



*Citation for published version:*

Pedon de Araujo, T, Moura Fittipaldi, I, Galindo Bedor, DC, Ludna Duarte, M, Cordery, S, Guy, R, Delgado-Charro, M, Pereira de Santana, D & Bastos Leal, L 2018, 'Topical bio(in)equivalence of metronidazole formulations in vivo', *International Journal of Pharmaceutics*, vol. 541, no. 1-2, pp. 167-172.  
<https://doi.org/10.1016/j.ijpharm.2018.02.032>

*DOI:*

[10.1016/j.ijpharm.2018.02.032](https://doi.org/10.1016/j.ijpharm.2018.02.032)

*Publication date:*

2018

*Document Version*

Peer reviewed version

[Link to publication](#)

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<https://doi.org/10.1016/j.ijpharm.2018.02.032>

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1 **TOPICAL BIO(IN)EQUIVALENCE OF METRONIDAZOLE FORMULATIONS *IN VIVO***

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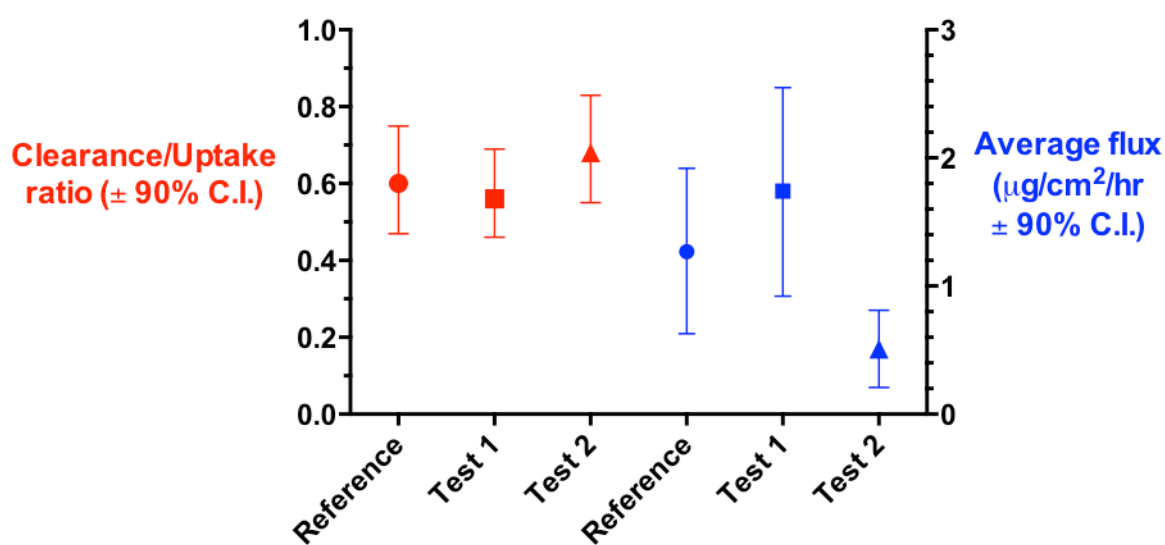
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11

12 **Graphical abstract**



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14

15 **ABSTRACT**

16 The topical bioavailabilities of metronidazole from a commercially available ‘reference’ product  
17 (Rozex<sup>®</sup>) and two extemporaneous test formulations were compared. With the reference drug product,  
18 a full skin pharmacokinetic profile, *in vivo* in human volunteers (following a 6-hour uptake and  
19 clearance over the subsequent 22 hours), was obtained using an improved stratum corneum (SC)  
20 sampling procedure. Then, a two-time point SC sampling method enabled the bio(in)equivalence of  
21 the test formulations to Rozex<sup>®</sup> to be evaluated. One test formulation was shown to be bioequivalent  
22 to Rozex<sup>®</sup>, both for uptake and clearance, whereas the other (more viscous and less spreadable)  
23 formulation was not. The delivery of metronidazole into the underlying viable epidermal tissue from  
24 Rozex<sup>®</sup> and from the equivalent test formulation was 2.5 to 3.5-fold higher than that from the  
25 inequivalent extemporaneous vehicle. The results highlight that the quantitative composition of a  
26 formulation, as well as its physical properties that influence events that take place at the vehicle-skin  
27 interface, can have a dramatic impact on the delivery of drug into the SC and subsequently to the viable  
28 skin layers below. The reproducible, sensitive and facile *in vivo* methodology employed may prove  
29 of particular value where regulatory approval of generic formulations lacks objective rigour.

30

31 **Keywords:** topical bioavailability; topical bioequivalence; skin; metronidazole; stratum corneum  
32 sampling *in vivo*; skin pharmacokinetics

33

34

## 35 **1. Introduction**

36 The development of methodology, both *in vivo* and *in vitro*, to determine the bioavailability of topically  
37 applied drugs, the site of action of which is on or within (or even just below) the skin, is the subject of  
38 considerable attention at the present time (Yacobi, *et al.*, 2014). A particular driving force for this level  
39 of interest is to establish reliable and validated approaches to assess the bioequivalence between topical  
40 drug products so that less expensive generic formulations can gain regulatory approval and lower the  
41 burden on healthcare budgets.

42 Currently, there is no standardised methodology for topical bioavailability or bioequivalence  
43 measurement, and different regulations apply in different countries. Most typically, the approaches  
44 adopted by the U.S. Food & Drug Administration (FDA) are dominant and are followed by other  
45 agencies such as the European Medicines Agency (EMA) and the U.K. Medicines and Healthcare  
46 products Regulatory Agency (MHRA). Specifically, in the majority of cases, clinical studies, which  
47 are usually expensive, prolonged and poorly discriminating, are required to establish bioequivalence  
48 (Shah *et al.*, 1998; Braddy *et al.*, 2015); a particular exception involves corticosteroid formulations,  
49 for which the vasoconstriction assay may be used (US FDA, 1995), and a few other exceptions have  
50 been granted for specific products (e.g., acyclovir ointment, a lidocaine patch and dapsone and  
51 ivermectin products) (Draft Guidance on Acyclovir, 2012; Draft Guidance on Lidocaine patch, 2016;  
52 Draft Guidance on Dapsone, 2017; Draft Guidance on Ivermectin products, 2017). Other countries  
53 have also adopted FDA standards but some, like Japan and South Africa, have also accepted the  
54 surrogate *in vivo* technique of stratum corneum (SC) sampling using tape-stripping (Braddy *et al.*,  
55 2015). In contrast, elsewhere, there exists essentially no requirement for the establishment of *in vivo*  
56 bioequivalence of topical products. For example, in Brazil, the principal requirements for the  
57 registration of a generic product are (a) pharmaceutical equivalence, and (b) that the composition of  
58 the generic formulation should contain excipients with the same function as those in the reference  
59 product (Brazil, 2011).

