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In vitro predictions of skin absorption of caffeine, testosterone, and benzoic acid: a multi-centre comparison study

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Abstract

To obtain better insight into the robustness of in vitro percutaneous absorption methodology, the intra- and inter-laboratory variation in this type of study was investigated in 10 European laboratories. To this purpose, the in vitro absorption of three compounds through human skin (9 laboratories) and rat skin (1 laboratory) was determined. The test materials were benzoic acid, caffeine, and testosterone, representing a range of different physico-chemical properties. All laboratories performed their studies according to a detailed protocol in which all experimental details were described and each laboratory performed at least three independent experiments for each test chemical. All laboratories assigned the absorption of benzoic acid through human skin, the highest ranking of the three compounds (overall mean flux of $16.54 \pm 11.87\ \mu g/cm^2/h$). The absorption of caffeine and testosterone through human skin was similar, having overall mean maximum absorption rates of $2.24 \pm 1.43\ \mu g/cm^2/h$ and $1.63 \pm 1.94\ \mu g/cm^2/h$, respectively. In 7 out of 9 laboratories, the maximum absorption rates of caffeine were ranked higher than testosterone. No differences were observed between the mean absorption through human skin and the one rat study for benzoic acid and testosterone. For caffeine the maximum absorption rate and the total penetration through rat skin were clearly higher than the mean value for human skin. When evaluating all data, it appeared that no consistent relation existed between the diffusion cell type and the absorption of the test compounds. Skin thickness only slightly influenced the absorption of benzoic acid and caffeine. In contrast, the maximum absorption rate of testosterone was clearly higher in the laboratories using thin, dermatomed skin membranes. Testosterone is the most lipophilic compound and showed also a higher presence in the skin membrane after 24 h than the two other compounds. The results of this study indicate that the in vitro methodology for assessing skin absorption is relatively robust. A major effort was made to standardize the study performance, but, unlike in a formal validation study, not all variables were controlled. The variation observed may be largely attributed to human variability in dermal absorption and the skin source. For the most lipophilic compound, testosterone, skin thickness proved to be a critical variable.

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1. Introduction

Reproducible data on percutaneous absorption in humans are required to predict the systemic risk from dermal exposure to chemicals, such as hazardous substances at the workplace, agrochemicals, and cosmetic ingredients (EC 2002; EEC 1991; SCCNFP 2003). In this context, there is a need for reliable in vitro models since the European Union advocates this approach and national legislation stipulates that animal experiments should be avoided whenever scientifically feasible. Furthermore, owing to the difference in skin structure, animal studies do not always reflect the human situation.

Absorption through the skin is the primary route of exposure for most pesticides both occupationally (Benford et al. 1999) and in residential settings (Ross et al. 1992). Despite the often relatively high dermal (and inhalation) exposure in occupational settings, regulations for pesticides and other chemical exposure have evolved from concern about the oral route of exposure. In the absence of reliable dermal absorption data, route-to-route extrapolation has been used to assess dermal risk. It should be noted that this extrapolation is not always straightforward in cases when differences in biotransformation exist between the oral and dermal route, excessive first pass effects occur and/or large differences in rate of absorption exist between the various routes of exposure. When no information is available on percutaneous absorption, risk assessments may assume an absorption percentage of 100%, a worst case scenario (EC 2002). This is a very conservative approach and a more accurate measure of absorption would have a major impact on risk assessments for many chemicals in regulatory toxicology. The specific need for a valid method of assessing human dermal absorption has led the OECD (2000a,b,c) and EPA (1996, 1999) to produce guidelines for in vitro and in vivo assessment of percutaneous absorption.

A review of available data from published literature on in vitro dermal absorption was performed under the auspices of the OECD in order to evaluate the performance of in vitro and in vivo percutaneous absorption measurements. It was concluded that evaluation of in vitro test methods from published literature was difficult (OECD 2000d) because studies containing direct comparisons of in vitro and in vivo measurements were very limited. There were too many variables, such as different species, thickness and types of the skin, exposure duration, and vehicles. Also, very few multi-centre studies have been performed (Beck et al. 1994) and these studies were limited in their approach (e.g., with respect to the number of laboratories involved). Therefore, no proper data on the intra- and inter-laboratory reproducibility of the in vitro methodology are available.

