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Application of dermal microdialysis for the evaluation of bioequivalence of a ketoprofen topical gel

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ABSTRACT

The purpose was to investigate dermal microdialysis (DMD) for the assessment of the bioavailability of a ketoprofen topical gel formulation and to evaluate this technique as a tool for the determination of bioequivalence. Four microdialysis probes were inserted into the dermis on the volar aspect of the forearms of 18 human subjects and the probes were perfused with normal saline for 60 min. A ketoprofen (2.5%, m/m) gel formulation (50 mg) was applied to the skin directly overlying the probes and samples were collected at 30 min intervals for 5 h. With the probes still in place in the dermis each site was scanned by ultrasound to determine the implantation depth of these probes. Ketoprofen concentration in dialysates was determined by LC–MS/MS. The area under the curve obtained from the concentration–time profiles from pairs of application sites in each subject was evaluated in order to assess bioequivalence. Ninety percent confidence intervals were calculated using the two one-sided test procedure and limits of 80–125% based on log-transformed data were used as acceptance criteria to declare bioequivalence. The intra-subject variability was 10% between probes whereas inter-subject variability was 68% ($n = 18$). Bioequivalence was confirmed with a power greater than 90%.

1. Introduction

Dermal microdialysis (DMD) is a relatively new application of microdialysis (MD) which allows continuous monitoring of endogenous or exogenous solutes in the interstitial fluid (ISF) of dermal tissue with minimal tissue trauma (Chaurasia et al., 2007). This technique involves the placement of small perfused membrane systems within the dermis (Fig. 1).

When a topical formulation is applied onto the skin and perfusate is pumped through the implanted membrane system, drug molecules from the topical formulation present in the dermal ISF diffuse into the lumen of the membrane, driven by the concentration gradient. This results in the presence of a net gain of drug in the perfusion medium (dialysate) which is collected at timed intervals and the drug concentration can be quantitatively determined (Chaurasia et al., 2007).

Since the first report of DMD, this technique has been used successfully in human volunteers to study the cutaneous release of histamine in response to various topical stimuli and the penetration of a number of topically applied organic solvents (e.g., ethanol and isopropanol) (Cross et al., 1998), to measure inflammatory mediators in the dermis, to study skin

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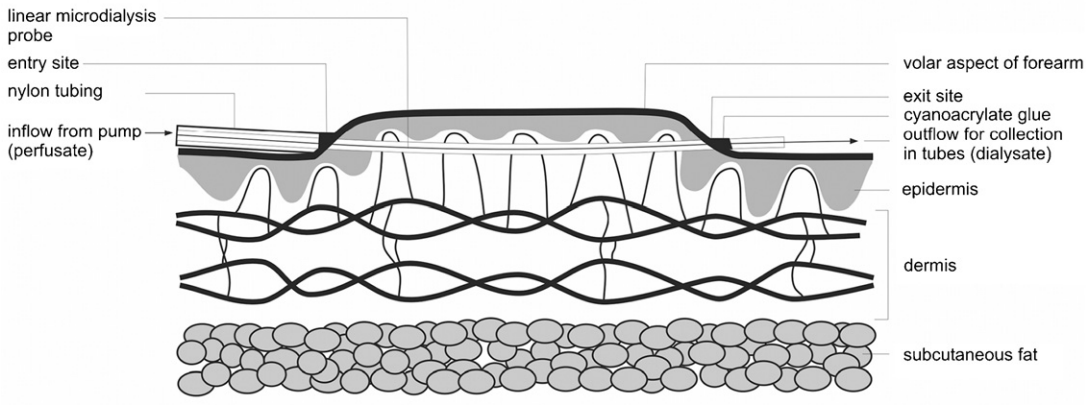

Fig. 1 – Membrane system implanted into the dermis. Modified from Benfeldt (1999).

metabolism, to determine the absorption of drugs or other agents in the skin and it has also been utilised as an alternate route of drug administration (Fulzele et al., 2007). Dermal tissue is an attractive sampling site since the tissue is relatively uniform with the extracellular fluid in constant equilibrium with the systemic circulation. Moreover, the implantation of the membrane system in the dermis involves a relatively simple procedure although training is imperative (Mathy et al., 2003a, b). DMD has been considered as a promising technique for the assessment of bioavailability and bioequivalence of topical formulations and has garnered a lot of interest amongst research scientists, dermatologists and the pharmaceutical industry (Benfeldt et al., 2007; Groth et al., 2006; Shah et al., 1998). The technique is minimally invasive and capable of producing concentration–time profiles from direct sampling in the dermis, the target tissue, and is therefore suited to study the local and/or regional delivery of drugs following topical administration.

