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Diclofenac in vitro microdialysis study comparing different experimental set-ups to improve quantitative recovery

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Abstract

Several studies investigated diclofenac tissue concentrations using microdialysis (MD). However, thorough evaluations of the optimal MD set-up for diclofenac are unavailable. Thus, this in vitro MD study aimed to compare different set-ups to improve quantitative recovery of diclofenac. In forward and reverse in vitro MD experiments with diclofenac at two concentrations (1 and 100 ng/ml), the perfusion solutions physiological saline 0.9% (PS) and human albumin 1% (HSA) were compared using tissue probes (10-mm membrane) and customized intravenous (iv) probes (30-mm membrane). Using PS, the mean relative recovery of diclofenac at 1 ng/ml was $1.6\% \pm 0.04\%$ and $3.12\% \pm 0.00\%$ with the tissue probe and the iv probe, respectively. The respective mean relative recovery for diclofenac at 100 ng/ml was $0.02\% \pm 0.01\%$ and $0.21\% \pm 0.11\%$. Using HSA, the mean relative recovery was $314\% \pm 25\%$ (tissue probe) and $1064\% \pm 97\%$ (iv probe) for diclofenac at 1 ng/ml and $444\% \pm 91\%$ and $1415\% \pm 217\%$ for diclofenac at 100 ng/ml. In reverse dialysis using PS, the mean relative loss of diclofenac was $99.2\% \pm 0.5\%$ (tissue probe) and $95.8\% \pm 1.7\%$ (iv probe). Using HSA, the mean relative loss was $-4.4\% \pm 7.2\%$ and $0.2\% \pm 7.5\%$, respectively. PS and HSA were not suitable perfusion solutions for quantification of absolute diclofenac concentrations. Despite methodological challenges, HSA may be used for comparative experiments or bioequivalence studies.

KEYWORDS
analgesics, diclofenac, microdialysis, pharmacokinetics, tissue concentrations

1 | INTRODUCTION

Diclofenac is a widely used and the most studied non-steroidal anti-inflammatory drug (NSAID) for topical use.[1] Supposed advantages of topical over systemic administration include greater pain relief at the treated site and lower probabilities of systemic side effects.[2]

Applying diclofenac topically obtains high local concentrations of diclofenac in the target tissues while maintaining low systemic exposure to the parent

compound and its metabolites, thereby reducing the occurrence of systemic adverse events. Diclofenac has been observed to preferentially distribute and persist in deep inflamed tissues, such as the joint,[3] where it is found in concentrations up to 20 times higher than in plasma.[4] Pharmacokinetic studies can also directly compare different formulations (e.g., spray gel instead of conventional gel[5]) or assess techniques to enhance delivery (e.g., iontophoresis[6]) at the site of interest. For such studies, microdialysis (MD) represents a well-established technique for determining free (i.e., unbound) tissue concentrations.[7] MD can measure unbound solutes in the surrounding medium (e.g., interstitial fluid) using passive diffusion across a semipermeable membrane. For this purpose, MD probes must be constantly perfused with solution, allowing the sampling of the substance-containing dialysate. Importantly, before conducting MD studies in humans, the feasibility and reliability of the MD set-up for each substance should be demonstrated in vitro.

Although no thorough evaluations of the optimal MD set-up for diclofenac have been published to date, several MD studies in humans have already been performed, with widely varying results.[6,8] Even though comparisons of results across studies may not be appropriate, the MD could have been further optimized for diclofenac because of its high plasma protein binding of 99.8%.[9] Thus, the selection of an appropriate perfusion solution is challenging. Additional factors such as membrane length, concentration dependency and consistency in long-term samples should also be considered.[10] A better understanding of these experimental variables might improve the design of future MD experiments with diclofenac.

To address these uncertainties and to improve the MD set-up for diclofenac, this in vitro MD study compared physiological saline 0.9% (PS) as perfusion solution with human albumin 1% (HSA), which has been proposed to prevent absorption problems and improve recovery.[10] Moreover, different diclofenac concentrations, probe types and sampling schemes were examined. We additionally aimed to evaluate ketoprofen as a potential internal calibrant for diclofenac and urea as a local blood flow marker, both of which might be valuable in human MD studies on diclofenac.

2 | METHODS AND MATERIALS

The study was conducted in accordance with the Basic & Clinical Pharmacology & Toxicology policy for experimental and clinical studies.[11]

2.1 | Substances

The substances used in the experiments included diclofenac (Deflamat® 75 mg/3 ml solution, Haupt Pharma Wuelfing GmbH, Gronau, Germany), ketoprofen (Profenid® 100 mg/2 ml solution, Sanofi-Aventis GmbH, Vienna, Austria), HSA (Human Albumin 'CSL Behring'® 20% solution, CSL Behring GmbH, Marburg, Germany), urea (Fagron Services B. V., Uitgeest, Netherlands) and PS (conventional physiological saline 0.9%). All experimental solutions were prepared by diluting and mixing these stock solutions.