60 In addition to SC sampling, other techniques being examined closely as surrogates (either alone or in  
61 combination) for clinical trials are *in vitro* skin permeation experiments and *in vivo* microdialysis,  
62 including open-flow microperfusion (Bodenlenz *et al.*, 2017; Yacobi, *et al.*, 2014). The former, of  
63 course, has been widely used in product development (both topical and transdermal) for many years  
64 and now seems likely – at least in some form – to be eventually recognised as a regulatory tool.

65 While recent data from open-flow microperfusion experiments appear to indicate a real step-change in  
66 the quality of microdialysis data (Bodenlenz *et al.*, 2017), there remains much to be done before one  
67 can envisage this technically highly-demanding approach as a routine method.

68 SC tape-stripping has had a chequered past, an FDA draft guidance having been withdrawn relatively  
69 quickly after its publication because of inconsistency in the results from two very qualified laboratories  
70 (US FDA, 1998). Further, despite a clear diagnosis and understanding of why this happened, in  
71 addition to well-supported demonstrations of the usefulness of an improved protocol (N'Dri-Stempfer  
72 et al., 2009) to distinguish bio(in)equivalence between anti-fungal (econazole) and non-steroidal anti-  
73 inflammatory (diclofenac) formulations (Cordery et al., 2017), the SC sampling method has yet to  
74 regain the confidence of the FDA, or of those regulatory agencies which follow its lead.

75 Nevertheless, the SC represents an accessible and easily interrogated skin compartment *in vivo*. The  
76 recent results from the diclofenac study (Cordery *et al.*, 2017), and their correlation with *in vitro* skin  
77 penetration data, demonstrate that the technique also has value for assessing the bioavailability of drugs  
78 with sites of action not only in the SC (such as econazole (N'Dri-Stempfer *et al.*, 2009)), but in skin  
79 layers below the barrier as well. For this reason, the improved tape-stripping method (N'Dri-Stempfer  
80 *et al.*, 2009) has been used in the research reported here which aimed to evaluate the bioequivalence  
81 (or not) of two extemporaneous metronidazole formulations to the marketed Rozex<sup>®</sup> product, the only  
82 topical formulation of this drug available in many countries (including Brazil). The 'generic'  
83 formulations contained the same concentration of metronidazole and the same excipients, but differed  
84 in their spreadabilities and viscosities from Rozex<sup>®</sup>. It follows that, in terms of the FDA's definitions  
85 (Chang *et al.*, 2013), the test formulations were Q1 (having the same components) with Rozex<sup>®</sup>, but  
86 not Q2 (i.e., same amounts of the same components) or Q3 (same amounts of the same components  
87 arranged in the same way).

88

89 **2. Materials and methods**

90 2.1 Human subjects

91 28 healthy volunteers, 19 females and 9 males, participated in the study. The mean (range) age, weight,  
92 and height of the subjects were 24 (21-32) years; 62.2 (53-78) kg; and 165 (156–175) cm, respectively.  
93 The study protocol (CAAE 34657814.2.0000.5208) was approved by the local ethics committee of the  
94 Universidade Federal de Pernambuco, Recife, Brazil. The subjects provided their informed consent  
95 prior to participating in the study.

96 2.2 Materials

97 Metronidazole was from Hubei Hongyuan Pharmaceutical, Hong Kong, China; Rozex<sup>®</sup> was purchased  
98 from Laboratoires Galderma, Alby-sur-Chéran - France; sodium hydroxide and methyl and propyl  
99 parabens were acquired from Vetec, Rio de Janeiro, Brazil; propylene glycol was obtained from  
100 Henrifarma, São Paulo, Brazil; and Carbopol was purchased from Fagron, Jundia, Brazil.

101 2.3 Formulations

102 Two extemporaneous formulations of metronidazole comprising the same drug concentration and the  
103 same excipients as the commercial product (Rozex<sup>®</sup>) were prepared (Table 1). The two test  
104 formulations differed only in the quantity of gelling agent used.

105

106 **Table 1:** *Composition (% w/v) of the extemporaneous formulations defined as Test 1 and Test 2.*

107

<b>Component</b>	<b>Test 1</b>	<b>Test 2</b>
Metronidazole	0.75	0.75
Propylene glycol	5.0	5.0
Carbomer	1.0	1.5
Methyl paraben	0.18	0.18
Propyl paraben	0.02	0.02
EDTA	0.05	0.05
NaOH 20%	qs pH 4.0	qs pH 4.0
Water	qs 100	qs 100

108

109 The apparent viscosities of the three formulations were determined on 15 g samples using a concentric  
110 cylinder-type rheometer (MCR 301, Anton Paar Brasil Ltda, Sao Paulo, Brazil) with ASTM spindle 7  
111 at 30 rpm and 25°C. The spreadability test was performed according to published procedures  
112 (Borghetti *et al.*, 2006).

113

114

#### 115 2.4 Stratum corneum (SC) sampling experiments

116 The principal experimental goal of this work was to determine, using the improved SC tape-stripping  
117 approach (N'Dri-Stempfer *et al.*, 2009), that has been validated for econazole (N'Dri-Stempfer *et al.*,  
118 2009) and, more recently, diclofenac (Cordery *et al.*, 2017), whether the two extemporaneous  
119 metronidazole test formulations were equivalent to Rozex<sup>®</sup>. The new protocol calls for an assessment  
120 of equivalence to be made at one so-called 'uptake' time, and one so-called 'clearance' time, a much  
121 less labour-intensive method than that initially proposed in the now-withdrawn FDA draft guidance  
122 (US FDA, 1998).