The objective of this study was to apply DMD and measure concentrations of ketoprofen in dialysate samples collected over a series of time intervals following the application of a gel formulation to the skin of human subjects in order to evaluate this technique as a tool for the assessment of bioequivalence for topical formulations intended for local and/or regional activity.

2. Materials and methods

2.1. *Chemicals*

Ketoprofen (99.4%) was purchased from Sigma–Aldrich (Atlasville, South Africa), acetonitrile 200 far UV ROMIL-SpS^{TM} Super Purity Solvent and methanol 215 ROMIL-SpS^{TM} Super Purity Solvent from ROMIL Ltd. (Cambridge, UK), isopropyl alcohol from Burdick & Jackson, Inc. (Muskegon, Michigan, USA) and acetone UL from Ultrafine Ltd. (Finchley, UK). Ethanol (96%, v/v) and ethyl acetate were of analytical grade and obtained from MERCK (Wadeville, South Africa).

2.2. *Test formulation*

Fastum® Gel (Adcock Ingram Ltd., Bryanston, South Africa) containing ketoprofen (2.5%, m/m) was purchased from a local pharmacy in Grahamstown, South Africa.

2.3. *Subjects*

The study population comprised 18 (nine females and nine males) healthy black subjects with ages ranging from 20 to 45 year and body weights ranging from 58.9 to 92.8 kg. The study protocol was approved by the Rhodes University Ethical Standards Committee (Grahamstown, South Africa) in accordance with the recommendations of the guidelines as set out in the Declaration of Helsinki (1964) and its amendments (WMA, 2004), and carried out in compliance with the guidelines on the conduct of clinical trials in South Africa as set out by the Medicinal Control Council (MCC) South Africa (SADoH, 2000). Volunteers were screened for ketoprofen allergy, pregnancy, concurrent drug administration and were only enrolled in the study after the successful completion of a pre-study medical assessment. All subjects gave written informed consent.

2.4. *Microdialysis system*

Linear DMD probes (Fig. 2) were prepared 24 h prior to use, from dialysis fibres (Haemophan® fibre dialyser Alwall GFS Plus 12,

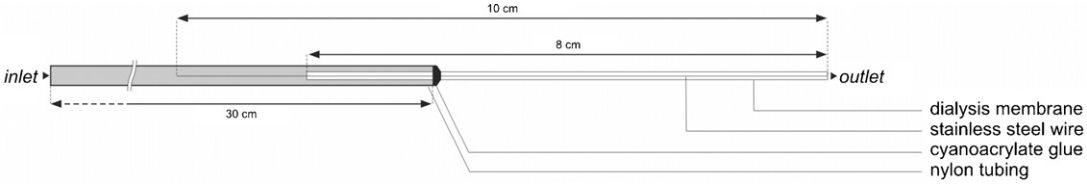

Fig. 2 – Linear DMD probe.