The purpose of the present research was therefore to assess intra- and inter-laboratory variability in determination of percutaneous penetration by in vitro methods on a larger scale than done previously. This report contains data generated by 10 independent laboratories from within the European Union, each testing the percutaneous absorption of three chemicals that are recommended by the OECD as suitable reference compounds for regulatory studies (OECD 2000c). The experimental conditions (amount applied, exposure time, vehicle, receptor fluid, preparation of membranes, and analysis) were standardized according to a detailed protocol that adopted many of the guidelines proposed by the OECD.

2. Materials and methods

2.1. Test substances and preparation of dose solutions

The test substances were chosen on the basis of their range in physico-chemical properties (Table 1) and their recommendation as reference compounds by the OECD (OECD 2000c). All participating laboratories used the same batches of test substances. Non-radiolabelled testosterone, caffeine, and benzoic acid were purchased from Steraloids (Newport, RI, USA) and Sigma Chemical Company by the study coordinator and were then supplied to the participants. [4-^{14}C]testosterone (53.6 mCi/mmol) and [1-methyl-^{14}C]caffeine (51.2 mCi/mmol) were purchased from Perkin–Elmer Life Sciences, while [ring-UL-^{14}C]benzoic acid (6.2 mCi/mmol) was obtained from Sigma Chemical Company. The dose solutions were prepared freshly by each laboratory in ethanol/water (1:1, v/v), yielding a concentration of 4.0 mg/mL for each compound. Participants with a license to handle radiochemicals prepared the dose solutions by mixing appropriate amounts of radiolabelled and non-radiolabelled test substances. The dose solutions were measured for exact total radioactivity prior to and directly after the application to the skin membranes. The radioactive concentration was approximately

Table 1
Test substances

Test substance	CAS No.	MW	log Po/w
Benzoic acid (benzenecarboxylic acid)	65-85-0	122.1	1.83
Testosterone (4-androsten-17β-ol-3-one)	58-22-0	288.4	3.32
Caffeine (3,7-dihydro-1,3,7-trimethyl-1H-purine-2,6-dione)	58-08-2	194.2	0.01

1 MBq/mL for testosterone and caffeine and approximately 4 MBq/mL for benzoic acid.

2.2. Preparation of skin membranes

Both human and rat skin membranes were prepared from frozen skin. Whole skin was cleaned of subcutaneous fat and the skin was stored at approximately −20 °C (participants 1 and 2 at approximately −70 °C) for a maximum period of one year. The supply and use of human and animal tissue was in full accordance with national ethical guidelines. Detailed information on the human skin source was recorded (Table 2). Most participants used human skin with a thickness between 0.7–1.1 mm, while one participant used skin that was 0.8–1.8 mm. Three laboratories used dermatomed skin with a thickness of 0.5–0.7 mm (participants 1 and 7) or 0.3–0.4 mm (participant 10). The range of skin thickness used by the various participants allowed for the assessment of the influence of skin thickness on the absorption characteristics of the test compounds. Skin from more than one donor was used in each experiment and each experimental group consisted of 5–7 skin membranes form different individuals. Rat full-thickness skin was used by participant 5 and was collected from the back (clipped carefully) of four weeks old male Sprague Dawley rats.

2.3. Diffusion cells and receptor fluid

Each participant used the diffusion cell that was established in their laboratory (details are shown in Table 3). For experiments with caffeine and benzoic acid, the receptor fluid consisted of saline (0.9% NaCl), while for experiments with testosterone, the receptor fluid consisted of saline (0.9% NaCl) + 5% Bovine Serum Albumin (BSA), adjusted to pH 7.4. For systems using flow-through diffusion cells, the flow of receptor fluid was approximately 1.5 mL/h.

