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RESEARCH PAPER

Comparison of Open-Flow Microperfusion and Microdialysis Methodologies When Sampling Topically Applied Fentanyl and Benzoic Acid in Human Dermis *Ex Vivo*

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ABSTRACT

Purpose The purpose of this study is to compare two sampling methods—dermal Open-Flow Microperfusion (dOFM) and dermal Microdialysis (dMD) in an international joint experiment in a single-laboratory setting. We used human *ex-vivo* skin and sampled topically administered Fentanyl and Benzoic Acid. The second purpose was to provide guidance to researchers in choosing the most efficient method for a given penetrant and give suggestions concerning critical choices for successful dermal sampling.

Methods The dOFM and dMD techniques are compared in equal set-ups using three probe-types (one dOFM probe and two dMD probe-types) in donor skin (n=9) - 27 probes of each type sampling each penetrant in solutions applied in penetration-chambers glued to the skin surface over a time range of 20 h.

Results Pharmacokinetic results demonstrated concordance between dOFM and dMD sampling technique under the given experimental conditions. The methods each had advantages and limitations in technical, practical and hands-on comparisons.

Conclusion When planning a study of cutaneous penetration the advantages and limitations of each probe-type have to be considered in relation to the scientific question posed, the physico-chemical characteristics of the substance of interest, the choice of experimental setting e.g. *ex vivo/in vivo* and the analytical skills available.

KEY WORDS dermal · *ex vivo* · human · microdialysis · open-flow microperfusion

INTRODUCTION

Traditional microdialysis (MD) is a sampling technique, which can be used *ex vivo* as well as *in vivo* and in all organs including the skin-dermal microdialysis (dMD). The technique has existed for dermal use since 1991 (1) and provides chronological, real-time pharmacokinetics of drugs and other substances. Dermal MD is a unique technique for *in vivo* sampling of topically as well as systemically administered drugs at the site of action, e.g. sampling the unbound tissue concentrations in the dermis and subcutaneous tissue Fig. 1. The method has undergone significant development, improvement and validation during the last decade and is a useful and safe tool in pharmacokinetic and pharmacodynamic studies (2). Sampling of large, highly lipophilic and/or protein-bound substances has, however, always been a challenge in MD when using the traditional MD probes and perfusates. Traditional probe membranes have a low MW cut-off, which may result in a negligible concentration of the

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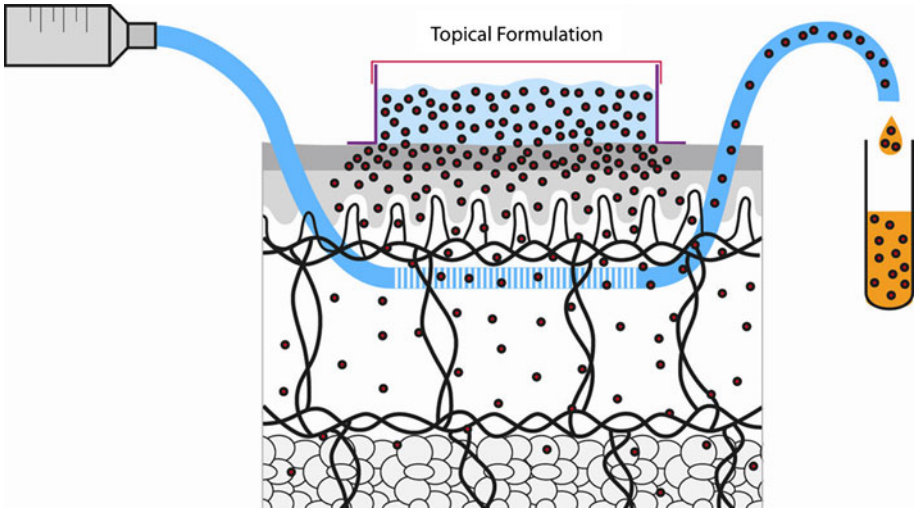


Fig. 1 Illustration of the microdialysis probe placed in the dermis, sampling increasing dermal drug concentrations following topical drug penetration (modified from Benfeldt and Serup 1999).

penetrant of interest in the perfusate (3). Lipophilic molecules have a low affinity for the traditional aqueous perfusate compared to that for the tissue or the probe material, which can hamper MD sampling (4). Improvements of the experimental set-up may prove necessary in order to achieve reproducible recovery of larger molecules and more lipophilic molecules. Sampling of proteins and peptides as markers of tissue homeostasis, dysfunction or repair can be done across membranes with higher MW cut-offs (5). The perfusate can be modified (6) by adding substances such as albumin (4,7), Intralipid® and Encapsin® (8,9). The dMD method has been employed for sampling of a large number of topically applied substances in both healthy and damaged/diseased skin of humans as well as animals (10).

The Open-Flow Microperfusion (OFM) sampling technique was introduced for metabolic research in 1997 (11) and has hitherto mainly been used for continuous glucose and lactate monitoring and for sampling of peptides and proteins in the subcutaneous and muscular tissues (12–17). In 2006 the method was adopted for dermal drug sampling (dOFM) (18). Dermal OFM probes are based on the OFM-technique and the design allows continuous tissue-specific sampling of all substances regardless of their physico-chemical properties, since there is no nominal cut-off value related to this probe-type Fig. 2. The open exchange area in OFM probes allows direct contact between the perfusate and the interstitial fluid thus, no dialysis or filtering of the sample occurs. However, due to this open exchange area the OFM probes require active push-pull pumps in order to avoid the loss of perfusate to the tissue and the risk of oedema formation.

Prior to dMD and dOFM studies, the relative recovery (RR) of the different probe-types has to be determined. The RR is the ratio between the concentration in the dialysate/sample fluid and the concentration in the peri-probe fluid expressed in percentage. The exchange/diffusion of a penetrant between the tissue and the perfusate (recovery or delivery) is determined by the probe membrane—pore area and MW cut-off value—as well as by the physico-chemical properties of the substance—size, charge, and solubility. The *in vitro* recovery studies are used to determine the basic efficacy of each probe type for sampling of the penetrant of interest and in order to evaluate the degree of non-specific adsorption. Very low recoveries will be associated with problems concerning analysis of low-concentration samples and increased variability.