2.2 | MD system

MD experiments were carried out using *tissue probes* (63 Microdialysis Catheter; M Dialysis AB, Stockholm, Sweden) with a cut-off of 20 kDa and a membrane length of 10 mm and *intravenous (iv) probes* (custom-made intravenous Microdialysis Catheter; M Dialysis AB, Stockholm, Sweden) with a cut-off of 20 kDa and a membrane length of 30 mm. Both probe types had a diameter of 0.6 mm. The shaft, the inlet and the outlet tubes consisted of polyurethane, and the membrane consisted of polyarylethersulphone. The thickness of the membrane was about 41 μm. All probes were perfused at a flow rate of 1 μl/min by a portable battery-driven precision pump (107 Microdialysis Pump; M Dialysis AB, Stockholm, Sweden). Each probe was placed in a separated glass vial containing approximately 6 ml of immersion solution (i.e., the surrounding medium). During the experiments, the vials were placed in a shaking water bath at approximately 37°C.

2.3 | Experimental design

Figure 1 summarizes the experimental design and lists the applied solutions. This study comprised four parts that differed with respect to the probe type (tissue probes in Parts 1 and 3; iv probes in Parts 2 and 4) and the basis of the perfusion solutions (PS in Parts 1 and 2; HSA in Parts 3 and 4). The HSA solution was prepared by diluting human albumin 20% with PS to a concentration of 1%. Each part tested three replicates of three different combinations of immersion and perfusion solutions (immersion solutions: 1-ng/ml diclofenac, 100-ng/ml diclofenac or PS only; perfusion solutions based on PS or HSA: 25-ng/ml ketoprofen + 1200-μg/ml urea or 100-ng/ml diclofenac + 25-ng/ml ketoprofen + 1200-μg/ml urea). Forward dialysis (FWD) experiments assessed the recovery of substances from the immersion solution

| Part 1 tissue probes (9x) Perfusion: saline	Part 2 iv probes (9x) Perfusion: saline	FWD of low diclofenac (3 probes)	Immersion solution: 1 ng/ml diclofenac
			Perfusion solution: 25 ng/ml ketoprofen + 1200 µg/ml urea
Part 3 tissue probes (9x) Perfusion: albumin	Part 4 iv probes (9x) Perfusion: albumin	FWD of high diclofenac (3 probes)	Immersion solution: 100 ng/ml diclofenac
			Perfusion solution: 25 ng/ml ketoprofen + 1200 µg/ml urea
		RD of diclofenac (3 probes)	Immersion solution: physiologic saline 0.9% only
			Perfusion solution: 25 ng/ml ketoprofen + 1200 µg/ml urea + 100 ng/ml diclofenac
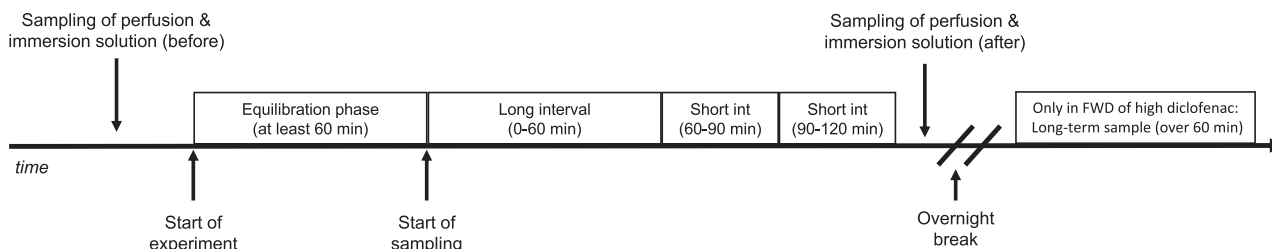
FIGURE 1 Summary of experimental set-up. Thirty-six probes were equally divided into four parts which differed concerning the probe type (tissue or iv probe) and the basis of the perfusion solutions (physiologic saline 0.9% or human albumin 1%). In each part, three different combinations of immersion and perfusion solutions were tested with three replicating probes (*right table*). Forward dialysis (FWD) experiments of diclofenac served at the same time also as reverse dialysis (RD) experiments of ketoprofen and urea (see perfusion solutions of FWD experiments). Each experiment followed the provided timeline. In addition, the probes in the high-concentrated diclofenac (100 ng/ml) immersion solution were kept in place overnight, and a long-term sample was obtained. In this case, the solutions were only sampled after the last sample

(i.e., influx of diclofenac), and retrodialysis (RD) experiments assessed the loss of substances out of the perfusion solution (i.e., outflow of diclofenac, ketoprofen and urea).

The respective perfusion and immersion solutions were sampled before and after each experiment. After an equilibration period of at least 60 min, three consecutive MD samples were collected at intervals 0 to 60, 60 to 90 and 90 to 120 min. In addition, the probes in the high-concentrated diclofenac (100 ng/ml) immersion solution were kept in place overnight, and a long-term sample was obtained the next day. The microvials were weighed before and after the sampling of the dialysate to assess the collected volume.

2.4 | Sample storage and analysis

All samples were immediately stored at $-70°C$ until analysis. Concentrations were measured by HPLC. The HLPC columns Acquity UPLC BEH C18 130A 1.7 µm 2.1 × 50 mm for diclofenac and ketoprofen and Xbridge BEH Amide 100 × 3 mm 2.5 µm for urea were used. Samples with concentrations above the upper limit of quantification (50 ng/ml for diclofenac and ketoprofen and 100 000 µg/ml for urea) were diluted and reanalysed. Values below the lower limit of quantification (LLOQ, 0.05 ng/ml for diclofenac and ketoprofen and 10 µg/ml for urea) were replaced by LLOQ/2 (i.e., 0.025 ng/ml and 5 µg/ml, respectively).

