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In vitro dermal penetration study of carbofuran, carbosulfan, and furathiocarb

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Abstract In this study, the dermal penetration rate of carbofuran, carbosulfan, and furathiocarb has been measured with rat abdominal skin using the static diffusion cell. The technical grades of three compounds were applied at different doses on skin surface mounted in static diffusion cell and incubated at 32°C for 48 h with shaking. The same procedures were carried out with furathiocarb EC (emulsifiable concentrate) and WP (wettable powder). At regular intervals, the receptor fluid in cell was sampled and analyzed by HPLC. Only carbofuran was found in carbosulfan- or furathiocarb-treated samples, suggesting they converted into carbofuran while passing through the skin layer. The quantity of insecticide penetrating skin increased with time and applied dose. The skin penetration rate increased with the water solubility of insecticides. The dermal penetration rates of carbofuran, furathiocarb, and carbosulfan were determined as 1.05 μg/cm^2 per h ($r^2 = 0.991$), 0.46 μg/cm^2 per h ($r^2 = 0.984$) and 0.14 μg/cm^2 per h ($r^2 = 0.967$), respectively. There was no significant difference in rate of skin penetration between furathiocarb EC (1.42 μg/cm^2 per h, $r^2 = 0.988$) and WP (1.35 μg/cm^2 per h, $r^2 = 0.982$), while furathiocarb technical grade showed a lower skin penetration rate. In vitro models may be used to predict percutaneous absorption and are useful in selecting safer formulations for field application of pesticide.

Keywords Penetration rate · Skin · Carbofuran · Carbosulfan · Furathiocarb

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Introduction

Drugs and xenobiotics in general may enter the body by ingestion, inhalation, or percutaneous absorption. Among these routes, dermal absorption has been known to constitute the major part of pesticide exposure because dermal contact with pesticides may occur during manufacturing, transportation, formulation, application, and harvesting. Skin is the largest tissue of the body, measuring about 2 m^2 in area in the adult (Pannatier et al. 1978), and acts as a primary protective envelope between man and environment. Exposure of the skin to chemicals can be potentially extensive and difficult to control. Thus, the absorption of chemicals through the skin is an important aspect of the risk associated with occupational, domestic and environmental exposures. Consequently, there is a great need to ascertain the capacity of a chemical to permeate the skin following dermal contamination. Using in vivo studies, predictions have been made for the toxicological consequences of dermal exposure, the risk involved, and the importance of protection. The preferred model is human skin, but the use of volunteers may not be ethically acceptable. A useful alternative is excised animal skin, and interest in developing methods to assess human exposure to pesticides has led to a search for a suitable animal model for dermal absorption studies (Moody and Ritter 1992; Scott et al. 1992).

Carbosulfan [2,3-dihydro-2,2-dimethyl-7-benzofuranyl (di-*n*-butylaminosulfenyl)methyl carbamate] and furathiocarb [2,3-dihydro-2,2-dimethyl-7-benzofuranyl 2,4-dimethyl-5-oxo-6-oxa-3-thia-2,4-diazadecanoate] are pro-insecticides that transform into carbofuran [2,3-dihydro-2,2-dimethyl-7-benzofuranyl *N*-methylcarbamate] in bio/environmental matrices (Fig. 1) (Lee and Choi 1995). Carbofuran acts as a substrate for acetylcholinesterases and initially forms a Michaelis-like reversible complex (Roberts and Hutson 1999). The insecticides including carbofuran have been used for many years throughout the world, and in Korea were

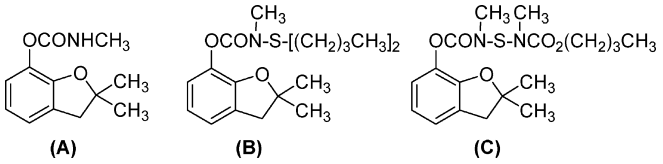

Fig. 1A–C Structures of carbofuran (**A**), carbosulfan (**B**), and furathiocarb (**C**)

employed to control soil-dwelling insects and foliar-feeding pests in rice, fruits, and vegetables (Korean Pesticide Industry Association 2000). There is considerable literature on their pharmacokinetics and metabolism in animals (Dorough 1968, 1970; Ivie and Dorough 1968; Metcalf et al. 1968; Roberts and Hutson 1999; Umetsu and Fukuto 1982). Carbosulfan was metabolized by two primary pathways in the rat. The initial oxidation of sulfur resulted in carbosulfan sulfone via the sulfoxide and eventually the sulfamide. The cleavage of the N–S bond gave carbofuran (Marsden et al. 1982). Furathiocarb was found to be metabolized to carbofuran and 3-hydroxycarbofuran following dermal treatment (Liu et al. 2001, 2002). However, virtually no information is available on the dermal penetration through rat skin, which could be an important factor for the human exposure risk assessment.