In this paper we compare the two sampling methods used in skin penetration studies: dOFM and dMD in human skin *ex vivo*. The comparison was made in an international joint experiment in a single-laboratory setting employing three sampling probes side-by-side in a direct investigation of the same penetration experiment. Two penetrants with different physico-chemical characteristics were used in the present study Table I. Fentanyl is a potent lipophilic synthetic opioid agonist used as an analgesic in the treatment of chronic pain (19,20). This drug has proven to be very suitable for transdermal delivery by patch technology. Benzoic acid is used in this study as a reference penetrant often used in skin penetration studies (21–23) and is recognized by the OECD as a test substance (24). The advantages and limitations of each method were studied in a practical, hands-on comparison with the overall purpose of providing guidance to researchers in choosing the most efficient method for a given penetrant and giving suggestions concerning critical choices for successful dermal sampling.

MATERIALS AND METHODS

Probe Types

The dOFM probes have a linear design for intradermal/subcutaneous use in humans (EN ISO 13485 quality, CE-labelled for human use, though not yet commercially available). The outer diameter (OD) is 0.32 mm and the length of the open exchange area of the probe is 15 mm. The inner lumen has a diameter (ID) of 0.25 mm and is PTFE-coated for minimal adsorption. A fine steel mesh with a mesh width of 0.1 mm

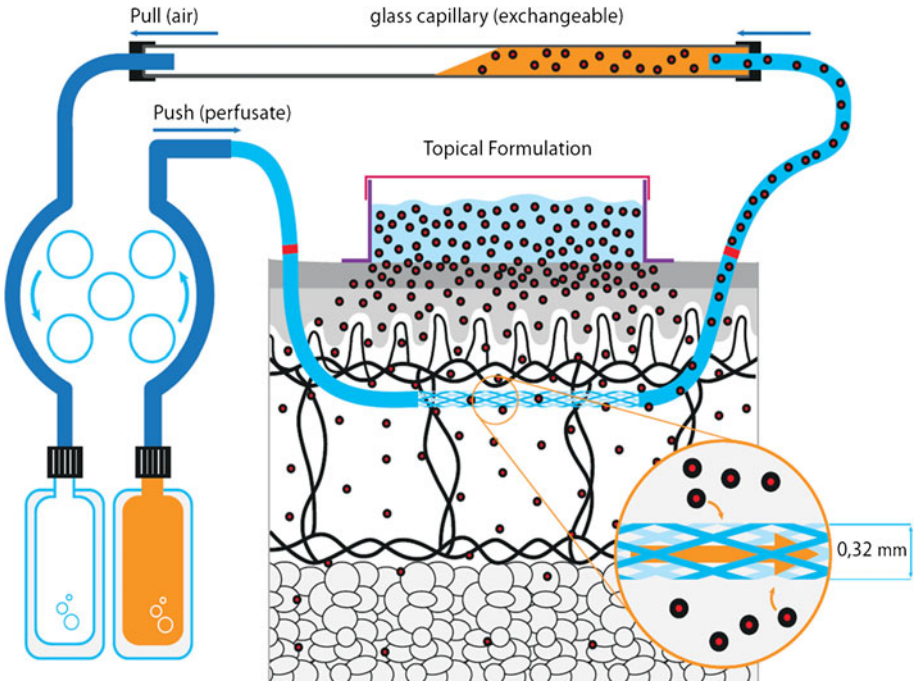


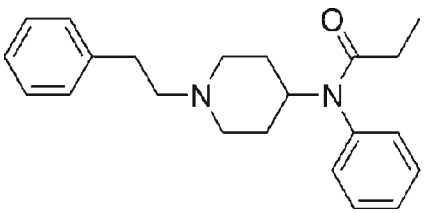
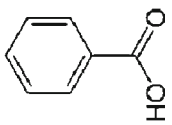
Fig. 2 Illustration of the Open-Flow Microperfusion probe placed in the dermis. The topically added penetrant is penetrating through the skin and sampled in the dermis. The push/pull system is connected to the probe and the samples are collected in an exchangeable glass capillary. Markings on the probe make a correct positioning in the skin easy.

serves as exchange area without cut-off between inner lumen and surrounding tissue. The probe has a small needle (50× 0.5 mm) attached at one end to ease implantation and minimize the skin trauma. In the current study the 0.5 mm needle was removed and the probes were all inserted via the larger CMA guide cannula (21-gauge, OD 0.8 mm), to give equal conditions to all probes. The sampling fluid was collected in 50 μL end-to-end glass capillaries (Hirschmann, Germany).

The CMA66 linear probe type is commercially available (CMA Microdialysis, Sweden) and like the dOFM probe CE-certified for human use. We used the 100 kDa CMA probe (10 mm membrane and an OD of 0.5 mm, Ref. No. 8010671), which was inserted by a 21-gauge (OD 0.8 mm; 50 mm length) guide cannula. Polyurethane inlet tube (ID 0.15 mm; 400 mm length) and polyurethane outlet tubing (ID 0.15 mm; 100 mm length) is provided for collection.

The linear 2 kDa MD probes were manufactured in the Danish laboratory by Benfeldt *et al.* Single hollow dialysis fibers of 0.22 mm OD (Gambro GFS 16+; Gambro Lundia AB, Lund, Sweden) with a nominal cut-off value of 2 kDa were used. This fiber is fragile and therefore a stainless-steel guide-wire (Sandvik Steel Wire, Norway, OD 0.10 mm) is placed inside the fiber for stability. Nylon tubing (Portex Nylon Tubing, Smiths Medical, USA, ID 0.50 mm and OD 0.63 mm) was glued to

Table I Physico-Chemical Properties of the 2 Penetrants

Name:	Fentanyl	Benzoic acid
CAS:	437-38-7	65-85-0
Formula:	C22H28N2O	C7H6O2
Structure:		
Mol. weight (g/mol):	336.5	122.1
LogP $_{ow}$*:	4.05	1.87
Usage:	Analgesic	Food preservative

* Values obtained from http://www.chemspider.com/Chemical-Structure.3228.html

one end of the fiber using cyanoacrylate glue (Loctite, Super Attak, Denmark). The membrane has no outlet tubing as such (except for the tubular membrane structure) and no fixed length, hence the membrane length accessible for drug diffusion is dependent on the insertion length in the skin. To reduce possible evaporation, the outlet membrane length was kept at a minimum. The probes were placed in an air-sealed container before use and moved to a container containing the perfusate 20 min prior to the experiment. Before connection to the pump, all dMD probes were carefully flushed to identify broken or blocked probes and to avoid air-bubbles blocking the probes. The dialysates from the CMA66 and 2 kDa probe outlets were collected in plastic vials (0.2 mL Domed Cap Maximum Recovery PCR Tubes®, Axygen, USA)

Penetrants

Fentanyl: 3 mL fentanyl was added to each penetration-chamber. The commercially available aqueous fentanyl solution (Janssen-Cilag, Belgium) was modified by ethanol (40 μg Fentanyl/mL in 20% ethanol) in order to assure skin penetration and to assure the recovery by the dMD probes. Benzoic acid: 3 mL aqueous benzoic acid solution (2.5 mg/mL) (Benzoic acid, Sigma-Aldrich, Germany) was added to the penetration-chambers in the benzoic acid set-up.