123 To select the most appropriate uptake and clearance times, a preliminary study was first performed to  
124 measure drug levels in the SC after a series of different uptake times (1, 2, 4 and 6 hours), and a series  
125 of clearance times (2, 6, 11, 14, 18 and 22 hours) post-removal of the Rozex<sup>®</sup> formulation. These  
126 experiments were conducted (on each of the 14 volunteers) following exactly the method of N'Dri-  
127 Stempfer *et al.* (N'Dri-Stempfer *et al.*, 2009) with the five specific refinements designed to  
128 significantly improve the quality and reproducibility of the data obtained: (a) a rigorous cleaning of  
129 excess drug product from the application site at the end of the uptake period; (b) retaining the quantity  
130 of drug recovered in the first two tape-strips as material that had been taken up and would ultimately  
131 become available in the underlying skin; (c) increasing the number of tape-strips removed to ensure  
132 collection of most (> 75%) of the SC; (d) controlling the tape-stripped skin area to avoid 'edge' effects  
133 and lateral spread of formulation (N'Dri-Stempfer *et al.*, 2009); and (e) combining tape-strips into  
134 groups for drug extraction and subsequent analysis to enhance quantitation.

135 The ventral forearms of the volunteers were first washed with water and gently dried with paper towels.  
136 Thirty minutes later, the Rozex<sup>®</sup> formulation was applied to 10 sites distributed over the two arms (one  
137 each for the four uptake times and the six clearance times); another untreated site was also delineated  
138 on each arm for tape-stripping to provide an analytical control. Each treated site (2.54 cm<sup>2</sup> in area)  
139 was demarcated with a circular template (Scotch Book Tape, 3M Co., St. Paul, MN, USA), and 143.5  
140 mg of Rozex<sup>®</sup> (i.e., 56.5 mg/cm<sup>2</sup>) was applied, achieving an even and complete coverage of the skin  
141 with the formulation. The sites were then occluded with a 4.9 cm<sup>2</sup> plastic chamber (Hill Top Research,  
142 Inc., Ohio, USA) to prevent any loss of the formulation from the skin surface. At each of the designated  
143 uptake times (1, 2, 4 and 6 hours), one plastic chamber was removed and the site cleaned of residual  
144 formulation with two isopropanol wipes (Biosoma<sup>®</sup> Laboratorios, São Paulo, Brazil). A smaller  
145 template (1.77 cm<sup>2</sup>) was then centered over the treated area and the SC was then repeatedly tape-  
146 stripped (Scotch Book Tape). A maximum of 30 tape-strips were taken during which regular  
147 measurements of transepidermal water loss (TEWL) (Tewameter, Courage & Khazaka GmbH,

148 Cologne, Germany) were recorded to assess the fraction of SC that had been removed. If TEWL  
149 reached more than 6-times the value observed before tape-stripping commenced, no more SC was  
150 removed as the barrier had by then been reduced to less than 25% of its normal function (Kalia *et al.*,  
151 1996; 2000).

152 Further, at 6 hours, all of the ‘clearance’ sites were exposed and subjected to the same cleaning  
153 procedure as described above. The skin sites were then left open to the ambient conditions before being  
154 successively tape-stripped at 2, 6, 11, 14, 18 and 22 hours later.

155 Having conducted this preliminary set of experiments, the bioequivalence protocol was then performed  
156 on Rozex<sup>®</sup> and the two test formulations using 6 hours for both the uptake and clearance times. In this  
157 case, after cleaning the volunteers’ forearms, formulations were applied to 12 treatment sites (3  
158 formulations per subject, and duplicate applications of each formulation on opposite arms); for each  
159 volunteer, on one ventral forearm, the 6 uptake sites to which the formulations were to be applied were  
160 randomly assigned between the wrist and the elbow fold; the 6 clearance sites on the opposite arm  
161 mirrored those used for uptake in each volunteer. An untreated site was again tape-stripped to provide  
162 an analytical control. Application and removal of the formulations, and the tape-stripping procedures,  
163 followed exactly the protocol described above except that only one uptake time (6 hours) and one  
164 clearance time (6 hours) were considered. Quantitative data on the number of tape-strips removed in  
165 the uptake and clearance ‘arms’ of the study are in Supplementary information, Table S1. Before any  
166 tape-stripping in the bioequivalence study, the volunteers were asked to report any adverse effects that  
167 they may have experienced, and the treated skin sites were inspected visually by the investigators.

## 168 2.5 Metronidazole extraction and analysis

169 The drug was extracted from tapes 1 to 14 individually by shaking with 1 mL of acetonitrile in a closed  
170 vial for 6 hours; tapes 15-17, 18-20, 21-23, 24-26 and 27-30 were grouped for extraction, and drug  
171 was extracted therefrom in the same way. Following filtration, the extraction samples were analysed  
172 for metronidazole with a previously validated high-performance liquid chromatography method with  
173 UV detection at 320 nm (Shimadzu Corp. (Kyoto, Japan) (Melo, et al. 2016). Separation was  
174 performed on a C18 reversed-phase column 150 x 4.60mm and a C18 (5µm) pre-column 4 x 4 mm (5  
175 µm) (Shimadzu Corp.) at 35°C. The mobile phase was an 88:12 mixture of 20 mM monobasic sodium  
176 phosphate buffer at pH 3.0 and acetonitrile; the flow rate was 1 mL/min, the injection volume 20 µL.

## 177 2.6 Interpretation of results

178 A non-compartmental analysis method was used to analyse the results from the preliminary SC  
179 sampling experiments (Phoenix WinNonlin Professional version 5.0, Certara, Princeton, NJ, USA).  
180 From the profiles of the quantity of metronidazole in the SC as a function of time, the following



181 'conventional' pharmacokinetic parameters were determined: (a) The maximum quantity of drug in  
182 the SC ( $A_{\max}$ ) was directly observed from the data. (b) The rate constant describing metronidazole  
183 elimination from the SC ( $k_e$ ) was determined from the slope of the linear regression of the 'clearance'  
184 phase of the log-transformed drug quantity *versus* time profile; the corresponding elimination half-life  
185 was found from  $t_{1/2} = \ln 2/k_e$ . (c) The area under the SC quantity of drug profile as a function of time  
186 ( $AUC_{0-\infty}$ ) was calculated using the trapezoidal method for that portion up to the last measured value  
187 ( $A_t$ ) and the standard extrapolation for that part from the final measurement to  $t = \infty$ , i.e.,  $AUC_{0-\infty} =$   
188  $AUC_{0-t} + A_t/k_e$ .

