co., Ltd. (Osaka, Japan). Other reagents and solvents were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), without further purification.

Normal human epidermal keratinocytes (HEK), serum-free medium for HEK (HuMedia-KG2), trypsin/EDTA solution, trypsin neutralizing solution and HEPES buffer were obtained from Kurabo Industries Ltd. (Osaka, Japan). Normal human dermal fibroblasts (HDF) were obtained from Toyobo Co. Ltd. (Osaka, Japan). Dulbecco's modified Eagle's medium (DMEM) was obtained from DS Pharma Biomedical Co. Ltd. (Osaka, Japan). Fetal bovine serum (FBS) was obtained from ICN Biomedicals, Inc. (Aurora, OH, USA). Trypsin was obtained from Invitrogen Corporation (Carlsbad, CA, USA). MTT reagent [3-(4, 5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide] was obtained from Sigma-Aldrich Co. LLC. (St. Louis, MO, USA).

Animals

Male hairless rats (WBN/Ila-Ht) weighing 220 to 260 g and male Wistar rats weighing 210 to 240 g were obtained from the Life Science Research Center, Josai University (Sakado, Saitama, Japan) or Ishikawa Experimental Animal Laboratories (Fukaya, Saitama, Japan) and Saitama Experimental Animal Supply (Sugito, Saitama), respectively. All animal feeding and experiments were approved by the Institutional Animal Care and Use Committee of Josai University.

Skin permeation study and measurement of biocide concentrations in skin

Abdominal skin was excised from hairless rats under anesthesia by an *i.p.* injection of pentobarbital (50 mg/kg). Tape-stripped skin was obtained by stripping the stratum corneum 20 times using an adhesive tape and then excising the area. Excess subcutaneous fat was trimmed off the excised skin, and the skin sample was mounted on a Franz-type diffusion cell (effective diffusion area; 0.64 cm^2) in which the receiver chamber was warmed to 32°C. We selected *p*-cresol, diazinon and resmethrin as model chemical compounds. The permeation experiment was initiated by applying PBS or kerosene (500 µl) containing one of the above biocides (*p*-cresol, diazinon and resmethrin concentrations were 100 mM, 0.164 mM and 0.59 mM, respectively) to the excised skin. PBS and kerosene were used as a hydrophilic and lipophilic vehicle, respectively. Applied concentrations of each biocide in the kerosene were determined by their solubilities in PBS. The skin temperature was maintained at 32°C during the permeation experiments, and PBS in the receiver compartment (3.8 ml) was stirred by a magnetic stirrer. The receiver solution was periodically sampled and the same volume of fresh PBS was added to the receiver cell to keep the volume constant. The experimental period was set as 90 min (*p*-cresol) or 24 hr (diazinon and resmethrin). Following the permeation experiments, the receiver sample was vortexed with the same volume of acetonitrile and centrifuged (15,000 rpm, 4°C, 5 min) for deproteination. The effective permeation area of hairless rat skin was then minced and homogenized using a Polytron PT-MR 3000 (Kinematica, Switzerland) at 10,000 rpm for 3 min in the presence of 4 ml PBS on ice. The homogenized sample was centrifuged (15,000 rpm, 4°C, 5 min), and the supernatant was collected in a new sampling tube. The sample was vortexed with the same volume of acetonitrile and centrifuged (15,000 rpm, 4°C, 5 min). The concentration of the biocide being examined was detected by an HPLC-UV or GC-MS system as described below.

An HPLC system equipped with an LC-10ATVP pump, SIL-10ADVP auto injector, SPD-10AVP UV/Visible detector, CTO-10ASVP column oven (all from Shimadzu Co. Ltd., Kyoto, Japan) and Superiorex ODS column (Shiseido Co. Ltd., Tokyo, Japan) packed with C18 silica reversed-phase particles was used for the quantitative analysis of *p*-cresol and diazinon. *p*-Cresol was detected by its absorbance at a wavelength of 280 nm with the mobile phase consisting of 40% acetonitrile in water. The flow rate, column temperature and injection volume were 1.2 ml/min, 40°C and 20 µl, respectively. Methyl 4-hydroxybenzoate was used as an internal standard. Diazinon was detected by its absorbance at a wavelength of 245 nm with the mobile phase consisting of acetonitrile, methanol and water (3:1:1 in the volume ratio) (Abu-Qare and Abou-Donia, 2001). The flow rate, column temperature and injection volume were the same as those in the *p*-cresol determination.