Perfusate

Fentanyl: Due to the high lipophilicity of fentanyl it was necessary to add 1% human serum albumin (Albunorm 200 g/L solution for infusion, Octapharma, Austria) to the traditionally used isotonic perfusate (ELO-MEL, Fresenius Kabi Austria; electrolyte composition in nM: 140 Na^+, 5 K^+, 2.5 Ca^{2+}, 1.5 Mg^{2+}, and 108 Cl^-) to facilitate the transport of fentanyl across the semi-permeable membranes in the case of the 2 kDa probe. The albumin content also gives the *ex vivo* perfusate a closer resemblance to the *in vivo* interstitial fluid.

Benzoic acid: The perfusate for the benzoic acid set-up was the traditionally used isotonic ELO-MEL.

The perfusion fluid was the same for all probe types and is uniformly referred to as 'perfusate'. The fluids obtained by dialysing probes and membrane-free probes are essentially different in composition. Thus, the dMD sample is referred to as 'dialysate' throughout the paper, whereas that of dOFM is termed 'OFM sample fluid'.

Skin

The skin was obtained from Caucasian women (n=9) undergoing abdomino plasty at the Department of Plastic and Reconstructive Surgery at the University Hospital in Odense, Denmark. The skin flap was left unprepared after surgery and the subcutaneous layer was left *in situ* on the flap to resemble the *in vivo* situation as much as possible. The skin was kept at −20°C thus avoiding the damaging effect of freezing the skin at a lower temperature (25) and was kept for periods not exceeding 12 months. This has been proven to maintain the barrier properties with no significant change in the water permeability (26). Only skin with a healthy appearance and no signs of skin surface abrasions was used. The donor was given complete anonymity and only age and surgery date was kept. The regional ethics committee approved the study.

Study Design

The *ex vivo* setting in this study was chosen to provide necessary experimental experience to improve the likelihood of unproblematic and reliable transfer from *ex vivo* sampling to *in vivo* conditions for future studies.

Recovery Study

In our recovery studies three probes of each type were placed in a beaker containing a medium of fentanyl or benzoic acid. The studies were performed over a concentration range of 1, 10, 100 and 500 ng/mL for fentanyl and 10, 100, 250, 500 and 1000 μg/mL for benzoic acid. The solutions were magnetically stirred at 200 rpm at room temperature (24°C). The experimental time was 4 h and dialysates/sample fluids were collected every 30 min and analyzed by HPLC.

Main Study

In each experiment skin from one donor was used and six probes of each probe-type (dOFM, 100 kDa CMA66 and 2 kDa dMD) were inserted in the dermis (Figs. 3 and 4). A total of nine experiments were performed and 54 probes of each type were used in these experiments. Three conventional multichannel peristaltic pumps (Minipuls 3; Gilson, Villier-le-Bel, France) were used to perfuse the 18 probes in each experiment. The dOFM probes were connected to the pump for rate-controlled perfusate inflow ('push') and on the outflow side to a sampling unit for rate-controlled sample withdrawal ('pull'). A pilot experiment confirmed that the membrane-free dOFM probes need to be connected to the pull mechanism to avoid loss and oedema in the tissue. The CMA66 and 2 kDa probes were also connected to the peristaltic pump, whereas the pull mechanism was not required for these probes to sample successfully. Prior to each experiment the pumps were prepared with peristaltic tubing, the perfusates were degassed at room temperature using an ultrasonic bath, and the flow rate was checked.

The donor skin was inspected for any visual damage and excluded from the study if this was suspected. Following a standardized gentle wash to clean the skin flap (using tap water and paper tissue), the flap was cut in two pieces, one half for each penetrant (fentanyl and benzoic acid). The donor skin

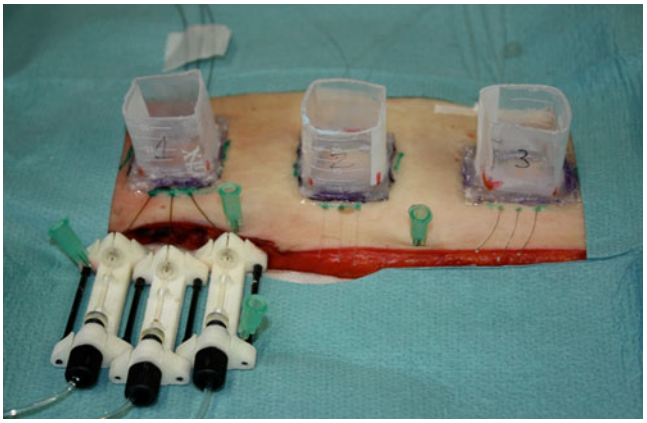


Fig. 3 Photo of the experimental set-up before adding test solution. The probes are positioned in the dermis and the donor chambers have been glued to the skin. Donor chamber 1 (left) is situated above the dOFM probes and the push-pull system with the sampling capillaries connected. Donor chambers 2 and 3 are situated above the CMA66 and the 2 kDa probes, respectively (the sampling vials have not yet been placed). The donor skin has been mounted without removing the subcutaneous fat. Test solution is added to the chambers and the chambers covered.