189 Analysis of the results from the bioequivalence protocol followed the published approach of N'Dri-  
190 Stempfer *et al.* (2008); briefly, a test formulation (Test 1 or Test 2) was considered bioequivalent to  
191 the reference Rozex<sup>®</sup> product if the ratio ( $\pm$  the 90% confidence interval) of the amount of drug in the  
192 SC from the test product to that from the reference formulation was within the range of 0.8 to 1.25.  
193 Determinations of bioequivalence (or not) were performed using (i) the drug amount in the SC after  
194 the 6-hour uptake period, and (ii) the quantity of metronidazole in the SC following the subsequent 6  
195 hours of clearance. Although the sum of the SC levels determined in uptake and clearance has also  
196 been reported as an additional metric in previous work (N'Dri-Stempfer *et al.*, 2008), there appears to  
197 be no clear mechanistic justification for doing so and such calculations have not been performed on  
198 the data obtained in this study.

199

### 200 3. Results

#### 201 3.1 Formulation characteristics

202 The measured physical properties of the test and reference (Rozex®) formulations are in Table 2.

203 **Table 2:** *Physical characteristics of the test and reference formulations studied (mean ± S.D.; n = 6)*

Formulation	Test 1	Rozex®	Test 2
pH	4.40 ± 0.03	4.59 ± 0.05	4.15 ± 0.07
Viscosity (Pa.s)	19.9 ± 0.33	22.2 ± 0.40	28.8 ± 0.63
Spreadability (cm <sup>2</sup> )	4.03 ± 0.12	2.59 ± 0.02	0.78 ± 0.07

204 The two test formulations differed only in the quantity of gelling agent; that is, the products could be  
205 considered, using the U.S. F.D.A terminology, as Q1 equivalent (same components), but Q2  
206 inequivalent (same components but not in the same quantities). The higher quantity of Carbomer in  
207 Test 2 led to a more viscous and less spreadable formulation than Test 1; the values for the two test  
208 vehicles bracketed those of the reference product.

#### 209 3.2 *In vivo* tolerability

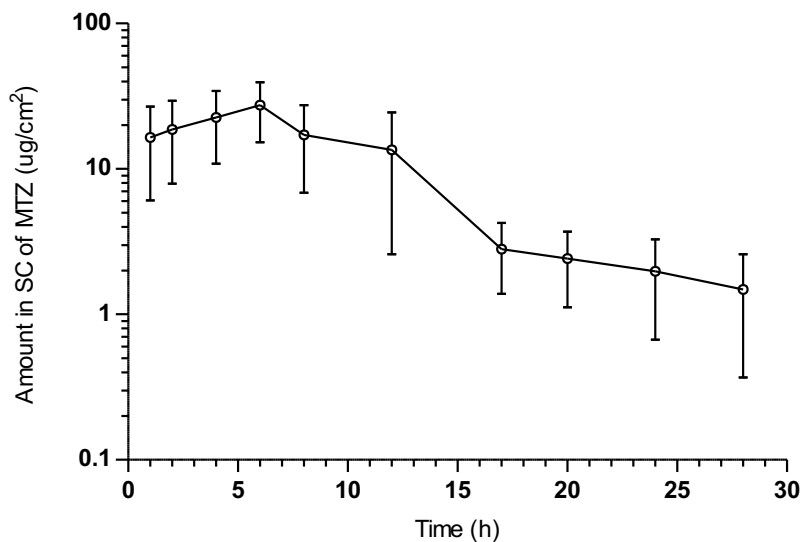
210 The distribution of skin types amongst the volunteers was: 3 of type II, 4 type III, 4 type IV and 3 type  
211 V. After 6-hour exposure to the formulations, no visible signs of irritation were observed in any  
212 volunteer. Similarly, at the 'clearance' sites, no redness at the treated skin sites had developed before  
213 SC sampling. Post-tape-stripping at both uptake and clearance sites, the skin was visibly irritated.  
214 However, the intensity of the reaction was no different at the control, untreated tape-stripped sites.  
215 Nonetheless, all volunteers fully completed the experiment.

#### 216 3.3 SC sampling *in vivo*: pharmacokinetic profile and bioequivalence assessment

217 The results of the preliminary series of experiments are summarized in Figure 1, which presents the  
218 average profile (derived from 14 subjects) of the quantity of metronidazole in the SC as a function of  
219 time over 28 hours. In this period, drug uptake was measured at 4 times over the first 6 hours and drug  
220 clearance was determined on 6 occasions over the subsequent 22 hours. As expected, the maximum  
221 amount of drug found in the SC ( $A_{max}$ ) was achieved after the longest uptake time (i.e., 6 hours); the  
222 mean ( $\pm$  S.D) value of  $A_{max}$  was 27.7 ( $\pm$ 10.1)  $\mu\text{g}/\text{cm}^2$ . Assuming a first-order clearance of  
223 metronidazole from the SC after the longest uptake time of 6 hours, linear regression of the log-  
224 transformed drug quantity *versus* time profile between 6 and 28 hours yields the average ( $\pm$  S.D, n =  
225 14) value for the elimination rate constant ( $k_e$ ) of 0.14 ( $\pm$ 0.03)  $\text{h}^{-1}$ ; the corresponding half-life is  
226 therefore 5.1 ( $\pm$  1.0) hours. The average  $r^2$  value of the 14 log-linear regressions was 0.90 with a  
227 standard deviation of 0.04. The mean ( $\pm$  S.D) measured area under the SC amount of drug *vs.* time

228 profile ( $AUC_{0-28h}$ ) was 288 ( $\pm 133$ ) ( $\mu\text{g}\cdot\text{h}/\text{cm}^2$ ) and, using the calculated  $k_e$ ,  $AUC_{0-\infty}$  was determined to  
229 be 299 ( $\pm 135$ ) ( $\mu\text{g}\cdot\text{h}/\text{cm}^2$ ).

230 **Figure 1:** Kinetic profile of the quantity of metronidazole in the stratum corneum *in vivo* during uptake ( $t \leq 6$   
231 *hr*) and clearance ( $t \geq 6$  *hr*) phases following topical application of Rozex®. Each data point represents the  
232 mean ( $\pm$  S.D.) value from 14 volunteers.