In the present study, the dermal penetration rate of carbofuran, carbosulfan, and furathiocarb were investigated in vitro, using glass diffusion cells, to elucidate the absorption rate of those pesticides via skin.

Materials and methods

Chemicals

Carbofuran (purity 99.7%), carbosulfan (99.3%), and furathiocarb (99.7%) were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). HPLC grade acetone and methanol were purchased from Duksan Co. (Ansan, Korea). Deionized water was obtained from a Milli-Q Plus analytical deionization system (Bedford, USA). All other chemicals and reagents were of analytical grade and were commercially available.

In vitro dermal penetration study

Male Sprague-Dawley (SD) rats, 5–6 weeks old, were received from SamTako Bio Korea (Osan, Korea). The rats were killed after diethyl ether inhalation. The hair of the abdominal region was removed with an electric clipper 1 day before the animal was killed, and there was no evidence of redness or irritation. The abdominal skin freed of subcutaneous tissue was stripped with scissors and visually inspected to check its integrity. Skin was used immediately after it was obtained. A Teflon O-ring was attached to the excised rat skin on the epidermal side with an adhesive agent. The static diffusion cell used was similar to that described by Tsuruta (1982). The skin membrane (3.14 cm^2) was clamped between the upper 'donor' chamber and the lower 'receptor' chamber, with physiological saline (18 ml) as the receptor fluid bathing the undersurface of the dermis.

The cells were supported in incubator set at 32°C, the same temperature found in vivo (Dick et al. 1997). The diffusion cells were permitted to equilibrate in a shaking incubator for 2 h to permit hydration of the skin membranes and permit the temperature at the skin surfaces to settle. Carbofuran, carbosulfan, and furathiocarb technical grades in acetone were applied on the skin surface in various amounts (2–150 mg, 100 µl), and furathiocarb emulsifiable concentrate (EC) and wettable powder (WP) were applied at two different doses. The donor cell was covered to avoid evaporation. The cell was constantly shaken at 600 rpm throughout the experiment. During the subsequent incubation for 48 h with shaking, the receptor fluid in cell was sampled (0.5 ml) at regular intervals. An equal volume of fresh receptor fluid was then returned to the receptor fluid to ensure a constant volume. Samples were stored at −20°C until analyzed by high-performance liquid chromatography (HPLC).

Instrumentation and chromatographic conditions

An HPLC system (Hewlett Packard series 1100) consisting of a variable wavelength detector, a carbamate analysis column (4.6 mm i.d. × 150 mm; 5-µm particle size, Pickering Laboratory, Mountain View, CA, USA) and a guard column (Novapak C_{18}; Waters, Milford, MA, USA) was employed for the quantitation of carbofuran, carbosulfan, and furathiocarb. The mobile phase was a mixture of methanol and water (70/30 v/v). The flow rate of the mobile phase was set at 1.0 ml/min and detection was performed at 280 nm. Chromatographic data were processed with HP Chemstation software, version A.04.01 (Hewlett Packard, Palo Alto, CA, USA).

Standard solutions and sample preparation

Carbofuran, carbosulfan, and furathiocarb (10.0 mg each) were placed in a 100-ml volumetric flask and the volume was brought to 100 ml with methanol. Solutions with known concentrations were prepared from the standard stock solution by serial dilution with methanol. The receptor solution sampled from the diffusion cell was passed through a 13-mm, 0.45-µm nylon membrane, and 20.0 µl was injected into the chromatograph. The identity of peaks was confirmed by co-chromatography with reference compounds.

Statistical analysis

Statistical analyses were performed using one-way analysis of variance (ANOVA) with a Duncan's multiple range test with respect to compounds and dose (SAS version 8.01; SAS Institute Inc., Cary, NC, USA). Statistical significance was set at $P < 0.05$.