(including subcutaneous tissue) was mounted/pinned on Styrofoam. The positioning of the penetration-chambers as well as the entry and exit points for the insertion of the probes was planned and outlined on the skin surface using a marker. To assure reproducibility an experienced person inserted all probes/guide cannulas (superficially in the dermis). All probes were inserted using 21-gauge (0.8 mm) guide cannulas, three under each outlined penetration-chamber area, nine probes in each half-flap and a total of 18 probes in each experiment/donor. Probes were never re-used. The probes were placed through the guide-cannula and carefully flushed to check functionality. When functionality was confirmed the guide-cannula was withdrawn leaving the probe in the dermis. The probes were then connected to the pumps, working with a flow-rate of 1 µL/min. When all probes had been flushed and tested for functionality, an equilibration period of no less than 30 min was started. The chambers were glued onto the skin and left to dry for 15 min before the penetrant solutions were added, following which the penetration-chamber were covered with Parafilm® to avoid evaporation. To keep the temperature around the flap constant, a lamp was placed high above each skin flap (preventing a direct hot heat source) and a thermometer below each flap secured a temperature of 25–28°C. This set-up may increase the thermodynamic activity in the solution, but this effect would then be similar for all probe-types.

The length of the permeable/membranaceous structure was 15 mm for the dOFM probe and 10 mm for the CMA66 probe. The 2 kDa probe was inserted through entry and exit points marked on the skin ~20 mm apart. Based on the penetration-chamber size of ~15 × 15 mm (see Figs. 3 and 4), and since the penetrating substances will not only penetrate vertically through the skin but also in a lateral direction, the length of exposed effective membrane was estimated to be 20 mm in total for the 2 kDa probe. An estimation of the effective membrane surface of each probe-type is shown in Table II. Across the probe wall/structure the interstitial fluid partially equilibrates with the perfusion fluid by diffusion (dMD) or convection (dOFM). Sample fluids from dOFM were collected in capillaries connected between the probe outlets on one side and the pump on the other for 'pull' functionality. Any sample evaporation was therefore prevented. Since the capillaries only had a volume of 50 µl they

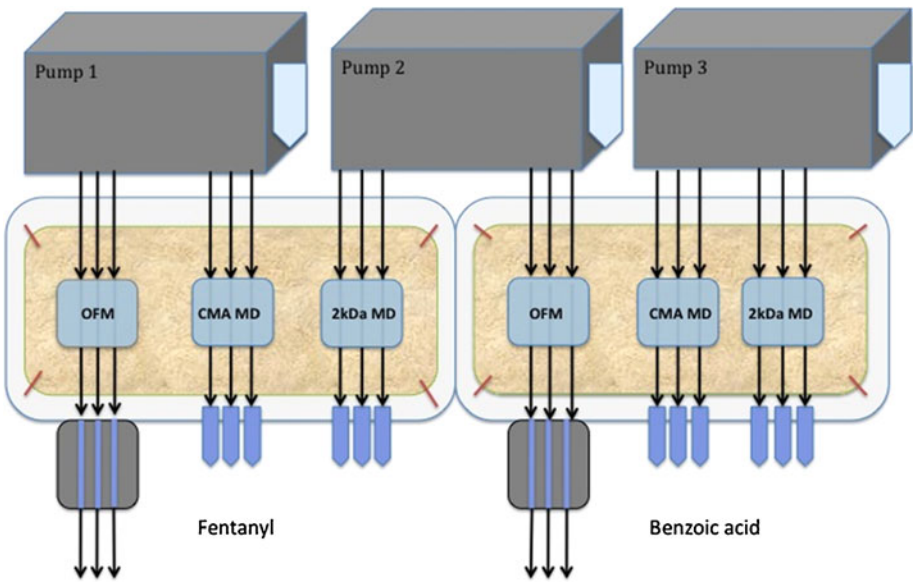


Fig. 4 The experimental set-up consists of three peristaltic pumps and one skin flap (which is divided in two halves). In each half flap three probes of each probe-type is inserted and one penetrant is applied in the penetration chambers glued to the skin surface. Fentanyl solution is added to the three penetration chambers on top of one flap and Benzoic Acid in the three penetration chambers on the other flap. The dOFM probes are connected to a push/pull pump function and glass capillaries whereas the other probe types (CMA66 and the 2 kDa) are collected in vials placed at the outlet.

Table II Specifics for the 3 Different Probe Types and Their Requirements. Membrane Length and Surface area of the 2 kDa Probe is Estimated Based on the Conditions in the Present Study

Probe type	Exchange surface length	Outer diameter	Surface area	Pump required	Nominal Cut off	Recovery %		Exchange surface
						Fentanyl	Benzoic acid	
OFM	15mm	0.32 mm	15.1 mm^2	Push-pull	None	96 ± 1.2	100 ± 0.1	 open sampling mesh
CMA 66	10 mm	0.50 mm	15.7 mm^2	Push	100 kDa	81 ± 5.5	101 ± 4.7	
2kDa MD	≈20 mm	0.22 mm	13.8 mm^2	Push	2 kDa	70 ± 2.2	79 ± 2.8	semi-permeable membrane

had to be changed every 40 min and the contents of three capillaries were pooled to gain 2-h vials. Dialysates from dMD probes were collected in plastic vials covered by Parafilm® to avoid evaporation. The vials were changed every 2 h. We collected 10 two-hour samples from each probe in all experiments from baseline to 20 h post-dose. Samples were frozen at −20°C immediately after sampling and until analysis.

Chemical Analysis

Fentanyl

The fentanyl concentration in the sample fluid was assayed by high performance liquid chromatography-mass spectrometry (HPLC-MS/MS) using an Ultimate 3000 HPLC system (Dionex, Vienna, Austria) coupled to a Quantum TSQ Ultra AM MS (ThermoScientific, Vienna, Austria). Xcalibur-Software 1.4 controlled the system.

The separation was performed using a Zorbax SB-C18 (35×0.5 mm (3.5 µm)) column (Agilent Technologies, Vienna, Austria) at a flow rate of 0.05 mL/min. The chromatographic eluents consisted of water (mobile phase A) and acetonitrile (mobile phase B), both containing 0.1% formic acid. For the quantification of fentanyl, a linear gradient from 0 to 90% mobile phase B in 0.3 min was applied using sufentanyl (Sufenta 0.05 mg/mL, Janssen-Cilag, Belgium) as internal standard (IS).

