Toxicology in Vitro xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

Percutaneous absorption of herbicides derived from 2,4-dichlorophenoxyacid: Structure–activity relationship

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ARTICLE INFO

Article history:
Received 11 December 2013
Accepted 17 April 2014
Available online xxxx

Keywords:
(2,4-Dichlorophenoxy)-esters
Percutaneous absorption
Esterases
log(k_{ow})
QSAR
Human

ABSTRACT

Ethyl to octyl esters of 2,4-dichlorophenoxy-acetic acids (2,4DAA), 2,4-dichlorophenoxy-propionic acids (2,4DPA) or 2,4-dichlorophenoxy-butyric acids (2,4DBA) are present in the most commonly used herbicides. Their use involves a significant risk of skin exposure, but little is known about the percutaneous flux of these substances. Studies have shown that percutaneous transition of esters may be dependent on their hydrolysis by esterases present in the skin. In this study, we describe *ex vivo* percutaneous absorption of seven pure esters (methyl to decyl) with a 2,4DA structure for rats ($n = 6$) and humans ($n = 7$). Esters were applied at 50 μL cm^{-2} to dermatomed skin (approximately 0.5 mm thick) for 24 h. The enzymatic constants for hydrolysis of each ester by skin esterases were determined *in vitro* using skin homogenates from both species. Structure–activity relationships linking the evolution of the *ex vivo* percutaneous flux of esters and the 2,4D structure with enzymatic (V_{max}; K_m) and/or physical parameters (molecular weight, molecular volume, size of the ester, log(k_{ow})) were examined to develop a good flux estimation model. Although the percutaneous penetration of all of the esters of the 2,4D family are "esterase-dependent", the decreasing linear relationship between percutaneous penetration and hyrophobicity defined by the logarithm for the octanol–water partition coefficient (log(k_{ow})) is the most pertinent model for estimating the percutaneous absorption of esters for both species. The mean flux of the free acid production by the esterases of the skin is not the limiting factor for percutaneous penetration. The rate of hydrolysis of the esters in the skin decreases linearly with log(k_{ow}), which would suggest that either the solubility of the esters in the zones of the skin that are rich in esterases or the accessibility to the active sites of the enzyme is the key factor. The structure–activity relationship resulting from this study makes it possible, in humans and in rats, to make a good estimate of the *ex vivo* percutaneous fluxes for all pure esters of this family of herbicides.

1. Introduction

Esters in the 2,4-dichlorophenoxy (2,4D) family are among the most frequently used herbicides, with a significant risk of skin exposure during their use. Many 2,4D derivatives are used as herbicides, either alone or in mixtures. These derivatives are mainly of three acidic structures (2,4-dichlorophenoxy(acetic, butyric or propionic) acid). For each one, a number of esters are available with an alcohol moiety with between 2 and 8 carbons.

QSAR (Quantitative Structure–Activity Relationship) models (Frasch, 2002; Potts and Guy, 1992) can be used to estimate the percutaneous flux of chemical substances using physicochemical parameters such as the molecular weight, the octanol/water partition coefficient (log(k_{ow})) and the substance's concentration. The Internet site for the US National Institute for Occupational Safety and Health (NIOSH) offers a tool based on QSAR models to calculate the permeability coefficient (K_p) of substances dissolved in water. (Skin Permeation Calculator, www.cdc.gov/niosh/topics/skin/skinPermCalc.html). However, this tool is difficult to use for esters from the 2,4D family, as these liquids are not very soluble in water with log(k_{ow}) values ranging between 3.1 (methyl ester) and 7.8 (decyl ester). The QSAR models currently available cannot calculate the percutaneous flux of pure substances, or of substances which are potentially metabolised during transdermal passage. The latter is also problematic, as studies of percutaneous penetration of esters have shown that it depends on skin esterases (Prusakiewicz et al., 2006). These enzymes can hydrolyse the esters in the 2,4D family, breaking them down to an acid and an alcohol (Beydon et al., 2010; Hewitt et al., 2000a; McCracken et al., 1993).