The GC/MS analysis of resmethrin was performed on an Agilent 6890N Gas Chromatograph (Agilent Technologies, Waldbronn, Germany) coupled to an inert Mass Selective Detector (MSD) (#5973) and 5% phenyl methyl polysiloxane capillary column (HP-5ms, 30 m × 0.25 mm i.d., 0.25 µm film thickness). The infusion method was splitless, injection volume was 2 µl, and flow rate was 1 ml/min. The inlet temperature, ion source temperature and quadruple temperature were 280, 230 and 150°C, respectively. The column temperature was increased from 50 to 170°C at 40°C/min, and then from 170 to 300°C at 6°C/min. Helium was selected as a carrier gas and selection ion monitoring (m/z of resmethrin; 123, 171) (Liu *et*

al., 2005) was used as a detection method.

Measurement of cell viability after exposure to *p*-cresol

HEK or HDF was cultivated in HuMedia-KG2 or DMEM containing 10% fetal bovine serum at 5% CO_2 atmosphere and 37°C. Cells were trypsinized in 80% confluence, and used for subsequent experiments.

Cells were seeded in 96-well microplates at 6.3×10^4 cells/cm^2 and pre-incubated for 24 hr. Various concentrations of *p*-cresol solution were prepared with the culture medium and exposed to cells for 3, 12, or 24 hr. MTT-medium (0.3 mg/ml) was then applied for 3 hr, the MTT-medium was removed, and 0.04 mol/l HCl in 2-propanol was added for 30 min to extract formazan converted from MTT. The extracted solution was measured using a microplate reader (SpectraMax M2e, Molecular Device Co. Ltd., Tokyo, Japan) at an absorbance of 570 nm (reference: 650 nm) to calculate the number of dead cells following exposure to *p*-cresol. Parameters in the Hill equation (TC_{50}, T_{max} and γ) were calculated by the obtained number of dead cells using the least squares method (eq. 1).

Measurement of total body clearance of *p*-cresol

To evaluate the elimination pharmacokinetics of *p*-cresol, *p*-cresol solution (100 μM) was intravenously administrated (250 μl) to male Wistar rats under anesthesia with ethylcarbamate (1.0 g/kg, *i.p.*). Blood was sampled from the jugular vein every 30 min, and centrifuged (15,000 rpm) for 5 min at 4°C to obtain plasma. The same volume of acetonitrile was then added to plasma for deproteinization.

Kinetic parameters (A, B, α and β) were calculated by data fit using the least squares method to the two-compartment model equation as follows:

$$C = A \cdot e^{-\alpha \cdot t} + B \cdot e^{-\beta \cdot t} \quad \text{(eq. 4)}$$

Statistical analysis

Data represented the mean ± S.E. A p value less than 0.05 represented a significant difference by the Student's *t*-test.

RESULTS

Skin permeation and concentration of *p*-cresol

Fig. 2 shows the time course of the cumulative amount of *p*-cresol that permeated through full-thickness or tape-stripped skin. The cumulative amount of *p*-cresol through full-thickness skin using kerosene as a donor solution was approximately 3-fold higher than that using PBS ($p < 0.01$). In contrast, the cumulative amount through tape-stripped skin from kerosene was not significantly different from that from PBS. These results demonstrated that the cumulative amounts through full-thickness skin were markedly lower than those through stripped skin (Bronaugh and Stewart, 1985).

Fig. 3 shows skin concentrations of *p*-cresol following its topical exposure for 90 min. The concentrations of *p*-cresol in full-thickness skin were 62.0 and 33.9 μmol/g after its application in kerosene and PBS solution, respectively. Kerosene resulted in a significantly higher concentration of *p*-cresol than that of PBS ($p < 0.01$). *p*-Cresol concentrations in tape-stripped skin after its application in kerosene and PBS were 51.7 and 37.0 μmol/g, respectively.