2.5 | Data analysis

Statistical and graphical analyses were performed using SPSS (SPSS® Statistics, Version 26.0; IBM, Armonk, NY, USA) and Microsoft Excel (Microsoft® Excel, Version 16, Redmond, USA). The relative recovery and loss rates (%), provided as mean ± standard deviation (SD), were calculated based on the measured concentrations in the immersion, perfusion and dialysate solutions using the following formulae:

$$\text{Relative recovery}\,(\%) = \frac{\text{Concentration}_{\text{dialysate}}}{(\text{Conc}_{\text{immersion before}} + \text{Conc}_{\text{immersion after}})/2} \times 100\%,$$

$$\text{Relative loss}\,(\%) - \left(\frac{\text{Concentration}_{\text{dialysate}}}{(\text{Conc}_{\text{perfusion before}} + \text{Conc}_{\text{perfusion after}})/2} \times 100\% \right).$$

The correction of the diclofenac concentration in the dialysate using the internal calibrant (i.e., ketoprofen) was calculated based on the following formula:

$$\text{Concentration}_{\text{diclofenac (corrected)}} = \frac{\text{Concentration}_{\text{diclofenac (dialysate)}}}{\text{Relative loss } (\%)_{\text{ketoprofen}}} \times 100\%.$$

The level of agreement and correlation between ketoprofen and diclofenac loss rates were assessed using the intraclass correlation coefficient (where values <0.5 indicate poor, 0.5 to 0.75 moderate, 0.75 to 0.9 good and >0.9 excellent reliability[12]) and Spearmen ρ (where values <0.4 indicate low, 0.4 to 0.7 moderate. 0.7 to 0.9 high and >0.9 very high correlation[13]). To assess the relative effect of the probe type and sampling intervals, ratios of means with 95% confidence intervals (95% CIs) were calculated according to Friedrich et al. and presented as forest plots.[14]

3 | RESULTS

3.1 | Recovery and loss of diclofenac and ketoprofen

Overall, the recovery rates were similar between the 1- and the 100-ng/ml concentrations of diclofenac (Table 1). Using PS, the recovery rates were low and ranged from 0.02% to 3.12%. The long-term samples, especially with the iv probe (mean 8.15%), were slightly higher. HSA as perfusion solution was associated with accumulation of diclofenac (i.e., the concentration of diclofenac was several times higher in the dialysate than in the immersion solution). Using HSA, the relative recovery rates ranged from 269% to 587% with the tissue probes and from 940% to 1674% with the iv probes.

Using PS as perfusion solution, high relative loss rates of diclofenac of almost 100% were observed (Table 2). In contrast, almost no loss was observed with HSA. Similarly, ketoprofen exhibited loss rates over 90% with PS (Table 3) with little difference between the probes. Using HSA, the relative loss rates of ketoprofen ranged from

−0.4% to 42.7% with a median of 27.8%, and loss rates with the iv probe were about twice as high as with the tissue probe (Table 3).

Because of the discrepancy between influx and outflow behaviour of diclofenac and ketoprofen in both set-ups, a meaningful correction of the diclofenac recovery using ketoprofen as an internal calibrant was not possible. Such a correction would on average underestimate the true diclofenac concentration by a factor of 55 with saline (mean ratio of calibrant-corrected dialysate concentration to directly measured immersion solution concentration: 0.018 ± 0.23) and would overestimate the true diclofenac concentration by a factor of 41 with albumin (mean ratio of calibrant-corrected dialysate concentration to directly measured immersion solution concentration: 41.0 ± 48.7). Instead, the correlation between diclofenac and ketoprofen loss rates is depicted in Figure 2. With respect to the HSA set-up, the intraclass correlation coefficient of 0.79 (95% CI [0.46, 0.92]; $p < 0.001$) and a Spearmen ρ of 0.69 ($p = 0.002$) indicate good reliability and moderate to good correlation between the loss rates of diclofenac and ketoprofen.

TABLE 2 Relative loss rates (%) of diclofenac using physiological saline and human albumin 1% as perfusion solutions

	Physiological saline 0.9% ($n = 9$)	Human albumin 1% ($n = 9$)
Tissue probe (10-mm membrane)	99.2 ± 0.5	-4.4 ± 7.2
iv probe (30-mm membrane)	95.8 ± 1.7	0.2 ± 7.5

Note: Values are mean $\pm$ standard deviation. The nine samples were obtained by using three microdialysis probes (three replicates) with three sampling periods each.

TABLE 1 Relative recovery rates (%) of diclofenac at different concentrations using physiological saline and human albumin 1% as perfusion solutions

	Physiological saline 0.9%		Human albumin 1%	
	Tissue probe (10-mm membrane)	iv probe (30-mm membrane)	Tissue probe (10-mm membrane)	iv probe (30-mm membrane)
1 ng/ml ($n = 9$)	1.6 ± 0.04	3.12 ± 0.00	313.6 ± 24.8	1064.3 ± 97.4
100 ng/ml ($n = 9$)	0.02 ± 0.01	0.21 ± 0.11	443.9 ± 90.7	1414.5 ± 216.6
100 ng/ml (long-term sample) ($n = 3$)	1.99 ± 1.04	8.15 ± 2.14	300.2 ± 11.5	443.6 ± 54.9

Note: Values are mean $\pm$ standard deviation. The nine samples (1 and 100 ng/ml) were obtained by using three microdialysis probes (three replicates) with three sampling periods each. The three long-term samples were obtained by using three microdialysis probes (three replicates) with one long-term sampling each.

