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ORIGINAL ARTICLE

METHOD OF STATIC DIFFUSION CELLS FOR ASSESSMENT OF PESTICIDES SKIN PERMEATION

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Summary

Usage and misusage of pesticides represent a health risk to military and civilian, especially to agricultural workers; also a possible terrorist threat is considered. The major route of low-volatility pesticides intoxication is percutaneous. Hence, skin permeation characteristics of pesticides are intensively investigated. In this study, *in vitro* measurement of skin permeation is presented on the example of pesticide paraoxon. Permeation experiments were performed in Franz-type of static diffusion cells using a pig skin. Paraoxon which permeated through the skin was determined enzymatically by modified Ellman's method. During 8 hours experiment, approx. 0.1 % of applied paraoxon has permeated through the skin. It was shown that pre-treatment by water simulating wet or sweated skin enhanced the paraoxon permeation.

Key words: Pesticides; diffusion cell; skin permeation; paraoxon

INTRODUCTION

The skin is at once crucial organ separating organism from its external environment and is important site of entrance for various chemical agents into the human organism [22]. Thus, skin permeability assessment represents a relevant part of medical research and development which leads to many practical applications as drug delivery or cosmetics. In the field of risk assessment, skin permeation characteristics of chemical warfare agents (CWA) are investigated. Except CWA also pesticides present a potential risk to civilians and could be even misused by terrorist [13]. We have been establishing *in vitro* method of CWA and pesticides skin permeation assessment at the Department of Toxicology since 2008. The purpose of this study was to introduce method of static diffusion cells. Model of pesticide paraoxon (diethyl 4-nitrophenyl phosphate) which is the active metabolite of the organophosphorus insecticide parathion was used as a skin penetrator. Furthermore, evaluation of skin permeability under the various physical conditions was assessed to find out if there is some difference in paraoxon permeability through standard ('dry') and modified ('wet') skin, simulating drenched or sweated skin.

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METHODS

Static diffusion cells

Static diffusion cell (Franz-type) consists of two main parts - a receptor and a donor chamber (Fig. 1 A). The skin is inserted between these two compartments [5, 12]. The dose of tested agent is applied into the donor chamber, placed upwards on the epidermal skin surface. Agent permeating through the skin is accumulated in the receptor chamber (under the dermal skin surface) which is filled by appropriate solution (receptor fluid; e.g. saline, ethanolic solution). The dermal surface of skin should be in direct contact with the receptor fluid without any artefacts as air bubbles. Magnetic stirrer is placed into each cell to provide system homogeneity. The set of cells (18 cells, Fig. 1. B) is connected with peristaltic pump and thermostat by the system of tubing which enabled the heating of the cell flow-jacket and the whole cell consequently. Samples of receptor fluid are collected through the sampling port connected to the receptor chamber.

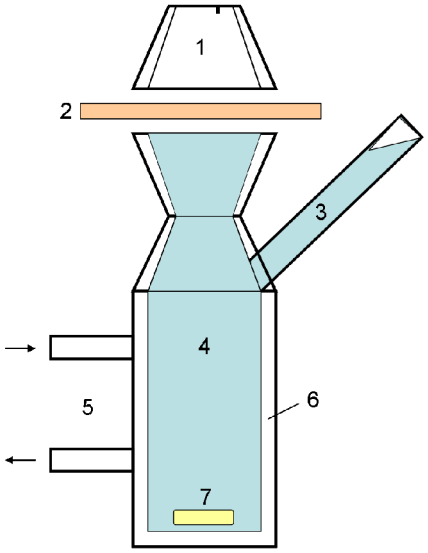

Figure 1. A - static diffusion cell: 1 – donor chamber, 2 – skin sample, 3 – sampling port, 4 – receptor chamber, 5 - manifold filling the water jacket, 6 – water (flow) jacket, 7 – magnetic stirrer.

Figure 1. B - set of static diffusion cells.

Skin

In this study the skin derived from the white domestic pigs was used (*Sus scrofa domestica*; female; b.w. 20 kg). The dorsolateral clipped skin was taken immediately after euthanasia of an experimental animal and stored at -20 °C. The day before the experiment, the skin was allowed to thaw in 8 °C for 24 hours. Afterwards the skin samples were sliced (thickness of 500 μm) using an electric dermatome (Humeca©). To elude damaged skin samples (intra-vital and post-mortal lesions) test of integrity is required [20]. One of recommended methods – measurement of electrical resistance (TER; 20) – did not perform reliably at our department. Thus, damaged skin samples were assessed retroactively by exclusion of significantly devious permeation rates (Dean-Dixon test).