Results

Method validation

A simple direct injection of the receptor solution of the diffusion cell resulted in a good HPLC chromatogram without any impurity peak. The linear range of the calibration curve for carbofuran, carbosulfan, and furathiocarb was 0.1–10 ppm and the correlation coefficients (r^2) were 0.998, 0.997, and 0.995, respectively. The limit of detection (LOD) was 0.1 ppm for those pesticides, with a signal-to-noise ratio of 3, indicating a sufficiently low limit of detection was achieved for routine analysis because the water solubility of carbosulfan, which is the lowest one of the three compounds, is 0.3 ppm (Tomlin 1997).

In vitro cutaneous metabolism of carbosulfan and furathiocarb

In the dermal penetration study of carbosulfan or furathiocarb using diffusion cell, only carbofuran was detected in receptor solution. This means that these insecticides were completely metabolized to carbofuran while passing through the skin layer. To check whether there is a possibility of hydrolysis of carbosulfan or furathiocarb in the receptor solution, the diffusion cell with or without excised skin was incubated at 32°C for 48 h with shaking after the receptor solution had been spiked with the insecticides. In the case of incubation without skin, they were not transformed to carbofuran (data not shown). However, furathiocarb was slowly transformed to carbofuran on incubation with skin (Fig. 2), and the same situation was found with carbosulfan (data not shown). This implies that the insecticides were hydrolyzed to carbofuran by some component (for example, enzyme) in excised skin.

In vitro dermal penetration rate of carbofuran, carbosulfan, and furathiocarb

The quantity of insecticides penetrating the skin increased with time for all cases, as shown in the plots of the cumulative amount of absorbed insecticides versus the incubation time (Fig. 3). The equation for the steady-state linear region (6–48 h) of each graph was obtained by the method of least-squares, and the penetration rate was obtained from the slope of the equation. The skin penetration rates increased with the application amount; however, the rate was near to the equilibrium value at application levels of more than 30 mg, as shown in the case of furathiocarb (Table 1). By applying the skin penetration rate (0.87 μg/cm^2 per h) at 30 mg level, the amount of furathiocarb that penetrated over the 48-h period was calculated to be about 0.43% of the applied dose. There was a good correlation ($r^2 = 0.968$, $y = 0.30 \log x + 0.25$) between water solubility (x, mg/l) and skin penetration rate (y, μg/cm^2 per h) of these three insecticides (Fig. 4), and a similar result was observed in the study of percutaneous absorption from organic solvents (Tsuruta 1975).

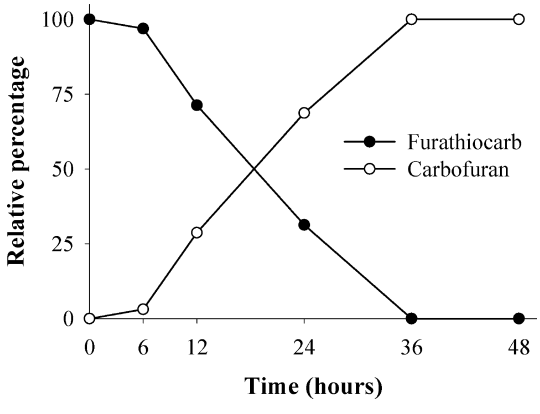

Fig. 2 The curve for the disappearance of furathiocarb and appearance of carbofuran in receptor solution of glass diffusion cell with excised skin, after spiking with furathiocarb

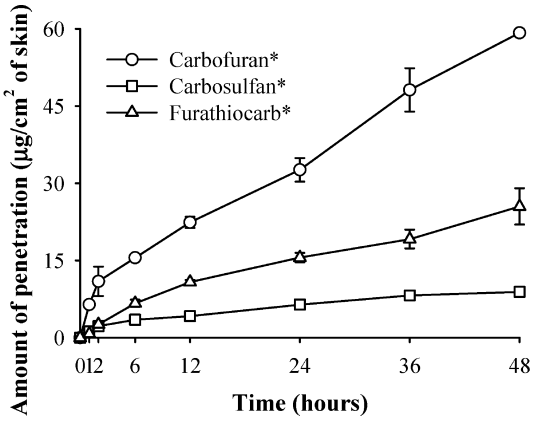

Fig. 3 Penetration curve for carbofuran, carbosulfan, and furathiocarb technical grades (2 mg, 100 μl application) through rat skin over 48 h. *$P < 0.05$, skin penetration rate is significantly different from the each other