TABLE 3 Relative loss rates (%) of ketoprofen (25 ng/ml) in reverse microdialysis experiments

	Physiological saline 0.9%		Human albumin 1%	
	Tissue probe (10-mm membrane)	iv probe (30-mm membrane)	Tissue probe (10-mm membrane)	iv probe (30-mm membrane)
Perfusion solution: ketoprofen + urea Immersion solution: diclofenac (n = 18)	98.6 ± 0.69	98.6 ± 0.46	19.26 ± 10.85	35.6 ± 4.37
Perfusion solution: ketoprofen + diclofenac + urea Immersion solution: physiological saline 0.9% (n = 9)	97.7 ± 0.5	98.3 ± 0.2	14.4 ± 4.7	28.2 ± 4.1
Perfusion solution: ketoprofen + urea Immersion solution: diclofenac (long-term sample) (n = 3)	98.7 ± 0.17	96.3 ± 0.57	8.33 ± 6.47	16.1 ± 3.8

Note: Values are mean ± standard deviation. The 18 samples (immersion solution: diclofenac) were obtained by using six microdialysis probes (three replicates) with three sampling periods each. The nine samples (immersion solution: physiological saline 0.9%) were obtained by using three microdialysis probes (three replicates) with three sampling periods each. The three long-term samples were obtained by using three microdialysis probes (three replicates) with one long-term sampling each. The loss rates of ketoprofen were assessed in different combinations of immersion and perfusion solutions. The *left column* indicates the compositions of the respective solutions.
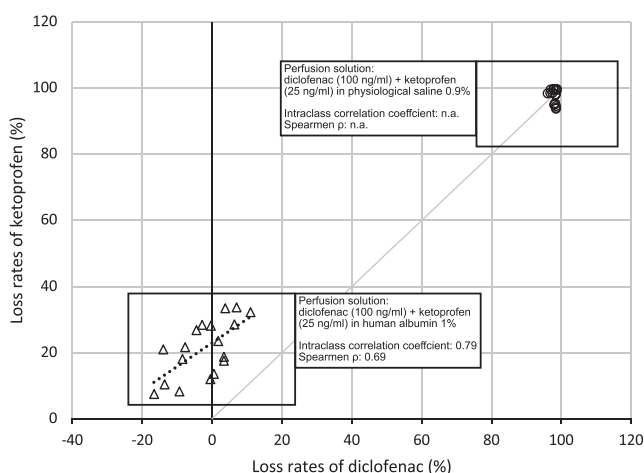

FIGURE 2 Correlation plot of relative loss (%) rates of diclofenac and ketoprofen. The light grey line symbolizes the perfect agreement ($y = x$). *Lower left quadrant*: Corresponding loss rates (%) of ketoprofen and diclofenac (*triangles*) using human albumin 1% as the basis for the perfusion solution. The dotted line indicates the linear regression line. In addition, the intraclass correlation coefficient and Spearmen ρ are provided. *Upper right quadrant*: Corresponding loss rates (%) of ketoprofen and diclofenac (*circles*) using physiological saline 1% as the basis for the perfusion solution. The regression line and the correlation are not provided because of the lacking range of the values

Because of the limited range of the loss rates (94% to 100%), no correlations could be established between diclofenac and ketoprofen in the PS set-up.

3.2 | Loss of urea

Urea, in contrast to diclofenac and ketoprofen, exhibited consistently high loss rates regardless of additional human albumin in the perfusion solution (Table 4). In the immediate samples (i.e., the two 30-min and one 60-min samples at the beginning of the experiment), the loss rates ranged from 79.8% to 99.6% with a median of 95.6% (mean 94.5%, SD 4.1%). The loss rates were lower in the long-term samples (median 82.6%, range 77.4% to 85.7%). The lower loss rates observed in the long-term samples were independent of the probe type and the perfusion solution.

3.3 | Comparison of tissue and iv probe

The two probe types showed overall similar magnitudes of diffusion rates for diclofenac (i.e., almost no recovery and almost complete loss with PS and very high recovery rates and almost no loss with HSA). However, the relative recovery rates were two to three times higher with the iv probes (Table 5). Using PS, the mean relative recovery rates were 2.6% ± 2.7% with the iv probe and 0.97 ± 0.9%. Using HSA, the mean relative recovery rates were 1124% ± 356% (iv probe) and 367% ± 90% (tissue probe). Such pronounced differences could not be observed concerning the loss rates; however, diffusion rates were still higher with

TABLE 4 Relative loss rates (%) of urea (1200 μg/ml) in reverse microdialysis experiments

	Physiological saline 0.9%		Human albumin 1%	
	Tissue probe (10-mm membrane)	iv probe (30-mm membrane)	Tissue probe (10-mm membrane)	iv probe (30-mm membrane)
Perfusion solution: urea + ketoprofen Immersion solution: diclofenac (*n* = 18)	94.2 ± 4.1	96.2 ± 2.4	93.6 ± 2.5	95.9 ± 0.8
Perfusion solution: urea + ketoprofen + diclofenac Immersion solution: physiological saline 0.9% (*n* = 9)	94.5 ± 4.2	96.7 ± 0.8	86.2 ± 5.3	96.4 ± 0.7
Perfusion solution: urea + ketoprofen + urea Immersion solution: diclofenac (long-term sample) (*n* = 3)	80.6 ± 3.2	82.2 ± 1.1	82.7 ± 2.3	83.0 ± 0.4

Note: Values are mean ± standard deviation. The 18 samples (immersion solution: diclofenac) were obtained by using six microdialysis probes (three replicates) with three sampling periods each. The nine samples (immersion solution: physiological saline 0.9%) were obtained by using three microdialysis probes (three replicates) with three sampling periods each. The three long-term samples were obtained by using three microdialysis probes (three replicates) with one long-term sampling each. The loss rates of urea were assessed in different combinations of immersion and perfusion solutions. The *left column* indicates the compositions of the respective solutions.