Skin permeation experiments

Set of cells with inserted skin, ready for experiment was left (heated and stirred) to equilibrate overnight. Next day 10 μl of paraoxon (90% chemical pure) was put on the middle of the skin surface (1.77 cm^2) as a liquid droplet. Furthermore, samples of receptor fluid (50 μl) were taken regularly each hour for 8 h and followed by enzymatic assessment of paraoxon.

Permeation of paraoxon was tested under different physical conditions – as a dry skin (2 experiments, 18 cells, 4 excluded) and a wet skin (1 experiment, 9 cells, 3 excluded). In the case of wet skin 300 μl of distilled water was pre-applied onto the epidermal surface 15 min before agent dosing. In both cases, the water bath temperature of 36 °C in the receptor chamber jacket provides a skin surface temperature of 32°C. The dry skin conditions were carried out as a two separate experiments (9 + 9 cells) to prove repeatability of measurements.

Assessment of paraoxon

Based on Ellman's method [11] and its modification [30], the concentration of agent in samples of receptor fluid was determined spectrophotometrically according to the ability to inhibit electric eel's Acetylcholinesterase (AChE, Sigma Aldrich). The calibration curve (Fig. 2) was obtained from separate experiment.

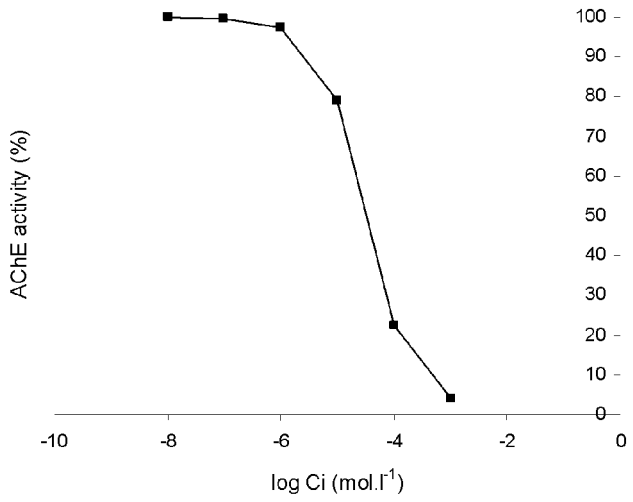

Figure 2. Inhibition of AChE by paraoxon - relationship between paraoxon concentration (Ci) and percentage of AChE activity. Time of incubation = 25 min.

According to paraoxon concentration in the receptor fluid, cumulative amount of paraoxon penetrated through the skin was expressed as a percentage of applied dose. The spontaneous hydrolysis of paraoxon in the receptor fluid during 8 h was considered to be insignificant (< 5%) based on the separate experiment (own unpublished data). Statistical analyse was performed using Mann-Whitney U test.

RESULTS AND DISCUSSION

Skin permeation of paraoxon

Applied paraoxon persisted on the epidermal surface as a small droplet the whole time of the experiment (8 h) without visible recession (permeation, evaporation). Wet skin conditions provided significantly higher permeation rate than dry skin conditions ($p<0.05$). Cumulative amount of paraoxon, penetrated in the 8th hour through the wet skin was more than five times higher than in case of dry skin conditions (Fig. 3). There was no significant difference ($p > 0.05$) between couple of dry skin experiments and they were evaluated as a single data-set (Fig. 3).