233

234 The results of the subsequent bioequivalence protocol are summarised in Table 3 and Figure 2. An  
235 analysis of variance followed by multiple comparison tests when appropriate indicates clearly that,  
236 while the average values of drug quantities in the SC (both in uptake and clearance) are not  
237 significantly different between the reference product and formulation Test 1, there is a significant  
238 difference between the reference and formulation Test 2. However, the ratios of drug amount in the  
239 SC in clearance to that in uptake did not differ significantly between the formulations.

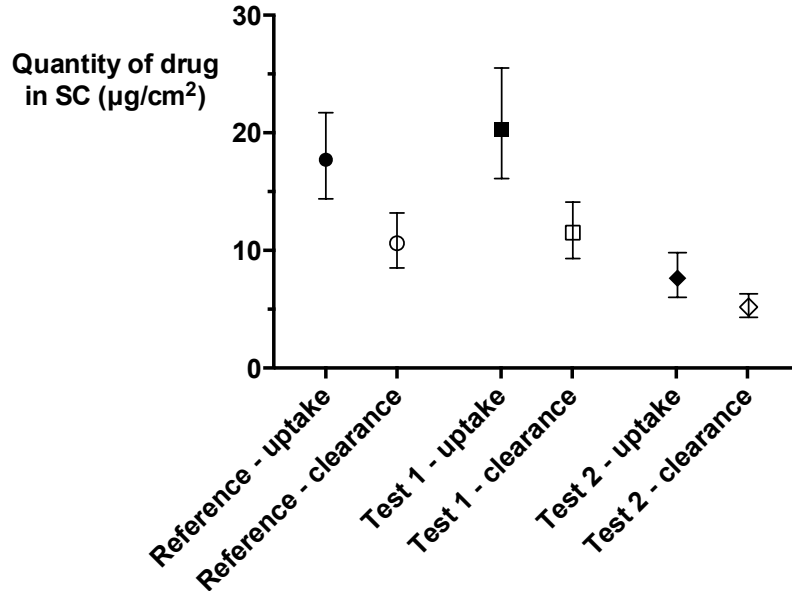
240 When the ratio of drug quantity in the SC following application of a test formulation to that after  
241 treatment with the reference product is determined during uptake and clearance, the results expressed  
242 as the mean values and the 90% confidence intervals are as shown in Figure 3. Traditionally, as used  
243 by the US FDA, for example, the average ratio and the 90% confidence limits must fall within the  
244 range 0.8 – 1.25 for a generic product to be considered equivalent to the reference (US FDA, 2007).  
245 It follows from the results in Figure 3, therefore, that formulation Test 1 was found to be bioequivalent  
246 from the data for uptake and for clearance. In contrast, formulation Test 2 was clearly inequivalent for  
247 uptake and clearance.

248

249 **Table 3:** Results of the two-point SC sampling bioequivalence protocol in vivo. The amounts of metronidazole  
 250 measured in the SC (geometric mean, and 90% confidence interval (C.I.); n = 14) during uptake and clearance,  
 251 together with the corresponding ratios of clearance-to-uptake for the reference and two test products tested.

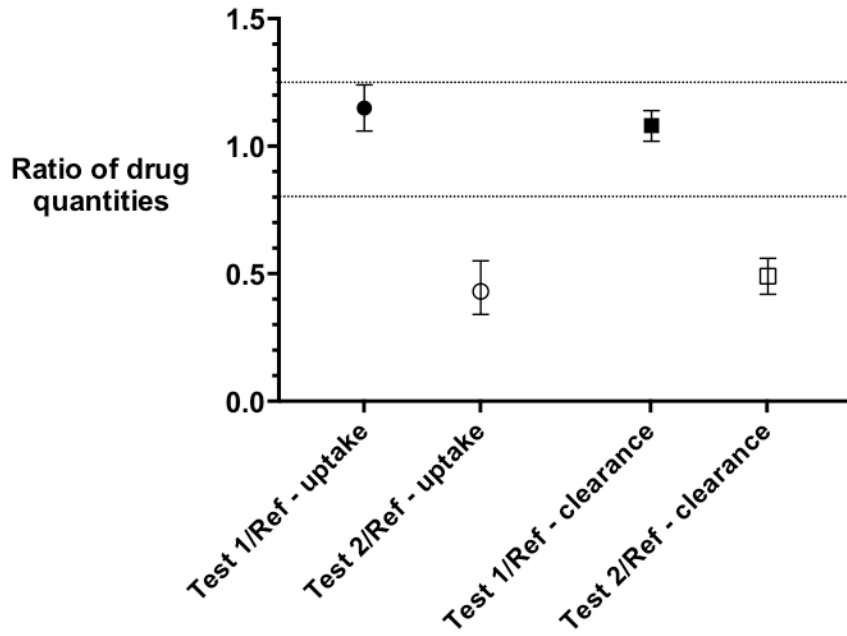
Formulation		Reference	Test 1	Test 2
Mass of Drug at uptake ( $\mu\text{g}/\text{cm}^2$ )	Average	17.7	20.4	7.8
	Lower 90% C.I.	14.5	16.2	6.1
	Upper 90% C.I.	21.8	25.7	9.9
Mass of Drug at clearance ( $\mu\text{g}/\text{cm}^2$ )	Average	10.6	11.6	5.3
	Lower 90% C.I.	8.6	9.4	4.4
	Upper 90% C.I.	13.3	14.3	6.5
Clearance/Uptake ratio	Average	0.60	0.57	0.70
	Lower 90% C.I.	0.48	0.46	0.57
	Upper 90% C.I.	0.76	0.70	0.86

258 **Figure 2:** The quantities of metronidazole measured in the SC (mean  $\pm$  the upper and lower 90% confidence  
 259 interval; n = 14) during uptake and clearance following application of the reference and test formulations.



260  
 261  
 262

263 **Figure 3:** Bioequivalence assessment of the extemporaneous gels compared with the reference listed product  
264 (Rozex®). The ratios (mean  $\pm$  the upper and lower 90% confidence interval;  $n = 14$ ) were determined using the  
265 quantities of drug in the SC during uptake and clearance. The 0.8 to 1.25 range for the ratio, for which  
266 bioequivalence is implied, are indicated on the graph.