Positive electrospray-MS/MS detection of fentanyl was performed using the following parameters: spray voltage 3.5 kV, capillary temperature 250°C, sheath gas pressure 50 AU, auxiliary gas 5 AU. Quantitation was performed in multiple reaction monitoring mode (collision gas pressure of 1.5 mTorr) for fentanyl (337.2→fragments 215.8, 187.8, 145.8, 104.8 m/z; collision energy 18, 22, 29, 35 eV, respectively) and the IS (387.2→fragments 354.8, 237.8, 139.8, 110.8 m/z; collision energy 17, 18, 26, 35 eV, respectively).

Fentanyl standard solutions and quality controls were prepared in 30% acetonitrile each day of analysis covering a range from 0.5–20 ng/mL. Samples were thawed and mixed shortly prior to sample processing (liquid-liquid extraction). Briefly, 70 µL ammonium hydroxide solution (15 mM) and 10 µl IS (10 ng/mL) were added to 90 µL sample. Liquid-liquid extraction of fentanyl was performed by adding 400 µl *tert.*-butyl methyl ether and whirl-mix for 10 s. After centrifugation at 1000 g for 2 min at 4°C the organic layer was transferred into 200 µl auto-sampler vial inlets and evaporated to dryness (UniVapo 100ECH, UniEquip, Germany; 1 h at room temperature). The dried extracts were reconstituted by dissolving in 20 µl reconstitution solution (acetonitrile:water 30:70 v/v). The auto sampler inlets were transferred into auto sampler vials, which were capped and placed into the auto sampler for injection of 7.5 µL of each sample. The preconcentration of fentanyl during sample processing lead to a final quantification range of 0.11–4.4 ng/mL.

Benzoic Acid

A LaChrom HPLC system (Merck Hitachi, Darmstadt, Germany) was used for quantitation of the benzoic acid concentration in the sample fluid. The separation was performed using an ACE C18 (150×4.6 mm) 5 µm column (Advanced Chromatography Technologies, Aberdeen, Scotland), installed with a C18 4×3.0 mm precolumn (Phenomenex, Torrance, Canada). The mobile phase consisted of a 70:30 v/v% (1/15 mol/l KH_2PO_4; pH=4.57) methanol and was used with a flow rate of 1.0 mL/min. UV-detection was performed at 225 nm. The limit of quantification (LOQ) was 10 ng/mL. The limit of detection (LOD) was 3 ng/mL. Standard solutions were prepared in ethanol. Standard curves were produced each day of analysis and covered a range from 5 to 50 µg/mL. The sample preparation consisted of a single dilution step and addition of internal standard; 10 µL sample +190 µL HPLC mobile phase

+ 50 μL 100 μg/mL sorbic acid (IS). If a sample concentration exceeded the range, a further dilution was performed. The sample was added into a 300 μL HPLC vial, whirl-mixed and a sample volume of 20 μL was injected onto the system.

Data Treatment and Statistical Analysis

The RR was established for each probe-type by initially calculating the average RR values of the three probes in each experiment. Afterwards the mean and standard deviation of these average values were calculated providing the mean RR value of the concentrations.

Data was plotted as the concentration of penetrant sampled as a function of time. The mid-point of the sampling interval was used as time-point for calculations and the standard deviation (SD) was calculated. The Area Under the time-concentration Curve (AUC) was determined for each probe and the mean was then determined from the different probe-types.

The absorption rate was determined by the slope of the curve showing the amount of penetrant absorbed over time per mm^2 of permeable probe surface. This is different from the normal absorption rate, which is calculated as the amount of substance per unit surface area of exposed skin per unit time. Since this study is a comparison of sampling methods the comparison is undertaken by applying the same amount of penetrant solution to skin areas of the same size and subsequently quantifying the content of penetrant in the samples from the dermis. As the different probes have different areas available for exchange, we will need to adjust for that to make fair comparisons. Therefore the amounts of penetrants sampled by the different probes were divided by the exchange area of the probes.

The lag-time from each individual probe/graph was found from the intercept of the linear part of the graph with the x-axis (27). From these values the mean lag-time was calculated.

Since technical challenges and probe failures were anticipated in this study, we chose to insert 3 probes and analyze only 2. In this way equal numbers of probes were included in the study leveling out the number of malfunctioning probes. To avoid selection bias, the probes were selected before chemical analysis.

The non-parametric Mann-Whitney *U* test was used to compare the medians for statistical differences. Alpha-errors less than 0.05 were considered significant. Results are presented as medians with the 25% and 75% percentiles in a table but as mean ± SD values in the graphs for a more intuitive and illustrative presentation.

Data and Sample Exclusion

Pre-analysis

Probes were excluded if there were obvious signs of malfunction (blocked probe, leaking tube connection, low sample volume).

Post-analysis

Data from probes were excluded if the result from a probe was >3 SD away from the mean of the comparable probes or the results indicated damage to the skin barrier. If there were signs of contamination by fentanyl or benzoic acid at specific singular time points these time-points were excluded.

RESULTS

For fentanyl the RR for the different probes were: dOFM: 96 ± 1.2%, CMA: 81 ± 5.5% and the 2 kDa: 70 ± 2.2% and for benzoic acid the RR for the different probes were: dOFM: 100 ± 0.1%, CMA: 101 ± 4.7% and the 2 kDa 79 ± 2.8%.

All three probe-types provided measurable concentrations of both fentanyl and benzoic acid in the dOFM samples and in the dialysates (Fig. 5a and b). Following topical application of the two penetrants a relatively rapid dermal delivery was seen, especially for benzoic acid (Fig. 5b). The physico-chemical difference between fentanyl and benzoic acid is reflected in the absorption profiles of the two penetrants (Fig. 5a and b). The small, only slightly lipophilic benzoic acid showed a faster penetration (shorter lag-time and higher absorption rate - Table III). The median AUC of fentanyl and benzoic acid are shown in Table III. There were no significant differences in AUC between the probe-types sampling fentanyl and benzoic acid, respectively (*P*-values all > 0.05). The median Cmax of fentanyl and benzoic acid sampled by the different probe-types showed no significant probe related differences (*P*-values all > 0.05) (Table III). The median lag-times (the time from the penetrant is applied onto the skin surface (t=0) to the time point when a quantifiable concentration is found in the samples) of fentanyl showed no significant differences between probe-types (*P*-values > 0.05) whereas the lag-time for benzoic acid in the 2 kDa probe was significantly shorter than that of the dOFM and CMA66 probes (Table III). The median absorption rates of fentanyl were not significantly different between the probe-types, but the absorption rate for benzoic acid was significantly higher when sampled by the 2 kDa probe than by the two other probe-types (Table III).