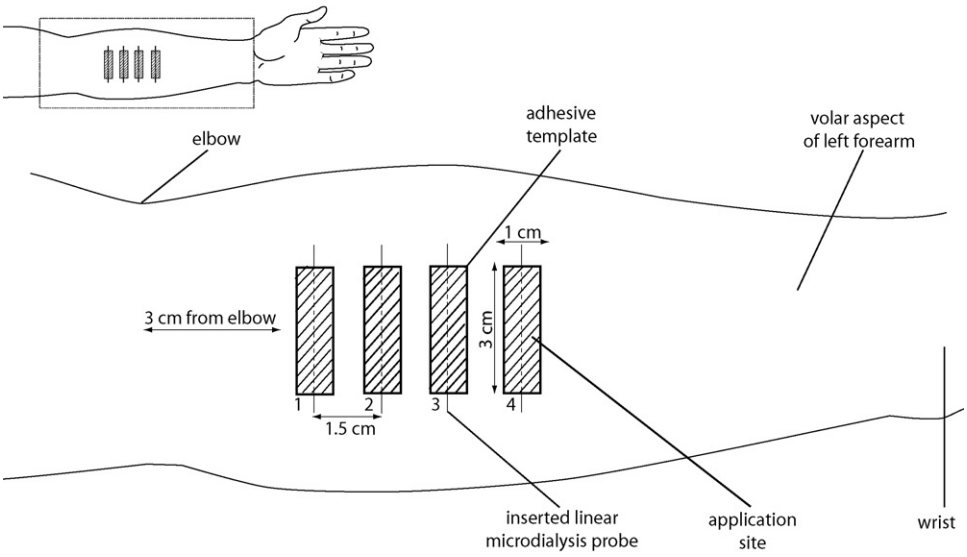

Fig. 3 – DMD study design employed. Experimental conditions: four probe insertions, four application sites, one probe per site, probes were 1.5 cm apart, probes covered approximately second quarter of the volar aspect of the forearm of each volunteer.

208 μm i.d., 216 μm o.d., 2 kDa, Gambro, Hechingen GmBH, Germany) re-enforced with an internal stainless steel (0.10 mm o.d.) wire (At Sandvik Benelux Steel, Leuven, Belgium) and the assembly glued (Loctite® Super glue gel, Scientific Laboratories Supplies Ltd., Nottingham, UK) to nylon inlet tubes (Portex® tubing (0.50 mm i.d., 0.63 mm o.d.)) also obtained from the latter suppliers.

Using tubing adapters (CMA/MD AB, Stockholm, Sweden) the inlet tubes were connected to 2.5-ml glass microsyringes (Exmire®, Aurora Borealis Control BV, Schoonebeek, The Netherlands) housed in a precision pump (CMA 400, CMA/MD AB, Stockholm, Sweden).

2.5. *In vitro recovery*

Standard solutions (1, 2 and 5 μg/ml) of ketoprofen were used to evaluate the linear DMD probes with a perspex MD cell constructed in our laboratories. The recovery of ketoprofen was evaluated for concentration dependency whereas the delivery of ketoprofen was evaluated for possible binding effect of ketoprofen to the probes. Recovery experiments were performed with the periprobe chamber containing a standard solution of ketoprofen in normal saline (sodium chloride 0.9%, m/v) (Bodene (Pty) Ltd., Port Elizabeth, South Africa) and the probes perfused with normal saline solution whereas the delivery experiments involved perfusing a standard solution of ketoprofen through the probes with the periprobe chamber unfilled (air filled). Agitation was provided by a stainless steel ball 440C (3 mm o.d., 0.0006 sphericity) obtained from Small Parts, Inc., Miramar, Florida, USA and the MD cell placed on a shaker (The Chemical Rubber Company, Cleveland, Ohio, USA) at ambient temperature (22 ± 0.5 °C). The outlet end of the probe was placed into a pre-weighed 300 μl sample insert with attached plastic springs and inserted into 9 mm, screw top, 12 mm × 32 mm amber sample vials (Waters® Corporation, Milford, Massachusetts, USA). The system was equilibrated at 1 μl/min for 30 min and six consecutive samples were collected.

The extraction efficiencies of ketoprofen by recovery (EE_r) and by delivery (EE_d) were calculated according to the following relationships:

$EE_r(\%) = C_d/C_s \times 100$ and $EE_d(\%) = ((C_p - C_d)/C_p) \times 100$ (Song and Lunte, 1999) respectively, where C_d and C_s are the dialysate concentration and concentration of the standard solution of ketoprofen in the periprobe and C_p is the initial perfusate concentration of ketoprofen.