Skin permeation and concentration of diazinon

No diazinon was detected in the receiver cell after the *in vitro* permeation experiment for 24 hr. Fig. 4 shows diazinon concentrations in skin after its exposure for 24 hr. Diazinon concentrations in full-thickness skin were 15.6 and 76.8 nmol/g and those in tape-stripped skin were 1.19 and 84.2 nmol/g after its application in kerosene and PBS solution, respectively. PBS as an application solvent led to a significantly higher concentration in full-thick-

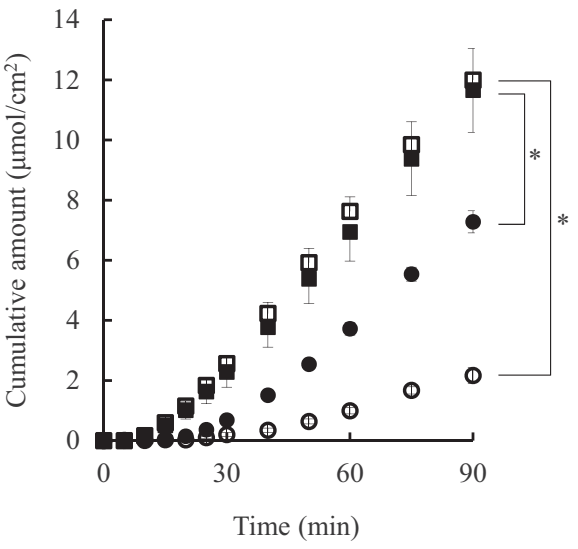


Fig. 2. Permeation profile of *p*-cresol through hairless rat full-thickness (circle) and stripped (square) skin. Closed and open symbols indicate kerosene and PBS, respectively, as a donor solution. Each data point shows the mean ± S.E. of 3 or 4 separate experiments. *; $p < 0.05$.

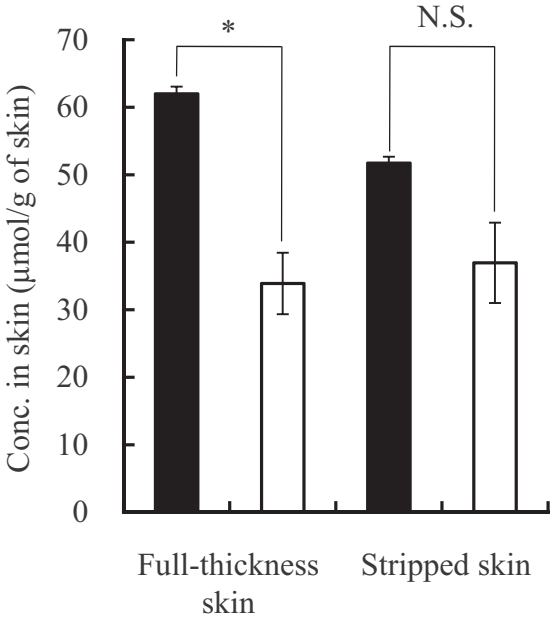


Fig. 3. Concentration of *p*-cresol in skin after the *in vitro* permeation experiment. A donor solution of kerosene (closed column) or PBS (open column) was used. Each column represents the mean ± S.E. of three separate experiments. *; $p < 0.05$, N.S.; no significant difference.

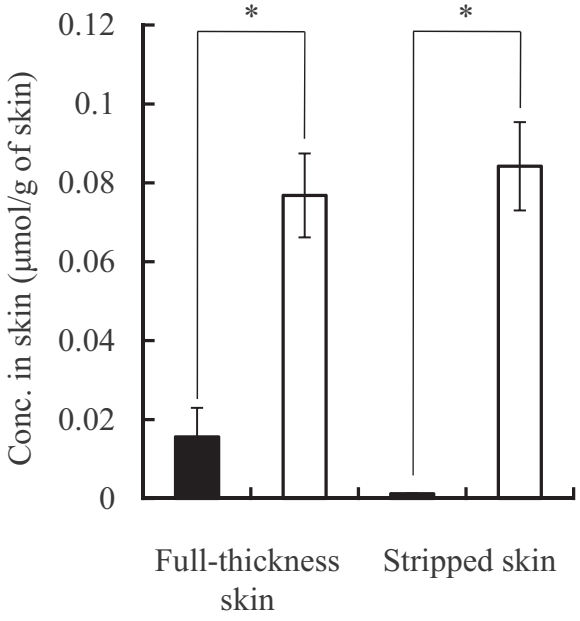


Fig. 4. Concentration of diazinon in skin after the *in vitro* permeation experiment. A donor solution of kerosene (closed column) and PBS (open column) was used. Each column represents the mean ± S.E. of six separate experiments. *; $p < 0.05$.