267  
268  
269  
270

271 **4. Discussion**

272 Measurement of the physical characteristics of the two test formulations confirms that their Q2  
273 inequivalence to Rozex® translates into evidence of Q3 inequivalence as well. A one-way analysis of  
274 variance followed by Tukey's multiple comparison test reveals that the pH, viscosity and spreadability  
275 values of each of the two test formulations differ significantly ( $p < 0.05$ ) from each other and from  
276 those of Rozex®, the reference product.

277 The preliminary SC sampling protocol produced a classic pharmacokinetic profile that adequately  
278 characterised the uptake and 'clearance' of metronidazole from this skin compartment after application  
279 of Rozex®. This type of information, derived using the improved and previously validated SC  
280 sampling methodology (N'Dri-Stempfer *et al.*, 2008), was effectively the intention of the original  
281 F.D.A. draft guidance on 'dermatopharmacokinetics' (or DPK) (US FDA, 1998). Clearly, however,  
282 with respect to using the approach for the assessment of bio(in)equivalence between different  
283 formulations, the protocol is extremely labour-intensive in terms of the sample handling and analytical  
284 chemistry involved (Pershing *et al.*, 2001).

285 It was for this reason that the simplified two-time point method was developed and successfully applied  
286 to the comparison of three econazole formulations (N'Dri-Stempfer *et al.*, 2009). In the  
287 bioequivalence set of experiments reported here, this protocol (using uptake and clearance times of 6  
288 hours) again generated reproducible data and differentiated between Rozex® and the two test  
289 formulations with a modest number (i.e., 14) of subjects (Table 3 and Figure 2).

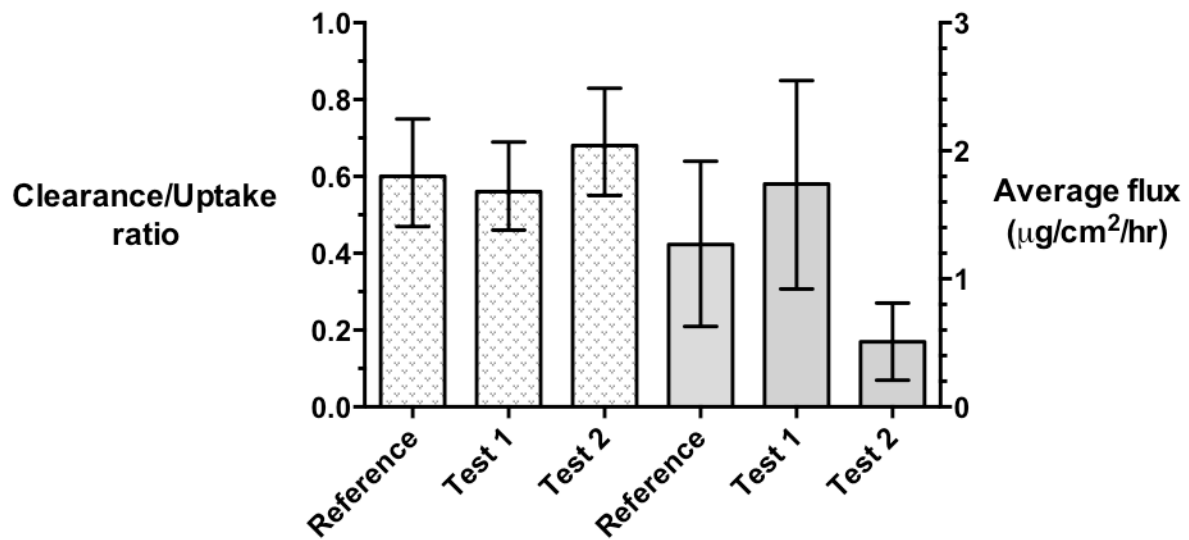
290 First of all, the clearance/uptake ratios across the three formulations were consistently around 0.6 (and  
291 not statistically different from one another) suggesting that the chosen timings for the two SC sampling  
292 events were well-chosen based on the preliminary experiment (Figure 1); that is, the uptake time  
293 ensured a significant presence of metronidazole in the SC after 6 hours, while the 6-hour clearance  
294 was sufficient for the drug level to have measurably decreased (by nearly 50%, consistent with the  
295 results from the preliminary experiment) but yet still be present in an amount well above the analytical  
296 limit of quantification. Furthermore, it has been shown that the  $\{\log(\text{clearance/uptake ratio})\}$  is  
297 proportional to the lag time for clearance (N'Dri-Stempfer *et al.* 2008, 2009). Therefore, the fact that  
298 the clearance/uptake ratios were essentially identical for the three products (as shown in Figure 4)  
299 indicates that the clearance rate constant from the SC is the same (i.e., the lag time and drug diffusion  
300 rates are similar).

301

302

303

304 **Figure 4.** Clearance-to-uptake ratios (stippled bars, left-hand axis; mean  $\pm$  the upper and lower 90%  
 305 confidence interval;  $n = 14$ ) of metronidazole delivered into the skin from reference and two test formulations;  
 306 and the corresponding estimated average fluxes ( $J_{av}$ ) of the drug (shaded bars, right-hand axis; mean  $\pm$  the  
 307 upper and lower 90% confidence interval;  $n = 14$ ) from the SC into the underlying viable skin tissue.



308

309 Second, using the uptake and clearance results, together with the corresponding 90% confidence  
 310 intervals, it was then possible to undertake a conventional bioequivalence assessment of the two test  
 311 formulations against Rozex<sup>®</sup> (Figure 3). It is evident from this analysis that the Test 2 formulation is  
 312 inequivalent to the reference product, regardless of whether the evaluation is performed using the  
 313 uptake data or the clearance results. In contrast, the Test 1 formulation was equivalent to Rozex<sup>®</sup>  
 314 based on either uptake or clearance data.