Thus, the mechanism by which esters cross the skin can be schematised as follows: first the ester penetrates the *stratum*

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http://dx.doi.org/10.1016/j.tiv.2014.04.014
0887-2333/

corneum and skin appendages such as hair follicles, which are of variable density depending on the species; the substance then reaches skin layers where many complex mixtures of water and lipids are present (Hadgraft, 1991; Moss et al., 2002); from there it can progress to zones rich in esterases such as the sebaceous glands or the basal layer. In these zones, the ester can be hydrolysed to produce an acid (Payan et al., 2001). This hydrolytic step differs between species (Beydon et al., 2010), and can influence the speed of absorption of the substance into the body, depending on the type and activity of the esterases present. The overall speed of passage of the ester and its corresponding acid through the skin to the microcirculation depends, among other things, on the relative solubility of each substance in the different layers through which it must pass as well as the rate of metabolism by the esterases.

Data on the percutaneous absorption of esters in the 2,4D family are necessary for risk assessment, but the percutaneous flux of these esters has been little studied in humans. The aim of this study was to check whether first-pass metabolism of esters exists. For a limited number of esters with three main acidic structures, we then investigated the relationship between the *ex vivo* percutaneous absorption flux and the chemical characteristics of the molecules studied and/or the hydrolytic capacity of rat and human skin. This structure–activity relationship was then used to estimate the percutaneous absorption flux in humans for all the esters in the 2,4D family.

2. Materials and methods

2.1. Material

2,4-Dichlorophenoxyacetic acid (2,4DAA) [CAS: 94-75-7] and 2,4-dichlorophenoxybutyric acid (2,4DBA) [CAS: 94-86-6] were supplied by Sigma–Aldrich France (purity > 98%). 2,4-dichlorophenoxypropionic acid (2,4DPA) [CAS: 120-36-5] was from Alfa Aesar France (purity = 98%). 4-nitrophenol (NP) [CAS: 100-02-7] and 4-nitrophenyl butyrate (NPBut) [CAS: 2635-84-9], used to quantify the esterase activity, were provided by Sigma–Aldrich France, as was the diisopropyl fluorophosphate (DFP) [CAS 55-91-4], used as an aspecific irreversible esterase inhibitor, and bis(4-nitrophenyl)phosphate (BNPP) [CAS: 645-15-8], used as a specific carboxylesterase inhibitor. All other reagents and solvents were purchased at analytical grade. The ester derivatives of 2,4DAA (methyl, ethyl, butyl, pentyl, hexyl, octyl, decyl) and of 2,4DBA (ethyl, pentyl, octyl) were synthesised in the laboratory according to the method described by Casal et al. (1999). Briefly, the acid and the iso-alcohol (Sigma–Aldrich, purity > 99%) were adsorbed onto a solid support (Sepiolite [CAS: 63800-37-3], Sigma–Aldrich France,). Synthesis was triggered by heating in a micro-wave oven for 10 min at 500 W. The synthesis yield was over 80%. The product of synthesis was extracted from the support using hexane, and esters were purified by liquid–liquid extraction in a hexane–NaOH 0.1 N mixture. The structure of the esters was verified by gas-phase chromatography coupled to mass spectrometry (GCMS-2010 Plus, Shimadzu, Japan) and the purity (>99%, acidic structure < 0.05%) was measured by high pressure liquid chromatography (HPLC Agilent Technologies 920-LC, Les Ulis, France). The chemical stability of the esters was assessed over 24 h at 37 °C in the solution used as receiver liquid in the diffusion cells.