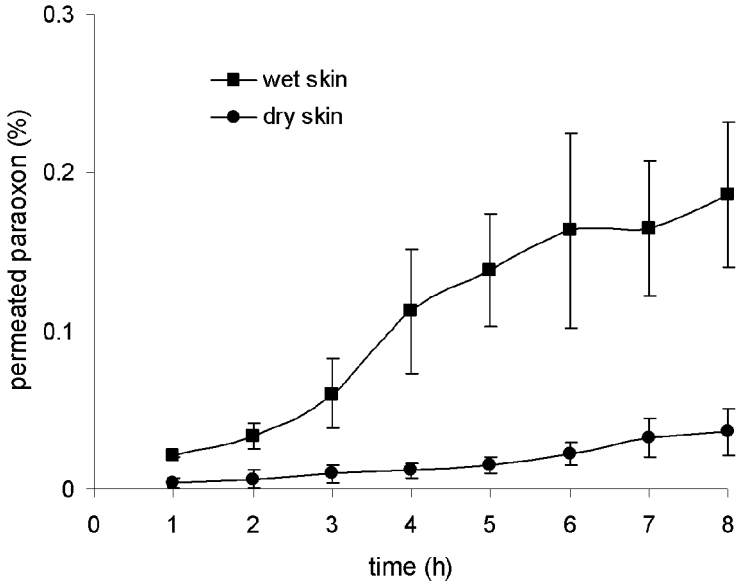

Figure 3. Cumulative amount of paraoxon (% of applied dose) permeated through the pig skin under dry (n = 14 cells) and wet (n = 6 cells) conditions. Mean ± SD.

The upper hydrophobic layer of dead cells – *stratum corneum* – is the principal barrier protecting the skin against permeation of various chemicals [10]. Inferior hydrophilic parts of epidermis and dermis represent another restriction for lipophilic agents as paraoxon. In this study paraoxon permeated through the dermatomed dorsolateral pig skin relatively gently as tenths of percent of applied dose which corresponds to study conducted on the pig ear skin [17]. Amount of paraoxon remained on the skin surface or retained in the skin was not measured. However, obviously the most of paraoxon stayed on the epidermal skin surface. Supposedly a certain amount of paraoxon generated a depot in the skin as was documented in case of other lipophilic agents, e.g. di(2-ethylhexyl) phtalate or pyrene [19, 21]. Wet skin provides several times higher permeability than dry skin. Increased absorption rates of water pre-treated skin were several times observed [15, 25]. Raising skin hydration could increase the skin permeability not even to hydrophilic but also to lipophilic permeants [29]. We also suggested a physical interpretation of water enhancing efect – undiluted viscous droplet of paraoxon occupied relatively small area on the skin surface (cca 0.16 cm^2), whereas paraoxon in the distilled water spilled over the several times larger area (almost whole skin surface 1.77 cm^2). Consequently, this led to the difference in permeation areas and skin absorption. Furthermore methodological artefact should be considered – spilled diluted paraoxon was more likely to achieve skin integrity defects.

Consequences of increased absorption of water pre-treated skin should be considered in individual and mass decontamination. Also decontamination treatments should be conducted in accordance with this finding.

Methodological considerations

Skin permeation is species specific and also individually dependent on gender, age of individual and anatomic localization [1, 2, 3]. Hence permeation experiments must be conducted and evaluated regarding this specifications [9, 20]. Experimental skin can be obtained from laboratory animals [4, 6, 17] or human, as a secondary product of plastic surgery [5, 7]. Alternatively, some types of synthetic skin are available [18, 24]. The pig skin used in this study is considered to be the most suitable animal model to predict skin absorption in man [23, 28]. In the field of organophosphorous chemicals research, no difference between pig and human skin absorption of nerve agent VX was found [8]. Whereas there was a significant difference between permeability of human abdominal and pig-ear skin to Sulphur Mustard [7].

Standard evaluation of permeation rates expressing the skin absorption as a permeation flux (J; $g.cm^{-2}.h^{-1}$) is recommended [9, 16, 20]. Permeation flux is calculated from steady state or pseudo-steady state conditions when the amount permeating per unit time unit is constant. Steady state conditions arise after lag phase – initial time necessary to achieve the steady state. In this study, the penetration rates were evaluated as relative values (%) considering the characteristics of measured data.

In vitro dermal permeation and decontamination experiments represent an alternative method to the experiments on animals. In comparison with *in vivo* studies, this method leads to a reduction of experimental animals and often to economic and time savings. There was approved correlation between *in vivo* and *in vitro* data, even supporting the permeation cell methodology in some studies [14, 19]. Whereas there were found also some discrepancies – *in vitro* could be more often overestimated [26, 27]. In some of the studies, *in vitro* data were compared to *in vivo* data, however obtained under different experimental circumstances, which led to wrong conclusions [26]. Therefore, further clearly defined comparative experiments are requested to evaluate the veracity of *in vitro* skin decontamination methodology.

Generally, method of static diffusion cells was shown as a useful *in vivo* alternative at Department of Toxicology. Analogous to paraoxon, other inhibitors of cholinesterases (pesticides, CWA) will be tested. Also agents without specific inhibition activity could be investigated using a different ways of identification in receptor fluid (HPLC, UV/VIS spectrophotometry etc.).

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