TABLE 5 Overall comparisons of relative recovery and loss rates between probe types (iv vs. tissue probes) and sampling intervals (30- vs. 60-min intervals)

		Probe types				
		iv probe	*n*	Tissue probe	*n*	Ratio of means [95% CI]
Relative recovery (%) in FWD	PS	2.6 ± 2.8	21	0.97 ± 0.9	21	2.7 [1.5, 4.9]
	HSA	1124 ± 356	21	367 ± 90	21	3.1 [2.6, 3.6]
Relative loss (%) in RD	PS	95.8 ± 1.7	9	99.3 ± 0.4	9	0.96 [0.95, 0.98]
	HSA	−0.1 ± 7.2	9	−4.4 ± 8.0	9	n.a.
		Sampling intervals				
		30 min	*n*	60 min	*n*	Ratio of means [95% CI]
Relative recovery (%) in FWD	PS	0.06 ± 0.07	24	0.04 ± 0.0	12	1.5 [0.9, 2.4]
	HSA	241 ± 282	24	255 ± 299	12	0.9 [0.4, 2.1]
Relative loss (%) in RD	PS	97.6 ± 2.1	12	97.5 ± 2.3	6	1.0 [0.98, 1.02]
	HSA	2.1 ± 4.8	12	−11 ± 5.1	6	n.a.

Note: Values are mean ± standard deviation. Because of partially negative values, meaningful ratios of the mean relative loss rates using HSA are unavailable. Abbreviations: FWD, forward dialysis; HSA, human albumin 1%; iv probe, intravenous probe; PS, physiologic saline 0.9%; RD retrodialysis.

the iv probe (PS: 95.8% ± 1.7% vs. 99.3% ± 0.35%; HSA: −0.1% ± 7.2% vs. −4.4% ± 7.9%).

3.4 | Comparison of short (30 min) and long (60 min) sampling intervals

The recovery rates of diclofenac were similar between the short (30 min) and long (60 min) sampling intervals (Table 5). The respective mean relative recovery rates of diclofenac were 0.02% ± 0.07% versus 0.04% ± 0.04% with PS and 241% ± 282% versus 255% ± 298% with HSA. The relative loss rates with PS were also similar between short and long sampling intervals (97.6% ± 2.1% vs. 97.5% vs. 2.3%). In contrast, the relative loss rates differed between the short and long intervals on average by 13.1% (2.1% ± 4.8% vs. −11.0% ± 5.1%).

3.5 | Quality and completeness of data

In total, all planned 120 samples (30 per Parts 1 to 4) could be obtained and analysed. One probe of Part 3 did not work in the first session; therefore, the experiment was repeated on another day. Because of technical problems, urea concentrations are unavailable for 5 of the 120 samples. Concentrations below the LLOQ were observed in 25 and 3 of 120 samples of diclofenac and urea, respectively. The 60-min samples weighed on average 53.1 ± 2.4 mg (PS: 51.1 ± 1.9 mg; HSA: 55.0 ± 0.8 mg). The 30-min samples weighed on average 26.2 ± 1.7 mg (PS: 25.0 ± 1.6 mg; HSA: 27.4 ± 0.7 mg). The respective concentrations of diclofenac in the immersion solution were on average lower than expected with 0.87 ± 0.37 (nominal 1 ng/ml) and 76 ± 35.7 ng/ml (nominal 100 ng/ml). Notably, the diclofenac concentrations in the immersion solution were consistently lower in the after sample in the high concentration (100 ng/ml), HSA set-up (tissue probe: before 79 ± 1.0 vs. after 48 ± 3.9, iv probe: before 76.3 ± 1.9 vs. after 21.7 ± 2.2). Such substantial differences were not observed in the other set-ups. The mean concentration of diclofenac in the perfusion solution was 116 ± 16.6 ranging from 94 to 161 (nominal 100 ng/ml). Ketoprofen showed a mean concentration of 28.7 ± 4.5 ng/ml (nominal 25 ng/ml) in the perfusion solution. The mean concentration of urea was 1185 ± 64.2 µg/ml (nominal 1200 µg/ml).

4 | DISCUSSION

4.1 | Discrepant recovery and loss rates of diclofenac and ketoprofen

This study aimed to improve the experimental set-up for diclofenac MD studies. To the best of our knowledge, no such methodological study has been published before. Two different probe types (i.e., 10-mm membrane tissue probe and 30-mm membrane iv probe) and two different perfusion solutions (physiologic saline 0.9% with and without human albumin 1%) were tested. The diffusion rates of diclofenac showed a clear pattern: almost no recovery but complete loss with PS and excessive recovery and no loss with HSA. A similar pattern was observed for ketoprofen. Surprisingly, we even found negative loss ratios using HSA as perfusion solution. Because this cannot reflect an actual increase of the amount of diclofenac (the immersion solution did not contain any diclofenac), there might have been a constant amount of diclofenac in the perfusion solution, combined with minimal loss of diclofenac-free fluid into the immersion solution.