2.4. Experimental design

All participating laboratories performed their studies according to a detailed study protocol in which the experimental design and parameters such as the dose of the test chemical, vehicle, duration of the experiment, preparation of the skin membranes, receptor fluid type, occlusion, temperature, sampling times, and number of replicates were defined. Skin membranes were thawed, mounted in the diffusion cell and the skin integrity was assessed by either visual assessment, permeation of tritiated water (cut-off $K_p > 3.5 \times 10^{-3}$ cm/h) or capacitance (cut-off: 55 nF), depending on the participant. Subsequently, the test substances were applied at a concentration of 4.0 mg/mL ethanol/water (1:1, v/v). The application volume was 25 μL/cm^2 which is considered the minimum volume necessary to produce a homogeneous distribution on the skin surface. This represented a finite dose (100 μg/cm^2), in order to mimic occupationally relevant situations. The exposure time was 24 h, during which the donor compartment remained occluded. Aliquots of the receptor fluid were collected at various time points (minimally at 1, 2, 4, 8, and 24 h post-dosing). For static cells, the original volume of the receptor fluid was restored by adding fresh receptor fluid to the receptor compartment directly after each sampling. In case of non-radiolabelled test compounds, the receptor fluid samples were stored at approximately −20 °C until analysis. At the end of the experiment, the test compound remaining at the application site was removed, using five cotton swabs dampened with ethanol/water (1:1, v/v), followed by one dry cotton swab. When a radioactive test compound was used, the cotton swabs, donor compartment rinse, receptor compartment rinse, and skin membranes [after digestion with 1.5 M KOH in water/ethanol (1:4)] were analysed for presence of the test compound by β-counting. Each laboratory performed 3–5 independent experiments for each test chemical.

Table 2
Details of source of human skin

Participant	Number of donors	Post-mortem/ surgical waste	Sex and age donor	Body site	Skin thickness (mm)
1. University of Newcastle, UK	17	Surgical waste	Female (20–59 y)	Breast	0.5
2. Instituto di Medicina del Lavoro, Italy	6	Post-mortem	Male (67–90 y)	Leg	0.7–0.9
3. Universita di Trieste, Italy	7	Post-mortem	Male, female (67–89 y)	Abdomen	0.8–1.8
4. TNO Nutrition and Food Research, The Netherlands	6	Surgical waste	Female (28–69 y)	Abdomen	0.7
6. Imperial College London, UK	3	Surgical waste	Female (29–50 y)	Abdomen	0.9
7. Health and Safety Laboratory, UK	3	Surgical waste	Female (26–60 y)	Abdomen	0.5–0.7
8. University of Southern Denmark, Denmark	22	Surgical waste	Female (16–68 y)	Breast, abdomen	0.7–1.1
9. University of Erlangen-Nuremberg, Germany	2	Surgical waste	Male, female (40–79 y)	Breast, leg	0.9
10. Huntingdon Life Sciences, UK	5	Post-mortem	Male, female (40–72 y)	Abdomen, leg	0.3–0.4

Participant No. 5 used rat skin.

Table 3
Details of diffusion cell systems

Participant	Diffusion cell type	Exposed skin area (cm^2)	Receptor compartment	Reference
1. University of Newcastle, UK	Flow-through	0.64	Volume: 0.25 mL; stirrer bar: yes	Clowes et al. (1994)
2. Instituto di Medicina del Lavoro, Italy	Flow-through	0.95	Volume: 3.5 mL; stirrer bar: yes	Reifenrath et al. (1994)
3. Universita di Trieste, Italy	Static	3.14	Volume: 15 mL; stirrer bar: yes	Larese Filon et al. (1999)
4. TNO Nutrition and Food Research, The Netherlands	Flow-through	0.64	Volume: 0.2 mL; stirrer bar: no	Bronaugh and Stewart (1985)
5. Institut National de Recherche et de Sécurité, France	Static	1.76	Volume: 5.15 mL; stirrer bar: yes	—
6. Imperial College London, UK	Flow-through	0.32	Volume: 0.4 mL; stirrer bar: no	Bronaugh and Stewart (1985)
7. Health and Safety Laboratory, UK	Flow-through	0.5	Volume: 0.35 mL; stirrer bar: yes	—
8. University of Southern Denmark, Denmark	Static	2.12	Volume: 17.7 mL; stirrer bar: yes	Nielsen and Nielsen (2000)
9. University of Erlangen-Nuremberg, Germany	Static	0.64	Volume: 5.0 mL; stirrer bar: yes	Franz (1975)
10. Huntingdon Life Sciences Ltd., UK	Flow-through	0.64	Volume: 0.25 mL; stirrer bar: yes	Clowes et al. (1994)