ness skin than kerosene ($p < 0.01$), and these results were markedly different from those for *p*-cresol.

Skin permeation study and concentration of resmethrin

No resmethrin was detected in the receiver cell after the permeation experiment for 24 hr. Similar permeation experiments were performed using a 10-fold higher donor concentration (5.9 mM) and 4% bovine serum albumin contained in the receiver solution (Scott and Ramsey, 1987) to help resmethrin protein binding in the receiver cell. In spite of these efforts, resmethrin and its metabolites did not permeate through the skin over 24 hr, and was not detected in the skin after its exposure for 24 hr.

Toxicity of *p*-cresol in cultured human skin cell lines

Fig. 5 shows the number of dead cells following the exposure of HEK or HDF to *p*-cresol for 3, 12 or 24 hr. The concentration of *p*-cresol applied was lower than the skin concentration obtained from the skin permeation experiment (≈ 60 μmol/ml). Table 3 shows the Hill parameters (TC_{50}, T_{max} and γ) calculated from the Hill equation (eq. 1) using the least squares method. The results obtained revealed that cell toxicity was closely dependent on the concentration of *p*-cresol applied.

The skin concentration of *p*-cresol after the 90 min exposure was higher than the TC_{50} calculated from the number of dead cells due to the exposure of the cell lines to *p*-cresol over 3 hr (Fig. 3 and Table 3). These results suggest that exposure to *p*-cresol may cause skin irritation.

Prediction of plasma concentrations of *p*-cresol after skin exposure

Fig. 6 shows the plasma concentration-time profile of *p*-cresol after its *i.v.* injection into rats. *p*-Cresol was rapidly eliminated from the systemic circulation. According to Akaike's Information Criteria (AIC), the elimination kinetics of *p*-cresol follow the linear two-compartment model. The elimination kinetic parameters (A, B, α and β) of *p*-cresol in rats were calculated as 0.12 mM, 0.06 mM, 0.38 min^{-1} and 0.09 min^{-1}, respectively. k_{el}, V and CL_{tot} were also calculated as 0.18 min^{-1}, 140 ml and 25 ml/min, respectively.