2.6. *DMD study design*

Subjects were admitted into the clinic (Biopharmaceutics Research Institute, Rhodes University, Grahamstown, South Africa) and remained there for the duration of the study. The forearms of each subject were washed with mild liquid soap (Medisan®, Designer Group, Edenvale, South Africa) and the subjects assumed a supine position with their arms placed on an armrest.

The wrists of the subjects were loosely restrained (Leukoband® S, BSN medical (Pty) Ltd., Pinetown, South Africa), to facilitate insertion of four probes into the dermis on the volar aspect of the forearm in each subject under the specific gel application sites (Fig. 3) as indicated by the shaded areas.

Ice packs (Medac (Pty) Ltd., Cape Town, South Africa) were placed directly over the area demarcated for the probes ~5 min prior to the insertion of the cannulae in order to induce a local anaesthetic effect. The DMD probes, which had previously been sterilised in ethanol (70%, v/v), were introduced through guide cannulae (21 G × 1½ in. disposable needles, Korea Vaccine Ltd., Seoul, Korea) which were subsequently withdrawn, leaving the DMD probe implanted within the dermis. The

insertions were guided by entry and exit sites marked on the forearms, ensuring that a length of 30 mm of the membrane portion of the MD probe was intra-dermally placed in each case. Pre-cut templates (25 mm × 45 mm with a fenestration measuring 10 mm × 30 mm in the centre) prepared from adhesive labels (Redfern Labels, Cape Town, South Africa), were positioned over the probes in the skin, covering the entry and exit sites as seen in Fig. 3. Following a successful probe leak test, both entry and exit sites were sealed and perfusion of the probes was initiated at 0.5 μl/min for 15 min and subsequently increased to 1.25 μl/min for 45 min. The baseline equilibration for 60 min was conducted in order for the insertion trauma to subside (Groth et al., 2006). The inlet tubes were secured with Micropore™ 1530 dressing tape (3 M, Isando, South Africa) and blank samples were collected prior to product application. The ketoprofen formulation (50 mg) was dispensed onto the application areas from preloaded Combitips® (1 ml) in a HandyStep® dispenser (Eppendorf®, Hamburg, Germany). No spreading of formulation was necessary since the entire surface area of each application site was covered. The entire study was conducted under filtered (deep golden amber) light (Lee Filters, Andover, Hampshire, England).

2.7. Sample collection and preparation

The samples were collected every 0.5 h for 5 h into labelled polyethylene centrifuge (1.5 ml) tubes (Eppendorf®, Hamburg, Germany). A total of 44 samples per subject were collected and the samples were stored in the dark at 4 °C and analysed within 24 h. Prior to analysis, ibuprofen (30 μl) in normal saline was added as internal standard to each dialysate and the samples were extracted with ethyl acetate, the extract blown down to dryness and reconstituted with methanol in sample inserts which were placed in sample vials, capped and vortexed.

2.8. Probe depth measurement

At the end of the sampling period, the probes were disconnected from the pump and the templates removed from the application sites. Excess formulation was removed using alcohol swabs and the depth of the probes measured in triplicate by ultrasound scanning at 20 MHz (Dermascan C®, Cortex Technologies, Hadsund, Denmark). The probe depth was measured, in the A-mode scan, as the vertical distance between the epidermis entrance echo and the echo of the DMD membrane (internal stabilizing steel wire). At the end of the study, the probes were withdrawn from the skin, the sites dressed with isopropyl alcohol (70%, v/v) swabs (Tyco Healthcare (Pty) Ltd., Midrand, South Africa) and each subject was provided with a tube of Biocort® cream (Adcock Ingram Ltd., Bryanston, South Africa) containing hydrocortisone (0.01%, m/m) to apply twice daily for 2 weeks as a prophylactic measure against post-traumatic skin inflammation. A post-study medical follow-up examination of the skin sites were performed weekly for 4 weeks for each subject.