Additionally, such small differences of a few percentage points are within the range of measurements errors.

Usually, quantitative MD experiments use a correction method based on the loss rate of the substance of interest in RD or a calibrant.[15] However, this method relies on the equivalence of influx and outflow. We observed a marked discrepancy between influx and outflow that disallows a meaningful recovery correction both with PS and HSA. One reason for the lacking recovery of diclofenac with PS could include unspecific binding of diclofenac to the membrane or other parts of the MD system.[16] This explanation is supported by (i) the high binding capacity of diclofenac (plasma protein binding of 99.8%[9]), (ii) the accumulation of diclofenac with HSA, which can compete with the unspecific binding sites and (iii) the increased recovery rates in the long-term sample, which might reflect some saturation of these unspecific binding sites (e.g., at the membrane or the plastic tube). Our results suggest that both PS and HSA do not allow for the exact determination of absolute diclofenac concentrations in in vivo MD experiments.

4.2 | Previous MD studies on diclofenac and outlook

Several MD clinical trials on diclofenac have already been conducted (Table 6). Most membranes had a molecular weight cut-off of 20 kDa, similar to ours. The probes were commercially available products from µDialysis® (Sweden) or CMA® (Sweden). The exact chemical compositions were not reported. The designs of the available clinical trials were heterogeneous in terms of dosage, frequency of administration, depth of MD probes, type of probe, sampling time points and other variables. Hence, the comparability of the studies from a MD perspective is scientifically questionable. Nevertheless, in these trials, high loss rates were observed and interpreted by the authors of these studies as indicative of correspondingly high recovery rates. Consequently, dialysate concentrations were only minimally corrected, and in one study, the correction was even omitted due to recovery rates above 85%.[19] If our findings were applicable to the MD methodology used in the previous studies, this correction method might have led to incorrect results when the determination of absolute concentrations in tissue was intended. In addition, the assumed subcutaneous tissue concentrations following topical diclofenac treatment varied considerably, ranging from 0.23 ± 0.66 (or not detectable) to 5000 ± 7600 ng/ml. In three studies, diclofenac was undetectable in most tissue samples. Two studies mentioned preceding in vitro experiments but only one publication provided actual results.[8] Using a

TABLE 6 Overview of clinical microdialysis studies on diclofenac

Author (year)	n	Preceding in vitro experiments	MD probe specifications	Perfusion solution	Parameter (tissue)	Concentration in ng/ml (mean ± STD)	Values % < LLOQ (LLOQ in ng/ml)	Treatment arms with diclofenac
Crevenna et al. (2015)[6]	8	Not reported	63 MD probes Cut-off: 20 kDa (μDialysis, Sweden)	Physiologic saline	Cmax (sc)	-	100% (0.5)	A: 50 mg (126 cm²) SD with iontophoresis B: 50 mg (126 cm²) SD w/o iontophoresis
Burian et al. (2013)[17]	12	Not reported	CMA 63 MD probes Cut-off: 20 kDa (CMA, Sweden)	Ringer's solution	Cmax (im)	0.23 ± 0.66	-	140 mg (topical) 2×/d for 3d
Riecke et al. (2011)[18]	14	Prior validation by GlaxoSmithKline (without further description)	Customized plasmapheresis hollow fibre probes diameter: 0.3 mm Cut-off: 100 kDa (CMA, USA)	Intralipid 20%[a]	Cmax (sc)	-	80% (0.5)	A: 35 mg (13 cm²) SD with iontophoresis B: 37 mg (13 cm²) SD w/o iontophoresis
Brunner et al. (2011)[19]	20	Not reported	Cut-off: 20 kDa (CMA, Sweden)	Not reported[b]	Cmax (sc)	A: 0.5 ± 0.4 B: 1.0 ± 1.3 C: 0.8 ± 1.0 D: 1.2 ± 1.5 E: 0.6 ± 0.5	-	A: 2.5 mg (100 cm²) 2×/day for 3d with menthol and eucalyptus oil B: 6.25 mg (100 cm²) 2×/d for 3d with menthol and eucalyptus oil C: 2.5 mg (100 cm²) 2×/d for 3d D: 6.25 mg (100 cm²) 2×/d for 3d E: 10 mg (100 cm²) 4×/d for 3d
Brunner et al. (2005)[5]	12	Not reported	CMA-10 MD probes (CMA, Sweden)	Not reported[b]	Cmax (sc)	A: 13.1 (9.3–33.6)[c] B: 1.9 (1.6–2.5)[c]	-	A: 48 mg (topical) 3×/d for 4d B: 50 mg (oral) 3×/d for 4d
Dehghanyar et al. (2004)[20]	6	Not reported	CMA-10 MD probes Cut-off: 20 kDa Outer diameter: 0.5 mm Membrane length: 16 mm (CMA, Sweden)	Ringer's solution	Cmax (sc)	A: 0.96 ± 1.05 B: 117 ± 249[d]	A: 70% B: 60% (1.02)	A: 60 mg (100 cm²) 3×/d for 4d B: 300 mg (100 cm²) SD

(Continues)

TABLE 6 (Continued)