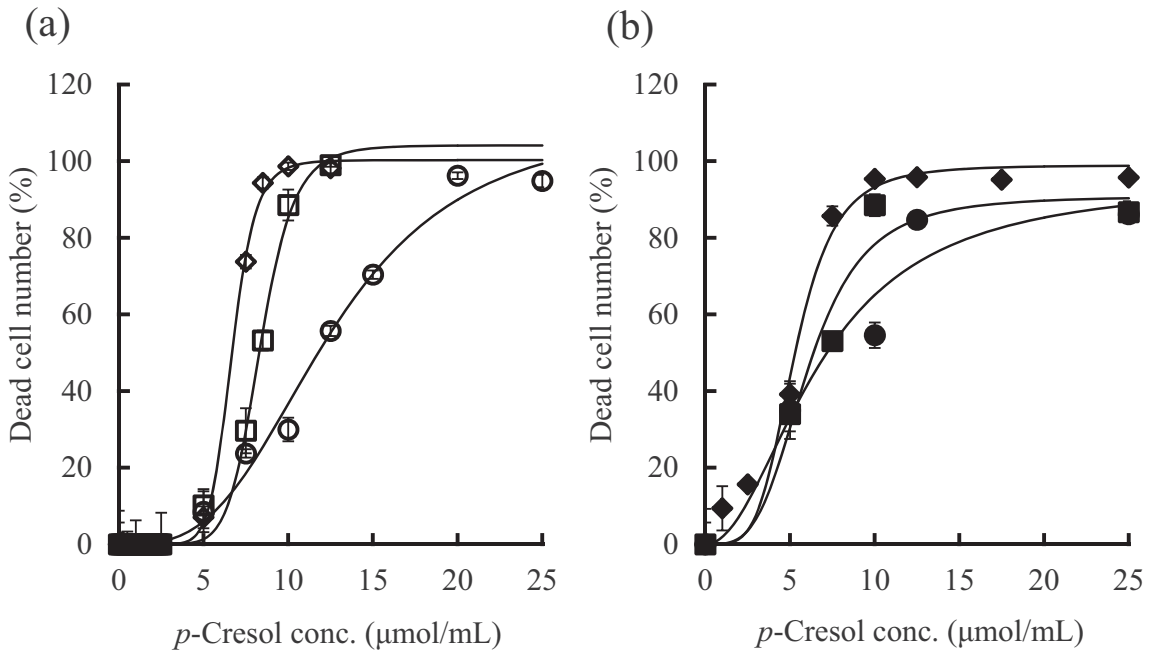


Fig. 5. Relationship between the number of dead cells and concentration of *p*-cresol applied on HEK (a) or HDF (b) for 3 (circle), 12 (square) and 24 (diamond) hr. Each value shows the mean ± S.E. of six experiments. Solid lines are fitted curves drawn by the least squares method to Hill equations.

Table 3. Irritation parameters for the 3-, 12-, or 24-hr exposure of HEK or HDF to *p*-cresol

Cell	Applied period (hr)	T_{max} (%)	γ	TC_{50} (μmol/ml)
HEK	3	111	3.12	12.5
	12	104	8.43	8.37
	24	100	9.69	6.69
HDF	3	94.9	2.03	6.80
	12	90.9	3.63	6.04
	24	98.9	4.37	5.27

When the skin permeation of chemicals reached a steady state, the rate becomes a zero-order process. We predicted steady-state plasma concentrations from equation 3, using both skin permeability coefficient and total body clearance. The plasma concentration was calculated to be between 0.14 and 0.46 mM when applied to the full-thickness skin, and 0.63 mM on the tape-stripped skin.

DISCUSSION

Many novel compounds are being synthesized every year to improve our daily life. However, concerns have been expressed regarding the safety of these compounds, especially with prolonged exposure. Skin exposure is one of the most important exposure pathways, together with oral and respiratory routes. The present study focused on the exposure of skin to chemical compounds that are used on a daily basis. *p*-Cresol, diazinon and resmethrin were chosen as model insecticide compounds and their skin permeability coefficients were compared to the desquamation rate of the stratum corneum, ca. 10^{-9} cm/sec in the order to predict their safety in the skin and the whole body. Although hair follicles and sweat glands are known to be skin permeation routes for water-soluble chemical compounds, the contribution of such appendage routes can be ignored especially for small and lipophilic compounds, which are easily distributed to the stratum corneum (Prausnitz and Langer, 2008). According to Potts and Guy (Potts and Guy, 1992), the permeability coefficient may be 690-fold larger when the *n*-octanol-wa-

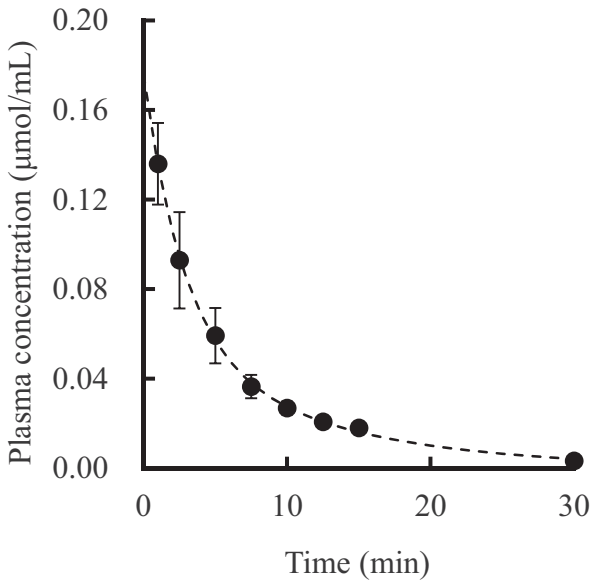


Fig. 6. Elimination kinetics of *p*-cresol after *i.v.* injections to rats. Each value represents the mean ± S.E. of four separate experiments. Broken line shows a theoretical curve drawn by toxicokinetic parameters estimated using the least squares method.

ter partition coefficient ($K_{o/w}$) changes from 0.1 to 1,000. Therefore, the importance of these appendage routes becomes negligible when the $K_{o/w}$ of *p*-cresol, diazinon and resmethrin is considered.