2.2. Preparation of ex vivo skin samples

Ex vivo percutaneous absorption was studied in line with guideline No. 428 of the Organisation for Economic Cooperation and Development (OECD, 2004). The animal house and the animals used for these experiments were approved by the French governmental authorities. Male Sprague–Dawley rats weighing 275-300 g were supplied by Charles River Laboratories (L'Arbresle, France). They were humanely sacrificed by intraperitoneal injection of pentobarbital (2×120 mg kg^{-1}, i.p). The dorsal skin was shaved and washed with 50% ethanol before removal by excision with a scalpel. Human skin from the abdominal region was donated by women of Caucasian origin (47–64 years of age) having undergone cosmetic surgery. For the two species, the hypodermis was separated from the dermis using a scalpel. Then the skin was thinned to around 500 μm with an electric dermatome (Zimmer, France). The thickness of the dermatomed skin was comparable for the two species, with a mean thickness of 0.503 ± 0.052 mm (n = 42) in rats and 0.441 ± 0.085 mm (n = 49) in humans. Each skin sample was placed on absorbent paper soaked in phosphate buffer (0.01 M, pH 7.2) and stored at −80 °C wrapped in aluminium foil until use. The effect of storage at −80 °C on percutaneous flux was assessed with two 2,4DAA esters (ethyl and octyl). The flux of the esters with stored skin was not significantly different from the flux measured with freshly-isolated rat (102 ± 25%, n = 6) or human skin (92 ± 7%, n = 4).

2.3. Set-up to measure ex vivo percutaneous absorption flux

Frozen skin samples were gently warmed to room temperature on absorbent paper saturated with RPMI 1640 (Gibco France). Discs of skin (15 mm diameter) were cut with a scalpel and their thickness was measured with a comparative device (Prost-Boutillon, Lunéville, France). Skin samples were then placed in glass diffusion cells with a useful area of 0.45 cm^2 and a diffusion chamber of 2000 μL. An RPMI 1640 solution complemented with 2% (w/v) bovine serum albumin (BSA) and 100 mg L^{-1} gentamycin was placed in the diffusion chamber as receiver liquid. The receiver liquid was continuously stirred with a Teflon-coated magnetic stirrer. After a 2-h equilibration period, the physical integrity of the skin samples was checked by measuring transepidermal water loss with a Tewameter TM210 (Courage + Khazaka, Germany) (Nangia et al., 1998). The surface temperature of the skin was maintained at 32.0 ± 0.5 °C. An automatic fraction collector (Gilson DA 204, Middleton, WI, USA) was used to sample 200-μL aliquots of receiver liquid over a 24-h period. An equivalent quantity of solution was added back to the receiver compartment to maintain a constant volume of receiver liquid.

2.4. Exposure and determining percutaneous absorption of 2,4D-family esters

Skin samples were exposed to 2,4D-family esters for 24 h. During this time, the donor compartment was uncovered. The dose of ester deposited was chosen such that 90% of the total amount of ester deposited on the skin remained at the end of the experiment (i.e., an "infinite" dose). Pure esters (liquid at room temperature) were applied to the surface of the epidermis using a Hamilton micro syringe at 50 μL cm^{-2}. The residual ester remaining at the skin's surface was removed at the end of each experiment by washing the skin three times with 500 μL pure acetonitrile. The disc of skin exposed to esters was then cut up and ground in 5 mL acetonitrile. Ester and acid concentrations were measured by HPLC in aliquots of receiver liquid, in the solution used to wash the skin, and in the skin homogenate. The percutaneous absorption flux was calculated based on the cumulative absorption curve for the receiver liquid over time.

The percutaneous absorption flux for each 2,4DA ester (methyl, ethyl, butyl, pentyl, hexyl, octyl, or decyl) was determined using seven human skin samples and six rat skin samples. The percutaneous fluxes for 2,4DP and 2,4DB structures were determined for

Please cite this article in press as: Beydon, D., et al. Percutaneous absorption of herbicides derived from 2,4-dichlorophenoxyacid: Structure–activity relationship. Toxicol. in Vitro (2014), http://dx.doi.org/10.1016/j.tiv.2014.04.014