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Quantitative assessment of non-steroidal anti-inflammatory topical products in nicotinate-induced erythema using tristimulus colour analysis

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Summary

The activities of several proprietary non-steroidal anti-inflammatory topical preparations in nicotinate-induced skin inflammation were studied in two comparative trials using a tristimulus colour analyzer: the first to observe intra-individual variation in a single subject and the second to study inter-subject variation with six volunteers. Objective parameters were derived from the prolonged multiple-point chromaticity coordinate a^* values obtained after occlusion. Statistical evaluation (ANOVA and Tukey's multiple-range test) of these parameters was performed. Using those parameters, non-steroidal anti-inflammatory formulations could be ranked according to their efficacies in suppressing the nicotinate-induced erythema.

Introduction

Topical non-steroidal anti-inflammatory formulations are designed to deliver therapeutic levels of the active ingredient to the inflamed tissues without elevating serum levels after application to the skin. This route of administration is an attractive alternative to the oral administration of non-steroidal anti-inflammatory dosage forms which have been associated with a high incidence of adverse reactions, particularly in the gastrointestinal tract (Szabo et al., 1989; Gabriel and Bombardier, 1990; Hawkey, 1990), and occasionally in tissues of the skin (Stern and Wintroub, 1989), renal system, bone cartilage, and others (Velo and Milanino, 1990). Ibuprofen creams and gels have recently been made available over-the-counter in the U.K. These and other non-steroidal anti-inflammatory topical products are more costly than the traditional over-the-counter rubefacients/ embrocations which act merely as counter-irritants (Editorial, *Lancet*, 1989).

Non-steroidal anti-inflammatory drugs (NSAIDs) possess heterogeneous chemical structures but many share similar features such as a hydrophobic moiety and an ionizable proton usually

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in the form of a carboxylic acid function. They also share at least one common mode of action, namely inhibition of cyclo-oxygenase which catalyzes the conversion of arachidonic acid to prostaglandins (PGs), thromboxanes and prostacyclin (Vane, 1971). Some of the latest NSAIDs also inhibit the lipo-oxygenase-catalyzed arachidonic acid metabolism, with and without concurrent cyclo-oxygenase inhibition (5-lipo-oxygenase inhibitors: McMillan et al., 1990; 12-lipo-oxygenase inhibitors: Higgs et al., 1979; Randall et al., 1980). At the molecular level, the synthesis of PGs and thromboxane from arachidonic acid is catalyzed by PG endoperoxide synthase, existing as a homodimer, which exhibits two catalytic activities, firstly a cyclo-oxygenase activity that inserts two molecules of oxygen into arachidonate, yielding the intermediate endoperoxide, PGG_2, and secondly a hydroperoxidase activity that reduces PGG_2 to PGH_2. These activities are present at separate, but interacting sites on the hemoprotein, PG endoperoxide synthase (Marshall and Kulmacz, 1988).

The screening of NSAIDs for evaluation of their anti-inflammatory actions has been conducted via in vitro systems (Cavey, 1989; Bouclier et al., 1990), using a wide range of experimentally induced inflammation in animal models (Bouclier et al., 1989; Trancik and Lowe, 1989) and in patients (Brooks et al., 1986). Attempts have also been made to rank the efficacy of these therapeutic agents for specific diseases like rheumatoid arthritis (Luggen et al., 1989; Cush et al., 1990).

Application of nicotinates caused rapid appearance of an erythema lasting 1–3 h, at the site of application, accompanied by an increase in skin temperature. The erythema is not associated with a wheal and is not directly associated with changes in permeability, pruritus or hyperalgesic effects which may involve an interaction with secondary mediators (Birnbaum et al., 1982; Harper et al., 1988). Biopsies of the dermal tissues 2 h after an application of tetrahydrofurfuryl nicotinate (Trafuril cream®) reveal polymorphonuclear leucocytes, characteristic of inflammation, in and around the cutaneous vasculature. These cells are absent after aspirin treatment (Winklemann and Wilhelmj, 1963). Prior administration of aspirin resulted in suppression of both Trafuril® inflammation and of the levels of prostaglandin E_2 and prostaglandin $F_{2\alpha}$ present in skin exudates as cyclo-oxygenase activity was inhibited (Plummer et al., 1977).

The aim of this study was to compare the activities of several non-steroidal anti-inflammatory topical formulations using a newly available method based on tristimulus colorimetry (Sugiyama and Tasaka, 1988). The Minolta Chroma Meter CR-200 was used to record the development of skin colour on sites pretreated with non-steroidal anti-inflammatory creams upon provocation with methyl nicotinate. Discriminative parameters were derived from the resulting chromaticity measurements and subjected to statistical analysis. The first part of the investigation was undertaken to study intra-individual variation in a single volunteer and the second to observe the variation of response in another six subjects.

Materials and Methods

Materials

The non-steroidal anti-inflammatory preparations used are listed in Table 1. Methyl nicotinate and its vehicle, propylene glycol, were purchased from Sigma Chemical Co. (St. Louis, U.S.A.).

Volunteers

Seven volunteers were recruited. They gave their written consent after approval was granted by the Research Ethics Committee, The Queen's University of Belfast, to conduct this trial. The participants did not suffer from any ailment and were not on any medication at the time of the study. They were rested for 15 min before the experiments.