Kerosene and PBS were used as solvents for *p*-cresol, diazinon, and resmethrin in the present study. Kerosene was selected because it has been used in spray formulations for pesticides. PBS was selected as sweat model because sweaty skins may touch biocide-containing walls or floorings where biocides were sprayed in houses. The permeability coefficient through human skin must be investigated in order to accurately evaluate the exposure of skin to chemicals. However, the amount of human skin is not enough for the first safety evaluation of many chemical compounds because of ethical issue. Hairless rat skin was used as a replacement for human skin in the present study, because the relationship between the skin permeability coefficients of these two species has already been established (Morimoto *et al*., 1992). It is important to consider that toxicity or irritation occurs in the viable epidermis and dermis, and not in the stratum corneum, which consists of dead cell layers. In other words, toxicity or irritation cannot be observed when only the skin surface is exposed to a chemical. Toxicities of chemical compounds occur when they reach to a certain skin layer.

The skin permeation of *p*-cresol was higher than that of diazinon and resmethrin (Fig. 2). The permeability coefficient of *p*-cresol, as calculated from equation 2, was between 10^{-5} and 10^{-6} cm/sec (Table 4). The ability of chemical compounds to penetrate skin is markedly reduced when the permeability coefficient is smaller than the desquamation rate. Because the permeability coefficient of *p*-cresol was much higher than the desquamation rate, it could be detectable in the skin (Fig. 3). Thus, we have focused on the skin irritation by *p*-cresol. In addition, it may permeate the skin and enter the systemic circulation causing systemic toxicity. Difference in the skin concentrations of *p*-cresol in full-thickness and stripped skins was comparatively smaller than that in its skin permeation. We have reported that steady-state skin concentration of topically applied chemical compounds could be determined by its partition coefficient from the vehicle into skin. Generally, lipophilic compounds show high partition into the stratum corneum as well as low partition into the viable epidermis and dermis. Therefore, concentration of *p*-cresol in the stratum corneum would be high due to its lipophilicity. This might be a reason for almost the same skin concentration in the full-thickness and stripped skins in spite of much higher permeation of *p*-cresol through the stripped skin than that through the intact skin.

On the other hand, neither diazinon nor resmethrin permeated the skin within 24 hr. The permeability coefficients calculated by the lower limit of quantification value (diazinon: 1.74×10^{-3} μM, resmethrin: 1.56×10^{-2} μM) were less than 10^{-9} cm/s for both diazinon and resmethrin. These permeability coefficients were lower or similar to the desquamation rate (Table 4). Chemical compounds with a permeability coefficient smaller than the desquamation rate, ca. 10^{-9} cm/sec, cannot penetrate the skin. The skin concentration of diazinon was very low after skin was exposed for 24 hr (Fig. 4). This might be due to high barrier to diazinon permeation in the viable epidermis and dermis located under the stratum corneum. Thus, diazinon might only permeate into the stratum corneum and hardly distribute and diffuse into the viable epidermis and dermis due to its high lipophilicity. On the other hand, the skin concentration of resmethrin was not detectable after skin was exposed for 24 hr. Although resmethrin has an ester moiety in its structure, no metabolites were detected in the receiver cell or skin. These data support that diazinon is known as a slight dermal-irritant (NPIC, Technical Fact Sheet: Diazinon, 2009), and that resmethrin is not a skin irritant (NPIC, Technical Fact Sheet: Resmethrin, 2008).