Of the 9 donors absorbing fentanyl one donor (donor 8) displayed lower penetration with AUC ranging from 2 to 5 ng*hr/mL and a significantly prolonged lag-time of 404–581 min in all probe-types. Another donor (donor 7) displayed a significantly increased penetration for fentanyl, most pronounced in the samples from the 2 kDa probe where the lag-time was as short as 5 min and the AUC 240 ng*hr/mL. These results are > 3SD away from the mean, and since this unusual penetration profile is seen for both 2 kDa probes, this could indicate a damaged skin barrier. We therefore excluded donor 7.

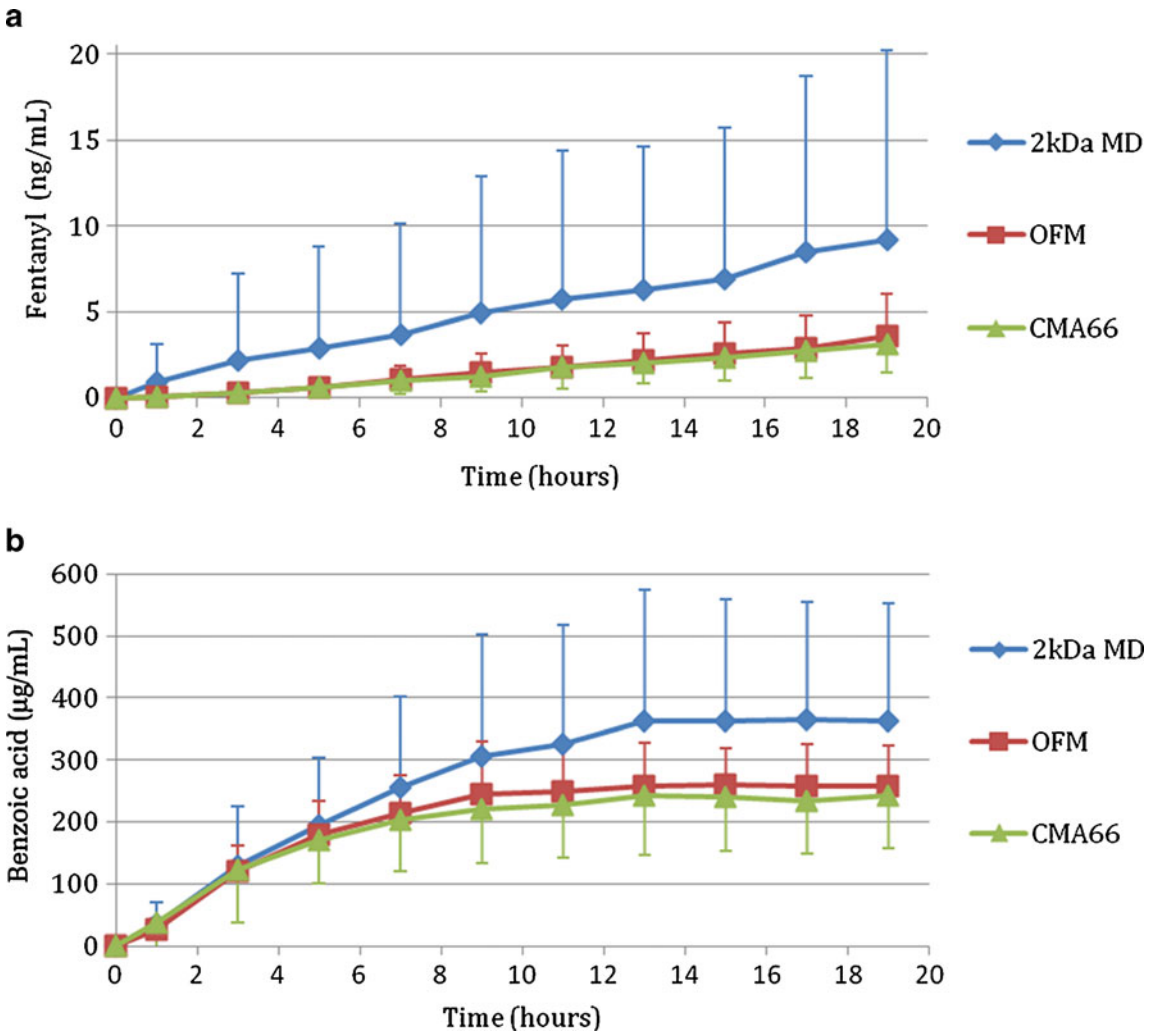


Fig. 5 (**a**) Mean concentration (mean ± SD) of Fentanyl sampled ($n=16$ for dOFM and CMA66; $n=14$ for 2 kDa) during 20 hours by the three probe-types. (**b**) Mean concentration (mean ± SD) of Benzoic Acid sampled during 20 hours by the three probe-types ($n=18$ for all probe-types).

For benzoic acid, the variability in penetration was generally lower than for fentanyl for all probe-types (Fig. 5a and b). The 2 kDa probes had much higher variations than the two other probe-types in the fentanyl experiments, whereas this was not as obvious in the benzoic acid experiments.

One donor (donor 1) displayed faster absorption than the remaining donors for all probe-types (large AUC, high absorption rate and short lag-time). The difference was more significant for the 2 kDa probe than for the dOFM and the CMA probes.

Table III Pharmacokinetics of the 2 Penetrants Shown for the 3 Probe Types Separately ($n=14$–16 for fentanyl $n=18$ for Benzoic Acid). Results are Stated as Medians with 25- and 75-Percentiles in Parentheses

	Fentanyl				Benzoic acid			
Median	AUC (ng*hr/mL)	Cmax (ng/mL)	Abs. rate (ng/mm^2/min)	Lag-time (min)	AUC (µg*hr/mL)	Cmax (µg/mL)	Abs. rate (µg/mm^2/min)	Lag-time (min)
dOFM	11.7 (5.6–25.6)	2.6 (1.3–6.3)	0.6 (0.4–1.5)	247 (132–402)	2123 (1450–2571)	279 (193–318)	195* (136–223)	76* (62–88)
CMA66	12.2 (5.3–18.8)	2.6 (1.5–4.0)	0.9 (0.4–1.3)	262 (157–325)	1787 (1314–2400)	222 (168–311)	135* (85–194)	74* (58–79)
2 kDa	27.9 (13.4–36.4)	6.2 (2.7–8.0)	1.8 (0.6–2.2)	274 (187–334)	2878 (1187–3925)	380 (169–520)	348 (193–466)	40 (31–51)

* significantly different from 2 kDa: $P<0.05$

During the pre-analysis phase significantly more probes were excluded due to malfunction when the perfusate contained 1% albumin. In particular the number of 2 kDa probes with malfunction was more than doubled (8 *vs.* 3 in the albumin-free studies).