In case of furathiocarb, four different doses and two different formulations were tested for the additional information. Total skin penetration ratio (y, percentage) of furathiocarb showed a linear relationship ($r^2 = 0.996$, $\log y = -0.86 \log x + 0.40$) with application amount (x, mg/cm^2) during 48 h (Table 2). Therefore, the penetration amount could be predicted for exposure levels between 0.6 and 47.8 mg/cm^2. To compare the penetration rate between formulations, furathiocarb EC and WP were also tried at two different levels of treatment. Furathiocarb EC and WP showed higher skin penetration rate than technical furathiocarb dissolved in acetone at same application level (2 mg, Table 1, Fig. 5). There was no significant difference ($P > 0.05$) in skin penetration rate between EC and WP formulations. However, it was impossible to detect the insecticide at 0.01 mg level due to the very low concentration.

Discussion

In the dermal penetration study of hydrophobic compounds, in general, organic solvents were needed as a vehicle to dissolve them. The selection of acetone as a vehicle in this study was based on its frequent use in the study of percutaneous absorption (Kao et al. 1984; Moody et al. 1994; Shah et al. 1981). Moody et al. (1992) reported that there was no significant difference between the dermal absorption obtained with acetone and that with aqueous vehicle in the study of a herbicide, 2,4-dichlorophenoxyacetic acid (2,4-D) dimethylamine. Another study showed that pre-exposure to acetone did not affect water permeability through skin relative to unexposed skin (Dick et al. 1997).

Table 1 The skin penetration rate of carbofuran, carbosulfan, and furathiocarb through excised rat skin. Penetration rates and correlation coefficients, the relationship between penetrated amount and incubation time for each insecticide, are expressed as mean values ± SD ($n = 3$) (*ND* not detected)

Compound	Amount applied (mg)	Skin penetration rate (μg/cm^2 per h)	Correlation coefficient (r^2)
Carbofuran technical	2	1.05 ± 0.02	0.991 ± 0.01
	15	1.52 ± 0.03^{a}	0.995 ± 0.02
Carbosulfan technical	2	0.14 ± 0.02	0.967 ± 0.02
	15	0.50 ± 0.07^{b}	0.975 ± 0.03
Furathiocarb technical	2	0.46 ± 0.07^{b}	0.984 ± 0.01
	15	0.75 ± 0.03^{c}	0.965 ± 0.03
	30	0.87 ± 0.06^{c}	0.966 ± 0.02
	150	0.88 ± 0.09^{c}	0.991 ± 0.03
Furathiocarb EC (10%)	2	$1.42 \pm 0.14^{a,d}$	0.988 ± 0.01
	0.01	ND	–
Furathiocarb WP (10%)	2	1.35 ± 0.12^{D}	0.982 ± 0.01
	0.01	ND	–

[a,b,c,d]Means indicated by the same letter are not significantly different at $\alpha = 0.05$ by Duncan's multiple range test

The excised skin has been used in many studies for the measurement of percutaneous absorption of chemicals (Bronaugh and Stewart 1985; Franz 1975; Moody 1997; Tsuruta 1982). It could reflect the living state (Franz 1975) and the data obtained correlated well with those from the in vivo method (Tsuruta 1977). In the present percutaneous absorption study of carbofuran, carbosulfan, and furathiocarb, the Tsuruta-type static diffusion cell with excised skin was successfully utilized with aid of HPLC, by which the cell fluid was analyzed directly. Physiological saline solution was used as a receptor fluid because it is convenient to use and the analytical method developed in this study can detect at least 0.1 ppm of parent compound in that receptor fluid. Even though there are no receptor fluids without any problems, the use of such a simple saline solution as receptor for hydrophobic compound could potentially be problematic because skin can act as a hydrophobic sink. This may explain the dependence of penetration rates on the water solubility of chemicals in the present work. Additionally, this could be account for the observation of carbofuran in the receptor fluid as a metabolite without any of the more hydrophobic parent compounds. Therefore, other receptor fluids such as 50% ethanol, albumin or surfactant-containing fluid could be recommended for obtaining complimentary results.