315 Third, it is apparent that the small difference in composition between the Test 1 and Test 2 formulations  
 316 (Table 1) can have a profound effect on topical bioavailability. Indeed, following the approach  
 317 described in a recent publication (Cordery *et al.*, 2017), the uptake and clearance amounts of the drug  
 318 in the SC can be used to estimate the average flux ( $J_{av}$ ) of metronidazole from the SC into the  
 319 underlying viable skin tissue (i.e., the site of action):

$$320 \quad J_{av} = (Q_{Up} - Q_{Cl})/\Delta t \quad (1)$$

321 where  $Q_{Up}$  is the mass per unit area of drug in the SC at the end of the 6-hour uptake period,  $Q_{Cl}$  is the  
 322 mass per unit area of metronidazole in the SC 6 hours after removal of the formulation, and  $\Delta t$  is the  
 323 time elapsed between the uptake and clearance measurements (i.e., in this case, 6 hours). The mean  
 324 values (and lower, upper 90% confidence intervals) of  $J_{av}$  for Rozex<sup>®</sup>, Test 1 and Test 2, calculated  
 325 from the data in Table 3, are 1.27 (0.63, 1.92), 1.74 (0.92, 2.55) and 0.51 (0.21, 0.81)  $\mu\text{g}/\text{cm}^2/\text{hr}$ ,  
 326 respectively (Figure 4). In other words, metronidazole delivery into the viable skin from formulation  
 327 Test 1 was >3-fold greater than that from Test 2 (significantly different with  $p < 0.01$ ), a clear reflection

328 of the differential quantities of metronidazole taken up into the SC rather than any difference in drug  
329 diffusion through the barrier (as seen by the consistency of the clearance-to-uptake ratios in Table 3).

330 In terms of the significance of these findings, perhaps the most important is that, in countries such as  
331 Brazil, the extemporaneous formulations studies here - being Q1 equivalent to Rozex<sup>®</sup> - would in  
332 theory be approvable generics despite, in the case of Test 2, clear inequivalence in terms of drug  
333 delivery to the skin. At the very least, therefore, studies such as the one presented here, offer a  
334 relatively straightforward *in vivo* methodology with which to compare the local bioavailability of a  
335 topical drug administered in a new formulation with that from the reference product.

336 Finally, it is worth pointing out that this research, like almost all recent efforts to address the issue of  
337 topical bioavailability/bioequivalence, has involved the single application of drug products to the skin.  
338 However, the treatment of major skin diseases involves repeated, chronic dosing and it may be argued,  
339 therefore, that it would be better to assess topical bioavailability/bioequivalence under multidose  
340 conditions (Wagner, 2013). This is particularly important for formulations which contain excipients  
341 that may exert a cumulative effect on skin barrier function. Further work designed to examine this  
342 issue in more detail is clearly warranted.

343



344 **5. Conclusions**

345 The delivery of metronidazole into the skin from a commercially available product, and from two  
346 extemporaneous formulations, was assessed by an improved stratum corneum (SC) sampling  
347 procedure *in vivo*, in healthy human volunteers. While the components of the three formulations were  
348 the same, the quantitative compositions, as well as their physical characteristics (including viscosity  
349 and spreadability) were different. It was shown that the uptake and clearance of the drug from one of  
350 the 'test' formulations were not significantly different from those of the 'reference' product. In  
351 contrast, the other 'test' formulation was clearly inferior to the 'reference'. Simple manipulation of  
352 the SC sampling data permitted the flux of metronidazole into the underlying viable skin compartment  
353 to be deduced; consistent with the bioavailability assessment, the rates of drug delivery from the test  
354 formulations were significantly different.

355

356

357 **6. Acknowledgements**

358 RHG and MBDC were Visiting Professors at the Universidade Federal de Pernambuco, Recife, Brazil,  
359 and were funded by the CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior).  
360

361 **References**

- 362 Bodenlenz, M., Tiffner, K. I, Raml, R., Augustin, T., Dragatin, C., Birngruber, T., Schimek, D.,  
363 Schwagerle, G., Pieber, T. R., Raney, S. G., Kanfer, I., Sinner, F., 2017. Open flow microperfusion as  
364 a dermal pharmacokinetic approach to evaluate topical bioequivalence. *Clin. Pharmacokinet* 56, 91–  
365 98.
- 366 Borghetti, G. S.; Knorst, M. T., 2006. Desenvolvimento e avaliação da estabilidade física de loções  
367 O/A contendo filtros solares. *Braz J Pharm Sci* 42, 531-537.
- 368 Braddy AC, Davit BM, Stier EM, Conner DP. 2015. Survey of international regulatory bioequivalence  
369 recommendations for approval of generic topical dermatological drug products. *AAPS J* 17, 121-133.
- 370 Brasil. Ministério da Saúde, Agência Nacional de Vigilância Sanitária, 2011. Resolução RDC nº 37,  
371 03 de agosto de 2011. Dispõe sobre o Guia para isenção e substituição de estudos de biodisponibilidade  
372 relativa/bioequivalência e dá outras providências. Brasília (DF): Ministério da Saúde, August 03 2011.
- 373 Chang RK, Raw A, Lionberger R, Yu L., 2013. Generic development of topical dermatologic products:  
374 Formulation development, process development, and testing of topical dermatologic products. *AAPS*  
375 *J* 15, 41-52.
- 376 Cordery SF, Pensado A, Chiu WS, Shehab MZ, Bunge AL, Delgado-Charro MB, Guy RH., 2017.  
377 Topical bioavailability of diclofenac from locally-acting, dermatological formulations. *Int J Pharm*  
378 529, 55-64.
- 379 Herkenne C, Alberti I, Naik A, Kalia YN, Mathy FX, Preat V, Guy RH., 2008. In vivo methods for  
380 the assessment of topical drug bioavailability. *Pharm Res* 25, 87-103.
- 381 Kalia, Y. N.; Pirot, F.; Guy, R. H., 1996. Homogeneous transport in a heterogeneous membrane: water  
382 diffusion across human stratum corneum in vivo. *Biophys J* 71, 2692-2700.
- 383 Kalia, Y.N., Alberti, I., Sekkat, N., Curdy C., Naik, A., and Guy, R. H, 2000. Normalization of  
384 stratum corneum barrier function and transepidermal water loss *in vivo*. *Pharm Res* 17, 1148-1150.
- 385 Melo EKS., Araujo TP., Dantas IMFS., Duarte ML., Andrade, ARB., Bedor, DCG., Santana D.P.Leal,  
386 LB.,2016. Criteria for the bioanalytical method and its application in dermatopharmacokinetic (DPK)  
387 study in pig skin (ex vivo) using metronidazole. *Afr J Pharm Pharmacol* 10, 817-822.
- 388 N'Dri-Stempfer B *et al.*, 2009. Improved bioequivalence assessment of topical dermatological drug  
389 products using dermatopharmacokinetics. *Pharm Res* 26, 316-328.
- 390 N'Dri-Stempfer B *et al.*, 2008. Optimizing metrics for the assessment of bioequivalence between  
391 topical drug products. *Pharm Res* 25, 1621-1629.
- 392 Pershing LK. Bioequivalence assessment of three 0.025% tretinoin gel products:  
393 Dermatopharmacokinetic vs. Clinical Trial Methods, Transcribed presentation to the Advisory  
394 Committee for Pharmaceutical Sciences Meeting. Rockville: Center for Drug Evaluation and  
395 Research, FDA; 2001; Nov. 29 presentation slides available at  
396 [http://www.fda.gov/ohrms/dockets/ac/01/slides/3804s2\\_02\\_Pershing/index.htm](http://www.fda.gov/ohrms/dockets/ac/01/slides/3804s2_02_Pershing/index.htm); transcript of