For the fentanyl studies, where the perfusate contained albumin, the following numbers of probes were malfunctioning: dOFM 2, CMA66 3 and 2 kDa 8. For the benzoic acid studies, where the perfusate did not contain albumin, the following numbers of probes were malfunctioning and therefore excluded: dOFM 1, CMA66 1 and 2 kDa 3.

DISCUSSION

This study represents the first comparison of the dOFM and the dMD methods. By using both methods in parallel in a human dermis *ex vivo* set-up it was possible to optimize the experimental conditions and to compare the advantages and challenges of the two methods.

Recovery Study

We established a high RR value for most of the probe-types. The RR is - as expected -lower for fentanyl than for benzoic acid as lipophilic penetrants generally have lower recoveries than less lipophilic penetrants (28). However, the RR of fentanyl is high considering its lipophilicity, which could be facilitated by the albumin content in the perfusate, since the addition of albumin is known to increase RR (4,29). The high RR of fentanyl in dOFM (close to 100%) is a feature of the dOFM open-mesh sampling in combination with the push-pull pump system (30). When sampling small penetrants like benzoic acid, the pump system has no significant influence on the RR.

Pharmacokinetics Obtained by OFM and MD

Benzoic acid reaches a steady-state level faster than the larger, more lipophilic fentanyl, which penetrates the skin slower, stays in the skin longer and does not reach a steady-state level during the 20-h sampling period (Fig. 5a and b). All three probes sample benzoic acid more effectively than they sample fentanyl in these *ex vivo* studies. The very high dermal concentration of benzoic acid is most likely due to the unique pharmacokinetic properties of this particular penetrant, which is known to exhibit fast and high penetration through the SC and has a low affinity for reservoir formations in the dermis (23,31,32).

The OFM and the CMA probes were in general unproblematic in practical set-up and the two probe-types showed concordance when sampling fentanyl as well as benzoic acid (Table III). When sampling benzoic acid, the 2 kDa probe gave quantitatively different results than the other probes when it came to absorption rate and lag-time but all probes showed concordance in the AUC and Cmax parameters.

The differences in absorption rate between the probe-types may relate to the different structure and material of the probes (Table II). The OFM probe mesh structure takes up about 50% of the surface area. In the 100 kDa and 2 kDa probes the area of exchange is more difficult to estimate, since the pores in the membrane are microscopic. However, the 'actual' area of exchange could be larger than for the OFM probe even though the calculated surface area of the OFM and the CMA66 probe is the same and the calculated surface area of the 2 kDa probe is smaller than for the other probe-types (Table II). Furthermore, membrane length itself has been described to affect the recovery (28,33) and the 2 kDa probe membrane is longer than the other probe-types.

The probe material also affects the propensity of the penetrants for adhering to the probe and thus the amount of penetrant recovered. Even though the RR is in theory independent of the concentration, exceptions have been observed (28) and described as a saturation of the membrane binding sites. Adsorption would therefore be expected to be most influential for the lower concentrations of lipophilic penetrants. Since our *in vitro* RR results (RR by gain) for the lowest fentanyl concentration (1 ng/mL) were higher than for the three higher concentrations, no adsorption tendency was found in our study. In view of the fact that we used albumin in the perfusate for *in vitro* RR (similar to how it was used in the *ex vivo* study) the albumin content may well have prevented adsorption. Finally, in the *ex vivo* study the 2 kDa probe has no outlet tubing, since the hemodialysis fiber continues from the outlet opening in the skin directly into the sampling vial, thus limiting the potential adsorption area.

For the lipophilic fentanyl the longer lag-time and the lower absorption rate than those found for benzoic acid was expected due to the reservoir effect of the skin. A lipophilic compound will easily cross the stratum corneum but the diffusion rate will decrease as it reaches the deeper, more hydrophilic epidermis. Substances that are soluble in the lipophilic layer as well as in the more aqueous layer and, at the same time, are small in molecular size have the fastest penetration through the skin barrier (34). The highest degree of absorption is associated with $\log P_{ow}$ values of 1–2 and decreases considerably when exceeding $\log P_{ow}$ of 3.5 (32,35). As expected, benzoic acid with a $\log P_{ow}$ of 1.87 penetrated the skin fast and with a short lag-time.

Variability

The inter-individual variability in skin penetration of fentanyl through human skin *in vitro* is around 40% (36). The inter-individual variability of results obtained using dMD in studies of topically applied drugs generally ranges from 30–

90% (37–40). In the present study the experimental variability for sampling varied between penetrants as well as between probes. The variability was higher for studies on fentanyl than for benzoic acid, and higher for the 2 kDa probe than for the other probe-types (Fig. 5a and b). We believe that this observation also reflects the high number of 2 kDa probes excluded when the albumin-containing perfusate was used. It seems that the 2 kDa probe, which has a narrower lumen and a much lower cut-off than the two other probe-types, is more susceptible to failure (by blockage/clotting or other) induced by the presence of albumin in the perfusate.

Experiments using dMD or dOFM have several sources of variability including inter-individual variability, day-to-day variability, intra-individual variability, variability in probe depth *etc*. For the 2 kDa probe variability in the effective length of the permeable section (intradermal membrane) is an added source of variability not present for the two other probe-types (Table II). Unfortunately we could not measure probe depth in the current study. Variability in probe depth has been demonstrated to affect the pharmacokinetic parameters of topically applied penetrants sampled by dMD *ex vivo* (41). However, the same study states that if all probes are inserted by the same experienced person, probe depth variability will be less than 0.1 mm and not influence the amount of penetrant sampled (41).