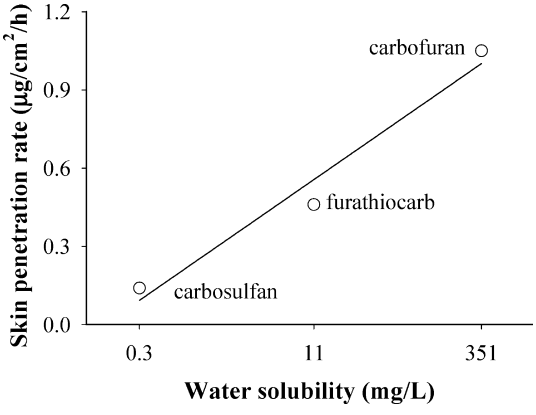

Fig. 4 Regression analysis of water solubility versus skin penetration rate of carbosulfan, furathiocarb and carbofuran

Mammalian skin is capable of many metabolic processes similar to those of other organs (Gysler et al. 1999; Moir et al. 1994; Pannatier et al. 1978; de Zeeuw et al. 1990), and it is, therefore, feasible that pro-insecticides, when applied to the skin, would be metabolized to various derivatives. In the dermal penetration study of carbosulfan or furathiocarb using the diffusion cell, these pesticides were metabolized to carbofuran while passing through the skin layer. This result is supported by studies that reported polyethylene glycol 300 mono- and di-stearate to be readily hydrolyzed by a guinea pig skin preparation (Pannatier et al. 1978), and diflucortolone valerate to be hydrolyzed by rat, guinea, and human skin preparations (Pannatier et al. 1978). Also, hydrolytic cleavage of the amide bond was observed in the study of percutaneous absorption of vanilloids using rat skin (Kasting et al. 1997). A toxicologically inter-

Table 2 Penetration of furathiocarb through excised skin in 48 h

	Furathiocarb value			
Total application amount (mg)	2	15	30	150
Application amount/area (mg/cm^2)	0.64	4.78	9.55	47.77
Skin penetration rate (μg/cm^2 per h)	0.46	0.75	0.87	0.88
Total penetration amount (mg)	0.08	0.09	0.10	0.15
Total skin penetration ratio (% of applied dose)	4.00	0.60	0.33	0.10

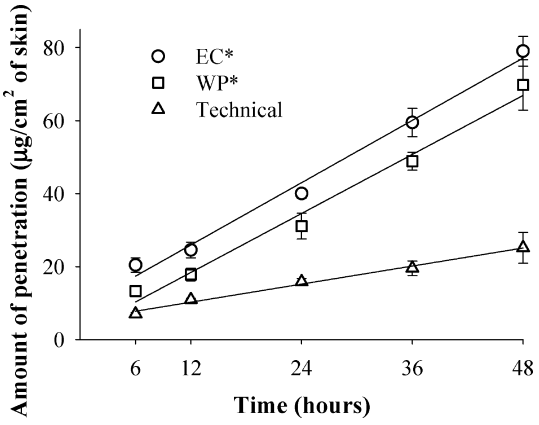

Fig. 5 Penetration curve for furathiocarb EC, WP, and technical grade formulations (2 mg, 100 μl application) through rat skin over 48 h $*P < 0.05$, skin penetration rate is significantly different from the corresponding technical grade value

esting observation in this study is the metabolism of parent insecticides to the more toxic carbofuran because the bioactivation process has occurred in the skin layer. Although it may be argued that skin viability should be maintained for in vitro studies (Collier et al. 1989), the present study showed that carbosulfan and furathiocarb was metabolized by skin, suggesting certain enzymatic reactions were supported in the skin for some period after death of the rat and mounting the tissue in the in vitro system.

Commercial formulations of furathiocarb (EC and WP) showed higher skin penetration rates than technical furathiocarb dissolved in acetone. It seems that surfactants in those formulations increased the penetration of furathiocarb in accordance with previously reported enhancing activity of several surfactants (Michniak et al. 1996). Such results could be useful in predicting the in vivo dermal toxicology of formulations, which is an important criteria in selecting safer formulation for field application.

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