Author (year)	n	Preceding in vitro experiments	MD probe specifications	Perfusion solution	Parameter (tissue)	Concentration in ng/ml (mean ± STD)	Values % < LLOQ (LLOQ in ng/ml)	Treatment arms with diclofenac
Burian et al. (2003)[21]	10	Not reported	CMA-70 MD probes Cut-off: 20 kDa Outer diameter: 0.6 mm Membrane length: 30 mm (CMA, Sweden)	Physiologic saline	Cmax (sc)	A. 46.1 ± 25.8 B: 11.4 ± 2.1	-	A: 65 mg (90 cm²) SD B: 100 mg (oral) SD
Müller et al. (1998)[22]	12	Not reported	Not available	Not reported[b]	Cmax (im)	220 ± 66.4	-	80 mg (200 cm²) 2×/d for 7d
Müller et al. (1997)[8]	20	Relative recovery of diclofenac in vitro: 64% (tested concentration range: 1000 to 25 000 ng/ml)	CMA-10 Cut-off: 20 kDa Outer diameter: 0.5 mm Membrane length: 16 mm (CMA, Sweden)	Ringer's solution	Cmax (sc)	5000 ± 7600[d,e]	45% (100)	300 mg (100 cm²) SD

Note: The specification of square centimetres implies topical administration on correspondingly large skin areas.

Abbreviations: Cmax, maximum concentration; d, day(s); im, intramuscular; LLOQ, lower limit of quantification; MD, microdialysis; sc, subcutaneous; SD, single dose; STD, standard deviation; w/o, without.

[a]Intralipid 20% (Fresenius Kabi, Uppsala, Sweden) containing purified egg phospholipids of the type used in parenteral nutrition.

[b]Presumably physiologic saline or Ringer's solution.

[c]Median (95% confidence interval).

[d]Standard deviation was calculated based on the standard error of the mean (STD = SEM × √n).

[e]Values estimated from line graph with error bars.

physiological solution as perfusion solution, this study reported in vitro recovery rates of 64% for diclofenac at concentrations ranging from 1000 to 25 000 ng/ml. It is unclear why such different recovery rates were observed. Compared with our experiments, the recovery assessment in this study was performed at markedly higher diclofenac concentrations (1000 to 25 000 vs. 1 to 100 ng/ml). Another obvious difference between this and our experimental set-up was the use of Ringer's solution as perfusion solution instead of PS. Only a few MD studies directly compared different physiological solutions (i.e., Ringer's solution and PS), but Hutchinson et al. observed similar recovery rates of glucose, lactate, pyruvate and glutamate using Ringer's solution and PS.[23] Therefore, it remains questionable whether Ringer's solution can so significantly improve the recovery of diclofenac compared with PS. A minor deviation from our design was the flow rate of 1.5 instead of 1 µl/min. However, our results do not suggest any concentration-dependent increase in relative recovery, and a higher flow rate would tend to result in lower recovery rates.[24]

Of the nine trials, three used Ringer's solution, two used physiological saline one used intralipid 20% solution, and three did not specify the perfusion solution used. We decided to use PS as the basis of the perfusion and immersion solutions. As suggested by Shippenberg and Thompson, the perfusate should ideally contain the exact concentration of all solutes that are present in the surrounding fluid, except for that compound to be sampled.[25] We also considered the drug label of this diclofenac product, which strongly recommended using only PS for the preparation of an infusion solution.

Available clinical trials aimed to compare two different treatments and, thus, could accept inaccurate absolute concentration values. In our study, we demonstrated an acceptably consistent accumulation of diclofenac using HSA (by factor 4 with the tissue probe and 14 with the iv probe). For a comparative study, this consistent accumulation using HSA is arguably more reliable than the low relative recovery rates observed with PS, which were well below 1%. We argue that the suitability of HSA for comparative study designs is of greater scientific relevance because absolute tissue concentrations, even if correct, are difficult to interpret. First, the concentrations required for effective cyclooxygenase-2 inhibition (as assessed by 50% reduction in the prostaglandin synthesis) ranged widely from 0.5 to 21 ng/ml in studies using different in vitro assays.[26,27] Second, the minimum tissue concentration that is ultimately required for a therapeutic effect remains unknown.[2] Thus, the real merit of diclofenac MD studies rather lies in the comparison between different administration methods (e.g., topical administration with or without iontophoresis[6]) or

pharmaceutical formulations (e.g., bioequivalence studies[7]). In view of our results, we propose that HSA is a more appropriate perfusion solution for such comparative or tissue bioequivalence studies on diclofenac. While MD is not sufficiently established to provide pivotal equivalence data for topical drugs, its supportive potential is recognized by health authorities like the EMA.[28]

4.3 | Clearance of diclofenac in long-term human albumin samples

Unexpectedly, the relative recovery rates with HSA were substantially lower in the long-term samples. Presumably, the MD using HSA exerted a clearance on the immersion solution. The effect of this clearance became apparent in the long-term samples, which were sampled approximately 20 h after the start of the experiment. Considering a flow rate of 1 µl/min and accumulation rates of roughly 400% and 1400% (tissue probe and iv probe, respectively), the MD cleared diclofenac out of the 6 ml of immersion solution at a rate of 4 and 14 µl/min over 1150 min on average. Accordingly, the respective half-lives would be approximately 1050 and 300 min. This clearance could also be evidenced by the difference between the before and after samples of the immersion solution. Consistent with this explanation, the decrease was considerably greater for the iv probe. These observations suggest that there might be no real decrease in relative recovery in the long-term sample but rather a decreased absolute recovery that was set in relation to the mean concentration of the immersion solution (i.e., the average of the before and after concentration). Even if this clearance calculation is just a rough estimation, the impact of the clearance of MD should be considered in long-running experiments with excessive recovery rates. Higher volumes of the immersion solution would attenuate this effect.