2.9. Dialysate analysis

Ketoprofen concentrations in DMD samples were quantitatively determined by an Acquity™ Ultra Performance Liquid Chromatography system (UPLC) (Waters® Corporation, Milford, Massachusetts, USA), coupled to an Acquity™ TQD tandem-quadrupole mass spectrometer (Waters® Corporation, Milford, Massachusetts, USA) using negative ion electrospray ionisation (NI ESI). Analysis was carried out with 5 μl samples on an Acquity™ UPLC BEH C_{18} column (100 × 2.1 mm i.d., 1.7 μm) stainless steel analytical column (Waters® Corporation, Milford, Massachusetts, USA) maintained at ambient temperature (22 ± 0.5 °C) using a mobile phase comprising acetonitrile/methanol/water (60/20/20, v/v/v) at a flow rate of 0.30 ml/min. The retention times of ketoprofen and ibuprofen were 1.07 and 1.49 min respectively with the multiple reaction monitoring transitions performed at 253.00 > 209.00 and 205.00 > 161.00 respectively. Calibration plots were linear over the range, 0.5–500 ng/ml and the method was accurate (99.97–104.67%) and precise with %R.S.D.s less than 2% and recovery of ketoprofen and ibuprofen from DMD samples were approximately 88% and 95% respectively.

2.10. Pharmacokinetic evaluation

The concentration–time profiles were generated by plotting ketoprofen concentrations at the mid-point between sample collections times and calculating the AUC from 0 to 5 h.

2.11. Bioequivalence assessment

Pharmacokinetic and statistical parameters to determine bioequivalence were calculated with a statistical package SAS® (SAS Institute Inc., Cary, North Carolina, USA). AUC_{0-5} was tested for comparable bioavailability using a two one-sided test procedure and bioequivalence was concluded if the 90% CI of the log-transformed AUC_{0-5} data for pairs of test/reference application areas were within the acceptance range of 80–125%.

Although the same ketoprofen product was applied to each site, in order to test the resulting data for bioequivalence, two sites on each subject were designated as test sites (T) and the other two sites as reference sites (R). The designation of the sites was performed according to the following randomisation sequence: A (TTRR/RRTT), B (TRTR/RTRT) and C (TRRT/RTTR). The means of two pairs of sites for each subject were used in the bioequivalence assessment. The means of probes 1 and 2 versus 3 and 4 were designated as sequence A. The means of probes 1 and 3 versus 2 and 4 were designated as sequence B. The means of probes 1 and 4 versus 2 and 3 were designated as sequence C.

3. Results

3.1. In vitro recovery

The recovery (EE_r) of ketoprofen from three different concentrations was independent of concentration and the data are presented in Table 1.

The delivery (EE_d) of ketoprofen from a standard solution (1 μg/ml) perfused through linear MD probes with air in the periprobe is presented in Table 2.

Table 1 – Recovery at different concentrations ($n = 3$).

Concentration (μg/ml)	Recovery (%)
1	67.50 ± 1.84
2	65.75 ± 1.63
5	69.20 ± 0.43
Mean ± S.D.	67.48 ± 1.72
R.S.D. (%)	2.55

Table 2 – Delivery in air ($n = 3$).

Sample	Mean delivery (%)
1	94.62
2	93.68
3	95.03
4	96.04
5	93.78
6	92.76
Mean ± S.D.	94.32 ± 1.16

3.2. *Clinical observations*

The insertion of the guide cannulae with the subsequent implantation of the probes was acceptable for all subjects and the use of ice was well tolerated and efficient as an anaesthetic. No local adverse reactions from the formulations were reported during the study and the weekly post-study medical examination showed no signs of residual inflammation, scarring or keloid formation at the sites of probe implantation.

3.3. *Probe depth measurements*

A white hyper-reflecting dot confirming the presence of the metal guide wire within the membrane was visualized *in situ* during the ultrasound scanning. The mean probe depths and %R.S.D.s obtained from all subjects are presented in Table 3.

Table 3 – Mean probe depth measurements ($n = 4$).