2.5. Analysis of non-radiolabelled test substances

The analysis of non-radiolabelled test substances in the dose solutions and receptor fluid samples was performed centrally: benzoic acid by the Health and Safety Laboratory (UK), caffeine by the University of Trieste (Italy), and testosterone by TNO Nutrition and Food Research (The Netherlands). Established protocols were used for the HPLC-UV analysis of benzoic acid (Phenomenex column, SphereClone ODS (2), eluent:methanol:phosphate buffer (pH 6) (4:6), flow 1 mL/min, $\lambda = 229$ nm), caffeine (Hypersil ODS column, eluent: methanol:water (1:3), flow 1 mL/min, $\lambda = 276$ nm), and testosterone (according to Bogaards et al. 1995). The amount of non-radiolabelled test substance was not determined in the skin tissue and therefore total recovery values were not calculated.

2.6. Analysis of radiolabelled test substances

Radioactivity measurements were made by individual participating laboratories. Radioactivity in the various samples (receptor fluid, skin, skin swabs, and cell washings) was determined by liquid scintillation counting. Receptor fluid samples were added directly to an appropriate scintillation fluid. For analysis of the skin membranes, an aliquot of the tissue digest (1.5 M KOH in 20% aqueous ethanol) was used.

2.7. Calculation of results

The calculations were performed using a standardized Excel spreadsheet prepared by the study coordinator. A cumulative amount absorbed per unit skin area versus time course was constructed from the amount of test substance in the receptor fluid and the maximum absorption rate was determined from the steepest, linear portion of the curve. The time to maximum rate, the percentage of the dose recovered in the receptor fluid in 24 h, the percentage in the skin membrane, and the percentage total recovery (for radiolabelled studies) was also calculated. The data of each laboratory were presented as mean ± standard deviation, together with the coefficient of variation (CV). The presence of the test compound in the skin membrane after washing the application area at 24 h was expressed by the ratio between the percentage of the dose in skin and receptor fluid [total penetration (TP)] and the percentage of the dose in receptor fluid (RF).

3. Results

The absorption of caffeine, benzoic acid, and testosterone through the skin was defined on the basis of maximum absorption rate, time to maximum rate, percentage dose recovered in the skin membrane (at 24h post-dosing), and percentage dose recovered in the receptor fluid (at 24 h post-dosing). The results of individual laboratory measurements are shown in Tables 4–6 and overviews of the mean values are given in Figs. 1–4.

3.1. Benzoic acid

The mean maximum absorption rate of benzoic acid through human skin membranes was 16.54 ± 11.87 μg/cm^2/h, while the amount in the receptor fluid after 24 h