4.4 | Ketoprofen as potential calibrant for diclofenac

Internal calibrants must exhibit highly concordant diffusion rates to provide a useful correction factor.[15] While we could not directly evaluate ketoprofen as a calibrant for diclofenac (due to the low recovery rates), we were able to demonstrate a moderate to good level of agreement between their respective loss rates. Such a level of agreement could potentially qualify ketoprofen as a suitable calibrant in a different set-up, in which equivalence between influx and outflow is given. In our experiments, ketoprofen could not be used as a calibrant.

Our findings suggest that thorough in vitro experiments, including FWD and RD experiments, should be performed before relying on calibration methods in vivo, especially if the compound of interest is highly protein bound. If there is no robust calibration method that allows accurate determination of concentrations of such highly protein-bound substances, this should at least be recognized, and interpretation of the results should be limited to comparative aspects. Our experimental set-up failed to provide a reliable recovery correction method using the RD and the internal standard method. Other established calibration methods include the low-flow rate method and the no-net-flux method.[15] The low-flow method is based on the inverse relationship between the flow rate and the relative recovery. The no-net-flux method, which has been proposed as the most robust calibration method,[29] uses a range of concentrations in the perfusion solutions. A regression plot of the difference between the perfusate and the dialysate concentrations indicates a specific concentration that results in neither loss nor recovery of the substance of interest in the dialysate sample (i.e., no-net-flux or $C_{perfusate} = C_{dialysate}$). This concentration then serves as an estimate for concentration in the surrounding fluid. Given the negligible relative recovery with PS and the excessive relative recovery with HSA in our diclofenac MD experiments, neither the low-flow nor the no-net-flux method would likely have produced meaningful results.

4.5 | Usability of urea in different MD set-ups

In contrast to ketoprofen and diclofenac, urea was unaffected by HSA in the perfusion solution. Urea as a local blood flow marker would be of considerable interest for studies investigating the interplay between tissue perfusion and drug disposition of diclofenac or other NSAIDs.[30] Here, we have demonstrated that urea can in principle be used for this purpose regardless of additional HSA in the perfusion solution.

4.6 | Difference between 10-mm tissue probes and 30-mm iv probes

The relative recovery rates observed for the iv probes with 30-mm membranes were approximately three times higher than for the tissue probes with 10-mm membranes with PS and HSA. This linear relationship is consistent with Fick's law (i.e., the diffusion rate is proportional to the respective diffusion area) and indicates that a state of complete equilibrium was not reached despite the

considerable accumulation in our experiments. In comparison, the loss rates of ketoprofen were approximately twice as high with the 30-mm iv probes as with the 10-mm tissue probes. In MD experiments on diclofenac, longer membranes yield higher recovery rates, and a direct comparison between the uncorrected results of two probes with different membrane lengths is not advisable.

4.7 | Limitations

Our study has several limitations. First, as with any in vitro MD study, the findings should be extrapolated to the clinical set-up with caution. Most importantly, our experiments used immersion solutions based on PS without additional protein. The total protein content of interstitial fluid is roughly 20 g/L (or 2%),[31] but the actual unbound fraction of diclofenac in interstitial fluid is unknown. It is possible that additional protein in the immersion solution would have resulted in different diffusion rates. Also, additional factors such as tissue trauma, different mechanical forces and varying fluid contents of tissue make clinical experiments inherently more variable.[25,32] Second, a constant flow rate of 1 μl/min was used in all experiments. Additional flow rates could have enriched our results. Third, a small number of the concentrations were below the LLOQ. However, it is unlikely that more accurate determinations of these low concentrations would have significantly affected the main outcomes. Fourth, the measured diclofenac concentrations of the immersion solution were considerably lower than the nominal concentrations (on average 76 ng/ml vs. nominal 100 ng/ml). Notably, the relative recovery calculations were based on the mean measured concentration of the immersion solution before and after the experiment and not on the nominal concentration.

5 | CONCLUSIONS

In the present study, we encountered methodological challenges that might also be relevant for other MD experiments. Despite the extensive literature on MD, diclofenac—and potentially other highly protein-bound compounds—require further evaluation to establish reliable and robust MD set-ups. Here, we presented our experiences with in vitro diclofenac MD experiments, including potential pitfalls: PS was in our set-up not a suitable perfusion solution for MD studies on diclofenac. Because PS has been used previously in several clinical trials, this finding is of relevance for future MD studies. Regarding HSA, accurate quantitative determination of absolute diclofenac concentrations is not possible due to

accumulation and the discrepancy between influx and outflow. Nevertheless, we conclude that HSA can be used for comparative experiments or bioequivalence studies. For these purposes, HSA is superior to PS as perfusion solution. Considering these findings, the optimal MD set-up for diclofenac remains to be found.

CONFLICT OF INTEREST
Marianna Armogida and Filip Sucharski are employees of GSK Consumer Healthcare SARL, Nyon, Switzerland. Sivasankari Saikumar is an employee of GlaxoSmithKline Consumer Pvt Ltd, Hyderabad, India. Marianna Armogida and Sivasankari Saikumar are stock shareholder in the company. Markus Zeitlinger has received consulting fees from GSK Consumer Healthcare independent from the present work. The authors report no other conflicts of interest in this work.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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