Subject	Probe depth (mm) (mean ± S.D.)	R.S.D. (%)
1	0.76 ± 0.08	11.09
2	0.65 ± 0.03	5.22
3	0.87 ± 0.05	6.23
4	0.66 ± 0.07	10.47
5	0.84 ± 0.08	9.00
6	0.64 ± 0.05	7.95
7	0.87 ± 0.03	3.15
8	0.77 ± 0.02	2.95
9	0.64 ± 0.06	9.27
10	0.72 ± 0.08	10.81
11	0.84 ± 0.13	15.57
12	0.70 ± 0.07	10.27
13	0.80 ± 0.07	9.23
14	0.67 ± 0.07	11.07
15	0.87 ± 0.05	5.38
16	0.79 ± 0.11	13.48
17	0.84 ± 0.06	7.29
18	0.75 ± 0.13	16.70

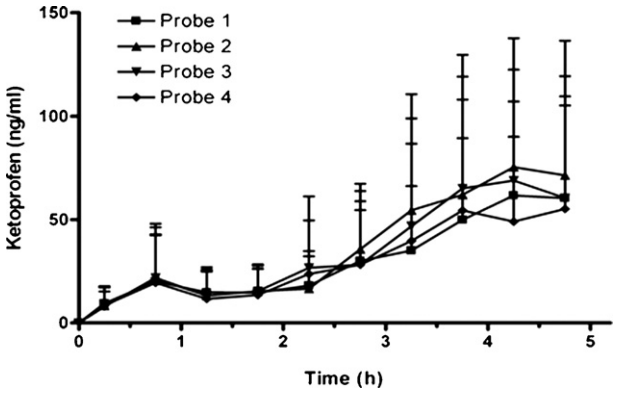

Fig. 4 – Mean dialysate concentration–time profiles (±S.D.) ($n = 18$). Experimental: four probe insertions, four application sites, one probe per site, probes were 3 cm apart, probes covered approximately two quarters of the volar aspect of the forearm of each volunteer, 18 subjects, formulation: Fastum® gel.

3.4. *Pharmacokinetics evaluation*

The mean dialysate concentration–time profiles (±S.D.) from four probes assessed for subjects 1–18 are illustrated in Fig. 4. The AUCs obtained from probes 1 to 4 were 141.95 ± 86.18 ngh/ml (ranged from 17.12 to 342.12; R.S.D. = 60.72%), 169.07 ± 115.47 ngh/ml (ranged from 20.61 to 415.14 ngh/ml; R.S.D. = 68.29%), 162.13 ± 121.93 ngh/ml (ranged from 18.52 to 460.16 ngh/ml; R.S.D. = 75.20%) and 137.83 ± 96.77 ngh/ml (ranged from 18.21 to 351.20 ngh/ml; R.S.D. = 70.21%) respectively. Inter-subject variability (%R.S.D.) for each probe was approximately 68% and the intra-subject variability (%R.S.D.) between probes was approximately 10%.

3.5. *Bioequivalence assessment*

The bioequivalence assessment of sequence A, B, and C from subjects 1 to 18 are presented in Table 4. The AUC_{0-5} CIs for all sequences were within the acceptance limits, although the power obtained for sequence C was low.

4. Discussion

The relatively high *in vitro* recovery of ketoprofen (94.32%) confirmed no significant binding of the drug to the probe as was shown by the delivery study in air (EE_d). Although ketoprofen is a hydrophobic drug (Dollery, 1991), the high recovery obtained suggests that measurable recovery would be expected *in vivo*. Recovery (EE_r) of ketoprofen was found to be independent of drug concentration in the range of ketoprofen concentrations studied.

In general, the DMD technique was well tolerated by all subjects with no residual scarring or inflammation after 4 weeks. Ice was effective as an anaesthetic which made probe implantation relatively simple and virtually pain free. Moreover, the baseline equilibration time (60 min) permitted during the study provided sufficient time for the temperature of application sites to be restored to ambient clinic conditions therefore

Table 4 – Bioavailability comparison of sequences ($n = 18$).