Table 4
Benzoic acid

Participant No.	Analysis	Experiment No.	No. of replicates	Species	Maximal absorption rate (μg/cm²/h)	Time to maximum rate (h)	24 h				Ratio TP:RF
							Skin content (% of dose)	Receptor fluid (RF) (% of dose)	Total penetration (TP) (% of dose)	Total recovery (% of dose)	
1	LSC	1	7	Human	31.56	0.8	3.6	53.1	56.7	72.8	1.03
		2	5	Human	38.20	0.5	1.9	65.9	67.8	85.2	
		3	7	Human	25.54	0.0	1.1	69.9	71.0	85.1	
		Mean ± SD			*31.77 ± 6.33*	*0.4 ± 0.4*	*2.2 ± 1.3*	*63.0 ± 8.8*	*65.2 ± 7.5*	*81.0 ± 7.1*	
		CV			*19.9%*	*93.3%*	*59.5%*	*14.0%*	*11.5%*	*8.8%*	
2	LSC	1	6	Human	7.36	2.5	5.7	72.5	78.2	87.8	1.10
		2	3	Human	2.94	3.5	10.5	27.7	38.2	44.8	
		3	3	Human	3.30	2.7	3.4	32.3	35.7	41.3	
		4	3	Human	8.46	3.2	3.6	92.7	96.3	105.8	
		5	3	Human	3.22	2.9	2.8	32.1	34.9	41.7	
		Mean ± SD			*5.06 ± 2.64*	*3.0 ± 0.4*	*5.2 ± 3.2*	*51.5 ± 29.4*	*56.7 ± 28.7*	*64.3 ± 30.4*	
		CV			*52.2%*	*13.3%*	*61.5%*	*57.1%*	*50.6%*	*47.3%*	
3	HPLC-UV	1	8	Human	6.63	2.0	—	86.6	—	—	ND
		2	7	Human	7.70	2.0	—	82.9	—	—	
		3	7	Human	7.72	1.2	—	80.5	—	—	
		Mean ± SD			*7.35 ± 0.62*	*1.7 ± 0.5*	—	*83.3 ± 3.1*	—	—	
		CV			*8.5%*	*26.6%*	—	3.7	—	—	
4	LSC	1	7	Human	21.46	1.1	3.4	85.2	88.6	90.9	1.04
		2	7	Human	21.76	1.1	3.3	87.4	90.7	94.9	
		3	7	Human	24.08	1.4	3.3	87.9	91.2	93.2	
		Mean ± SD			*22.43 ± 1.43*	*1.2 ± 0.2*	*3.3 ± 0.1*	*86.8 ± 1.4*	*90.2 ± 1.4*	*93.0 ± 2.0*	
		CV			*6.3%*	*14.4%*	*1.7%*	*1.6%*	*1.6%*	*2.2%*	
5	LSC	1	8	Rat	19.46	0.5	6.8	84.9	91.7	97.3	1.05
		2	8	Rat	22.53	0.8	4.1	91.6	95.7	99.2	
		3	8	Rat	21.65	0.5	3.7	93.0	96.7	99.1	
		Mean ± SD			*21.21 ± 1.58*	*0.6 ± 0.2*	*4.9 ± 1.7*	*89.8 ± 4.3*	*94.7 ± 2.6*	*98.5 ± 1.1*	
		CV			*7.4%*	*28.9%*	*34.6%*	*4.8%*	*2.7%*	*1.1%*	
6	LSC	1	7	Human	4.09	0.0	5.2	30.1	35.3	41.0	1.12
		2	7	Human	6.27	0.0	5.0	35.2	40.2	49.3	
		3	7	Human	9.64	0.5	4.6	62.9	67.5	70.4	
		Mean ± SD			*6.67 ± 2.80*	*0.2 ± 0.3*	*4.9 ± 0.3*	*42.7 ± 17.7*	*47.7 ± 17.4*	*53.6 ± 15.2*	
		CV			*41.9%*	*173.2%*	*6.2%*	*41.5%*	*36.5%*	*28.3%*	
7	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
8	LSC	1	7	Human	4.94	0.0	3.0	69.4	72.4	76.7	1.06
		2	7	Human	2.87	2.0	8.6	72.2	80.8	88.8	
		3	7	Human	4.70	1.0	2.2	65.3	67.5	73.9	
		4	7	Human	4.15	1.8	2.7	68.4	71.1	94.4	
		Mean ± SD			*4.17 ± 0.92*	*1.2 ± 0.9*	*4.1 ± 3.0*	*68.8 ± 2.8*	*73.0 ± 5.6*	*83.5 ± 9.8*	
		CV			*22.2%*	*75.8%*	*73.2%*	*4.1%*	*7.7%*	*11.7%*	
9	HPLC-UV	1	7	Human	21.39	0.5	—	81.9	—	—	ND
		2	7	Human	28.46	0.8	—	86.4	—	—	
		3	7	Human	24.51	0.8	—	102.5	—	—	
		Mean ± SD			*24.79 ± 3.54*	*0.7 ± 0.2*	—	*90.3 ± 10.8*	—	—	
		CV			*14.3%*	*24.7%*	—	*12.0%*	—	—	
10	LSC	1	5	Human	31.57	0.8	1.3	79.0	80.3	88.2	1.02
		2	6	Human	31.09	0.8	1.6	74.3	75.9	82.0	
		3	7	Human	27.68	0.9	1.3	80.2	81.5	93.2	
		Mean ± SD			*30.11 ± 2.12*	*0.8 ± 0.1*	*1.4 ± 0.2*	*77.8 ± 3.1*	*79.2 ± 2.9*	*87.8 ± 5.6*	
		CV			*7.0%*	*6.9%*	*12.4%*	*4.0%*	*3.7%*	*6.4%*	

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