Table 4. Relationship between permeability coefficient and the desquamation rate

	$P_{kerosene}$ (cm/sec)	P_{PBS} (cm/sec)	P_{des} (cm/sec)
p-Cresol	1.91×10^{-5}	5.97×10^{-6}	1×10^{-9}
Diazinon	$< 7.28 \times 10^{-10}$*	$< 7.28 \times 10^{-10}$*	1×10^{-9}
Resmethrin	$< 1.82 \times 10^{-9}$*	-	1×10^{-9}

Full-thickness skin permeability coefficients (P; cm/sec) of biocides were predicted from *flux* at the steady-state or limit of quantification (LOQ) value.
* calculated from LOQ

As explained above, safety issues have to be considered for several chemical compound when its skin permeability coefficient exceed the desquamation rate. To predict the skin safety or irritation, Hill parameters were determined using the relationship between skin concentrations of *p*-cresol and cell toxicity (Table 3). The TC_{50} shifted to a lower concentration with an increase in the exposure period of *p*-cresol (Fig. 5). However, the mechanism of cell toxicity must be the same in spite of the exposure period, because the Hill coefficient (γ) was not significantly different. The skin concentration of *p*-cresol (Fig. 3) was higher than TC_{50} (Table 3) calculated from the cell toxicity test; therefore, skin irritation must be considered following exposure to *p*-cresol. This finding confirms that *p*-cresol is known as a skin irritant chemical (NIOSH, ICSC, 2008). The present study focused on the consumer's situation. In addition, we examined acute toxicity in this study, because biocides are used very frequently but for a very short period. As explained in the introduction section, chronic exposure to *p*-cresol should be considered for industrial workers.

p-Cresol permeated through skin to reach the systemic circulation. We predicted the steady-state plasma concentration of *p*-cresol following its exposure to skin by the skin permeability coefficient and total body clearance from equation 3. The elimination rate of *p*-cresol from the systemic circulation was high in rats (Fig. 6). When a wide area of skin (*i.e.*, 100 cm^2) is exposed to a high concentration of *p*-cresol (*i.e.*, 100 mM) for a long duration (over the lag-time; *i.e.*, 20 min), the plasma concentration of *p*-cresol was calculated as 0.48 mM, which is quite low. The bioaccumulation of *p*-cresol must be low because of its low partition coefficient to *n*-octanol (OECD Screening Information Data Set, 2003), and the LD_{50} was previously shown to be 207 mg/kg (NIOSH, 2009 <http://www.cdc.gov/niosh-rtecs/GO62CCF8.html>). When we administered this dose to rats (body weight: 300 g) through an *i.v.* bolus injection, its plasma concentration was calculated as 4.3 mM. This value exceeded the one predicted by the present study. Thus, it is easy to predict that serious adverse effects are not obtained by exposing skin to *p*-cresol.

Based on these results, the amount of chemical compounds in skin can be predicted by comparing skin permeability coefficient and desquamation rate of the uppermost stratum corneum layer. A more accurate safety prediction can be performed after skin exposure using the T_{max} model. The comparison of skin permeability coefficient, desquamation rate and total body clearance can be used to predict the topical and systemic responses of irritants as well as medicines. Although calculating skin permeability is of importance when predicting the safety and effectiveness of chemical compounds, enzymatic biotransformation was not observed for the chemical compounds used in the present study. Amide compounds and ester compounds may be easily metabolized by enzymes such as *N*-acetyltransferase and general esterases, which are present in the skin. Enzymatic degradation in the skin plays an important role when skin is exposed to chemicals because the metabolites themselves may be the true irritants into skin. Therefore, the skin permeability coefficients of chemicals and their metabolites should be evaluated to predict the safety of chemical compounds. However, in the present study, we only focused on the skin permeation of non-metabolized compounds through skin. Further experiments will be needed to clarify the skin permeability coefficients of chemicals and their metabolites to predict their safeties.

In conclusions, the concentrations of chemical compounds were determined in topically exposed skin by skin permeation experiments. The dermatokinetics of chemical compounds were analyzed by comparing skin permeability coefficients with the desquamation rate of the stratum corneum. The safety of topically exposed chemical compounds was predictable by their cytotoxicity assays. The steady-state plasma concentrations of topically exposed chemicals were predicted by their toxicokinetics/pharmacokinetics. These present analyses using skin permeability represent a promising method not only

for safety, but also effectiveness evaluations of chemical compounds.

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