Sequence	PK parameter	Units	Arithmetic means (mean ± S.D.)		%Ratio (S1/S2)	90% CI (lower limit, upper limit)	Power of ANOVA (%)	ANOVA CV (%)
			Test	Reference				
A	AUC_{0-5}	ngh/ml	155.51 ± 98.89	149.98 ± 107.27	106.16	(97.4, 115.7)	92.88	14.88
B	AUC_{0-5}	ngh/ml	152.04 ± 99.23	153.45 ± 103.93	99.01	(89.9, 109.1)	95.95	16.72
C	AUC_{0-5}	ngh/ml	139.89 ± 87.28	165.60 ± 116.67	86.69	(80.4, 93.5)	53.99	13.04

not affecting either diffused drug concentration or pharmacokinetics.

In order to employ DMD routinely to assess bioequivalence of topical formulations intended for local and/or regional delivery, a validated model is necessary to show proof-of-concept. Hence, the same topical gel formulation was used as both the test and reference formulation. In this way, since bioequivalence is expected when paired sites designated as test and reference are compared, the ratios of AUC_{0-5} (T)/AUC_{0-5} (R) should provide 90% CIs within the acceptance range of 80–125%.

The dose applied was dispensed to sufficiently cover each site without having to spread the formulation over the area. Preliminary investigations showed that the use of a glass rod to spread the formulation removed in some cases as much as 60% of the intended dose resulting in considerable dose variability. Moreover, control of pressure during spreading is challenging. Preliminary investigations to provide full coverage of the application sites indicated that 50 mg of the gel was necessary for each area (10 mm × 30 mm). This strategy served to circumvent the above-mentioned problems by ensuring consistency of dose without the need to spread with associated variations in pressure during application.

The probe depth measurements showed that very consistent implantation depths were obtained within the dermis of all subjects and was unlikely to contribute towards variability. No correlation between probe depth measurements and AUCs was observed.

The data indicated dermal penetration of ketoprofen to varying degrees between subjects although a clear maximum concentration of ketoprofen could not be unequivocably established within the 5 h study duration. Hence C_{max} could not be used as a bioavailability parameter in the bioequivalence assessment. Although the concentration–time profiles showed high inter-subject variability (68%), more pronounced between 2 and 5 h, the study was associated with low intra-subject variability (10%). The high inter-subject variability is presumably associated with individual differences of the SC of the subjects and is not entirely unexpected (Benfeldt et al., 2007; McCleverty et al., 2006). Whereas C_{max} is normally included in the assessment of bioequivalence for drugs intended for systemic absorption, it remains questionable whether such a parameter is appropriate for products not intended for the systemic circulation. In this respect, a well-established precedent to waive such a requirement has long been used in the assessment of topical corticosteroid formulations where only a single criterion, the area under the effect curve (AUEC) is used to determine bioequivalence between such topical preparations (FDA, 1995).

Although bioequivalence was met for all three sequences, sequence C was associated with a relatively low power. The low power for AUC_{0-5} may be due to the location of the application sites being compared which involved comparing data from sites 1 and 4 (outside sites) versus 2 and 3 (inner sites). Possible differences in vasculature may account for the variable diffusion patterns between the inner and the outer sites. Although it is possible to improve the power of sequence C with the use of more subjects, modification of the study design may also be an important consideration. Since the least variable and thus strongest results were achieved with application sites 2 and 3, implantation of two probes in each of those sites instead of a single probe at the two outer sites (1 and 4) may be more appropriate (Fig. 3).

5. Conclusions

Although all sequences indicated bioequivalence, the power of the study was >90% for two out of the three study sequences (A and B), with the other sequence (C) being associated with a somewhat reduced power. The location of the application sites was seen to be an important consideration and a study modification to incorporate the implantation of two probes at sites 2 and 3 is proposed to minimise variability. Since paired applications of ketoprofen gel used in this study consisted of the same preparation, bioequivalence was expected and subsequently proven. AUC_{0-5} was used as the main bioequivalence parameter. The data from the study of the ketoprofen gel formulation provide the basis for and validates the use of DMD to assess bioequivalence of topical formulations applied to the skin and subsequently sampled in the target organ, the dermis. However, care must be taken to optimise the study design and application of the topical products to sites on the skin which have been appropriately identified.

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