397 presentation slides available at  
398 [http://www.fda.gov/ohrms/dockets/ac/01/transcripts/3804t2\\_01\\_Morning\\_Session.pdf](http://www.fda.gov/ohrms/dockets/ac/01/transcripts/3804t2_01_Morning_Session.pdf) pp. 31-47

399 Shah V. P., Flynn G.L., Yacobi A., Maibach H. I., *et al.* 1998. Bioequivalence of topical dermatological  
400 dosage forms-methods of evaluation of bioequivalence, *Pharm Res* 15, 167-171.

401 US FDA, 2007. Approved drug products with therapeutic equivalence evaluations (Electronic Orange  
402 Book), U.S. Department of Health and Human Services, Food and Drug Administration, Center for  
403 Drug Evaluation and Research, Office of Pharmaceutical Science, Office of Generic Drugs.

404 US FDA, 1995. Guidance for Industry: Guidance: topical dermatologic corticosteroids: in vivo  
405 bioequivalence, Office of Generic Drugs, Center for Drug Evaluations and Research. [cited 2017 Dec.  
406 16]. Available from:  
407 <https://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UC>  
408 [M070234.pdf](https://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UC/M070234.pdf)

409 US FDA, 1998. Guidance for Industry: Topical dermatological drug product NDAs and ANDAs-in  
410 vivo bioavailability, bioequivalence, in vitro release, and associated studies. [cited 2017 Dec. 16].  
411 Available from: <https://www.fda.gov/OHRMS/DOCKETS/98fr/3659bg.pdf>

412 US FDA, 2012. Food and Drug Administration, Office of Generic Drugs. Draft guidance on acyclovir  
413 ointment. [cited 2017 Dec. 16]. Available  
414 from:<http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/>  
415 [ucm296733.pdf](http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm296733.pdf)

416 US FDA, 2016. Food and Drug Administration, Office of Generic Drugs. Draft guidance on lidocaine  
417 patch. [cited 2017 Dec. 16]. Available from:  
418 <https://www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation/guidances/ucm086>  
419 [293.pdf](https://www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation/guidances/ucm086293.pdf)

420 US FDA, 2017. Food and Drug Administration, Office of Generic Drugs. Draft guidance on Dapsone.  
421 [cited 2017 Dec. 16]. Available from:  
422 <https://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UC>  
423 [M428205.pdf](https://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UC/M428205.pdf)

424 US FDA, 2017. Food and Drug Administration, Office of Generic Drugs. Draft guidance on ivermectin  
425 cream topical. [cited 2017 Dec. 16]. Available from:  
426 <https://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UC>  
427 [M573031.pdf](https://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UC/M573031.pdf)

428 Wagner N. 2013. Practical applications to evaluate topical drug products in patients. Product Quality  
429 Research Institute (PQRI) Workshop on Evaluation of New and Generic Topical Drug Products --  
430 Current Challenges in Bioequivalence, Quality, and Novel Assessment Technologies, Rockville, MD,  
431 March 11-13, 2013.

432 Yacobi, A., Shah, V.P., Bashaw, E.D. *et al.*, 2014. Current challenges in bioequivalence, quality,  
433 and novel assessment technologies for topical products. *Pharm Res* 31, 837-846.

434 **Supplementary information**

435 Manuscript Number: IJP-D-18-00081

436 Title: Topical bio(in)equivalence of metronidazole formulations in vivo

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440

441 Table S1

442 Number of tape-strips removed in the uptake and clearance ‘arms’ of the bioequivalence protocol.

<b>No. of subjects (N) and % of subjects from whom less than 30 tape-strips were taken in uptake and clearance ‘arms’ of the bioequivalence study</b>						
	<b>UPTAKE</b>			<b>CLEARANCE</b>		
	<b>T1</b>	<b>REF</b>	<b>T2</b>	<b>T1</b>	<b>REF</b>	<b>T2</b>
<b>N</b>	4	5	4	8	7	7
<b>%</b>	29	36	29	57	50	50
<b>Individual no. of tape-strips collected from these individuals</b>						
	<b>UPTAKE</b>			<b>CLEARANCE</b>		
	<b>T1</b>	<b>REF</b>	<b>T2</b>	<b>T1</b>	<b>REF</b>	<b>T2</b>
	21	22	19	18	17	13
	23	25	20	17	16	14
	26	25	24	15	19	19
	22	23	25	19	20	22
		19		20	18	18
				18	25	15
				19	15	22
				20		
<b>Mean</b>	23.0	22.8	22.0	18.3	18.6	17.6
<b>S.D.</b>	2.2	2.5	2.9	1.7	3.3	3.7
<b>%CV</b>	9.4	10.9	13.4	9.1	17.8	21.0

443 In neither uptake nor clearance was there any obvious difference between products in the number of  
 444 tape-strips removed for those subjects requiring less than 30 strips to remove the bulk of their stratum  
 445 corneum. Therefore, none of the excipients (alone or in combination) used in the three formulations  
 446 are believed to undermine the cohesivity of the skin barrier (as has been reported in other situations –  
 447 see, for example, Cordery *et al.*, 2017).

448

449