'Abnormal Absorbers'

When samples from one individual donor deviate significantly from other donors, they are often called outliers. In many cases they are removed from the study causing 'better' results with less variability. However, this need not be correct and will potentially ignore important information on individuals with a deviating susceptibility. Removal of outliers should therefore only be done after careful consideration and with plausible arguments. We observed a significantly deviating absorption profile of donor 7 in the 2 kDa probe. Reasons for 'abnormal absorption' could be local damage to the skin barrier inflicted either prior to (e.g. shaving) or during surgical procedures (e.g. pulling on the skin) (37,42,43). Donor 7 could also be an "extensive absorber". An extensive absorber is an individual that for known or unknown inherent reasons absorb a specific chemical significantly faster than the average person (36). This increased susceptibility to dermal exposures may pertain to single chemicals, but most often to groups of chemicals with similar physicochemical properties. In the present experiments it is too late to recheck skin integrity, and we are not able to conclude whether the skin is damaged or if donor 7 is an extensive absorber. However, since the deviating penetration profile is not as significant in the other probe-types using the same donor, damage to the skin is most likely and justifies the exclusion of donor 7. Donor 8 has a characteristic absorption profile too. All probe-types sample an almost negligible amount of fentanyl in this donor, but the absorption of benzoic acid in donor 8 is close to average. Donor 8 could be a "poor absorber" of fentanyl. Extensive and poor absorbers have been described previously in an *in vitro* study of fentanyl in Franz diffusions cells (36). If poor or extensive absorbers exist this could indicate that the absorption profile is not only related to the donor but also to the penetrant of choice.

Methodological Advantages and Challenges

The challenge was to sample the lipophilic fentanyl and while doing so evaluate and compare two different sampling methods. Prior to the present study we investigated the feasibility of fentanyl sampling by the dOFM probe (*ex vivo*) and the 2 kDa probe (*in vitro*). Studies were conducted with fentanyl in a pure aqueous solution (50 μg/mL) and using a traditional hydrophilic perfusate (Ringer solution) without albumin (results not shown). The dOFM results showed stable recoveries, whereas the 2 kDa probe showed unstable and low recoveries. In order to permit a comparison of the three probe-types, the experimental conditions were optimized by adding ethanol to the fentanyl solution (40 μg/mL fentanyl in 20% ethanol) and adding 1% albumin to the perfusate. The addition of albumin to the perfusate is known to enhance recovery of both protein-bound and lipophilic substances in dMD (4,7) and adding ethanol to the topical solution is known to increase drug penetration across the skin barrier (44,45). Under these specific *ex vivo* conditions, analyses of the dialysate and OFM samples demonstrated stable and reproducible fentanyl concentrations for all probe-types. No modifications were needed in the benzoic acid set-up since benzoic acid is far less lipophilic and easily penetrates the skin and readily enters the perfusate.

The OFM and CMA66 probes were more robust to work with, whereas the 2 kDa probes were more vulnerable to mechanical damage and to blockage by air-bubbles or albumin in the perfusate. On the other hand the 2 kDa probe is less expensive, since it is made in the laboratory. However, it is not CE-certified. For preclinical purposes, however, a non CE-certified probe could be a relevant choice in order to reduce expenses related to the study.

The OFM and the 2 kDa probes are easy to place exactly under the penetration chamber, as the OFM probe has preset markings for visual positioning of the exchange area right under the planned drug application site and the 2 kDa probe has an infinite membrane length.

The dMD set-up was less demanding than the dOFM procedures both technically and labour-wise. The dOFM method requires push-pull pump system to avoid fluid loss and formation of oedema in the dermis, whereas only a

simple push pump was required for the dMD probes used in our study. The dOFM technique is more demanding analytically (both technically and labor wise) due to presence of larger molecules and smaller proteins in the samples. An overview of advantages and challenges of the different probes is given in Table IV.

The present study does not allow us to conclude on the specific influence of MW or lipophilicity as our penetrants vary in both MW and $\log P_{ow}$. However, the MW of the two penetrants are both < 500Dalton, as topical drugs penetration is in general limited to substances below this size, described by Bos *et al.* as "the 500 Dalton rule" (46). However, the purpose of the present study was to compare dMD and dOFM by sampling a lipophilic as well as a less lipophilic penetrant and, based on the samples analysed and the hands-on experience, to evaluate the performance of each method. Future studies should include penetrants with variable logP and similar MW in order to selectively study the impact of lipophilicity on the efficacy of the sampling devices.

CONCLUSION

The comparison of the dOFM and dMD methodology demonstrated that both techniques are suitable for *ex vivo* dermal sampling of topically applied benzoic acid in a pure aqueous solution and fentanyl in an ethanol-containing solution and a perfusate including 1% albumin. Under these conditions the three probe-types show concordance in AUC and Cmax for both benzoic acid and fentanyl.

The technical aspects of using the different probe-types implicate important differences in relation to handling, vulnerability and experimental set-up. This should be considered in relation to study type, penetrant/drug and analytical techniques available as well as experience.

For studies of lipophilic compounds the OFM method can be expected to be advantageous despite the technical and analytical challenges described in the current study. Our study needs to be repeated with more lipophilic drugs as well as conducted under *in vivo* conditions in order to confirm the present experimental observations.

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Table IV Advantages and Limitations Related to the Different Probe Types. The + and ÷ Indicates if the Probe has the Mentioned Defining Feature and + or ++ Indicates if it is More or Less Characteristic for the Probe-Type

Method and probe comparison			
Probe type	dOFM	CMA66 100 kDa	MD 2 kDa
Minimally invasive, no loss of body fluid volume, thus also for paediatrics and small animals	+	+	+
Sampling at the site of action	+	+	+
Continuous sampling	+	+	+
Multiple application sites	+	+	+
Good reproducibility	+	+	+
Sampling of drug and metabolites	+	+	+
Sampling of hydrophilic drugs	+	+	+
Sampling of very lipophilic drugs ($\log P > 4.5$)	++	+	÷[1]
Sampling of large molecules	++	+	÷
Protein-free samples	÷	÷	+
Cessation of enzyme degradation in the samples	÷	÷	+
Push-pull required	+	÷	÷
Robust when handled	+	+	÷
Position marks on the probe for easy placement	+	÷	÷
Available as CE-certified product for human use	+[2]	+	÷
Low cost due to manufacture in the lab.	÷	÷	+

[1] Sampling can be facilitated by altering the perfusate, but may still prove difficult

[2] The OFM probe is not yet commercially available

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