Application of the creams

Each template was constructed using a piece of PVC film (Schwan Stabilo overhead projection transparency film, 0.08 mm thick, A4 size, Cat. No. 7248/100), a piece of Melinex polyester film (ICI Type S12 μm) and two layers of double-sided adhesive tape of 5 cm width. The PVC film was first sandwiched between the tiers of adhesive

TABLE 1

Non-steroidal anti-inflammatory formulations used

Code [a]		Topical product	U.K. manufacturer
1	2		
A	–	Traxam gel (felbinac 3% w/w)	Lederle Labs
B	C	Proflex cream (ibuprofen 5% w/w)	Zyma (UK) Ltd
C	–	Parfenac cream (bufexamac 5% w/w)	Lederle Labs
D	–	Difflam cream (benzydamine hydrochloride 3% w/w)	3M Riker
E	D	Feldene gel (piroxicam 0.5% w/w)	Pfizer Ltd
F	A	methyl salicylate ointment 50% w/w BP	Evans Medical Ltd
G	B	Voltarol gel (equivalent to diclofenac sodium 1%)	Geigy Pharmaceuticals
H	–	Aqueous cream BP	CP Pharmaceuticals Ltd

[a] Codes were assigned to the products used in the two trials, 1 and 2, respectively.

tape and square holes, each measuring 1.5 × 1.5 cm, marked with ink and cut out using a scalpel. The occlusive Melinex® film was then placed over one of the adhesive layers. A short length of cream extruded first from each tube was discarded. The creams were filled into standard 1 ml disposable syringes immediately before use, to minimise interaction between the plastic surfaces of the syringe and the formulation. The quantity of each cream dispensed onto the respective square of the template was determined by weight difference of the loaded syringe before and after extrusion (Fig. 1). The location of the application sites was demarcated on the ventral surface of each forearm with ink to allow for the exact positioning of the template. Baseline chromaticity readings were taken at each designated site before application of the template. The allocation of formulations to application sites was randomised with each cream applied to four areas on both arms in the single-subject study and two areas on both arms of each individual in the multiple-subject study. After the period of occlusion, the template was gently removed and the skin surfaces were lightly wiped, washed with soap and lukewarm water and patted dry with soft paper tissues to remove any traces of the creams. The skin may often appear puckered and red around the application sites, but this normally resolved within 15–20 min. The temperature of the forearm skin was 31–33°C, and room conditions were set at 22–24°C and 35–40% relative humidity.

The instrument

The Minolta® Chroma Meter was used to take measurements of chromaticity of the treated skin areas in the $L^*a^*b^*$ colour space. The reflective colour of any surface can be quantified in a three-dimensional coordinate system, with the L^* axis specifying the relative brightness of colour ranging from black to white, the a^* axis, the colour hue related to redness and greenness, and the third chromaticity axis b^*, the colour range from blue to yellow. The Chroma Meter is simple to use, with a weight of less than 2 kg. The measuring head of the Chroma Meter had been adapted by attaching a pre-moulded foam casing with a square aperture of 2 × 2 cm to it, so that the treated sites to be monitored were at a fixed distance of 1 cm from the end of the probe and no direct pressure was applied on the areas. The instrument was calibrated using the white calibration plate (CR-A43) at the start of each measuring session. Three chromaticity measurements were made at each site at each measurement

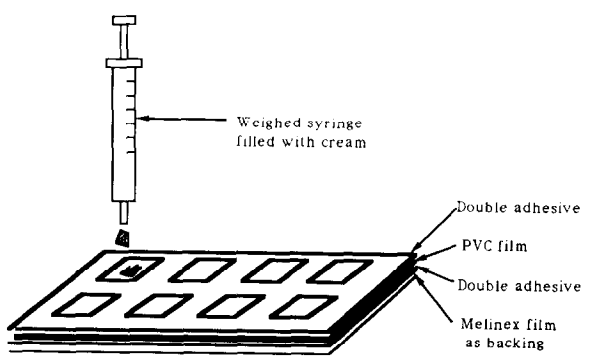

Fig. 1. Preparation of template for application of test formulation.

time, shifting and positioning the measuring head on the target sites on the ventral surface of the forearm. The average time required for the Chroma Meter to re-set itself between measurements was 3–4 s. Overall, less than 7 min were required for measurements at each of the 32 sites (4 s × three consecutive readings × 32 areas) in the single-subject study and eight sites (4 s × three readings × eight areas) in the multiple-subject experiment. Measurements were made in a sequential manner, returning to the same site every 15 min. Applications were randomised.

Data treatment

Each chromaticity coordinate is expressed as a two-decimal number. The a^* (or the red-green) axis was selected to monitor the response during which the treated areas would develop redness due to vasodilation. The data obtained were output to a personal computer, and the a^* coordinates plotted vs time. The area under the response-time curve [AUC] was computed using the trapezoidal rule while the slope of the cumulative chromaticity coordinate value-time plot between 30 and 90 min [Slope 30–90 min] was calculated by simple linear regression analysis (see the Appendix for a discussion on summary measures for serial data and for an ad hoc computer program).

The resulting parameter values were subjected to statistical analysis of variance using multiple regression and the Tukey's multiple comparison test using Minitab® (Release 7.2) and SAS/STAT® Release 6.0 programs, respectively.

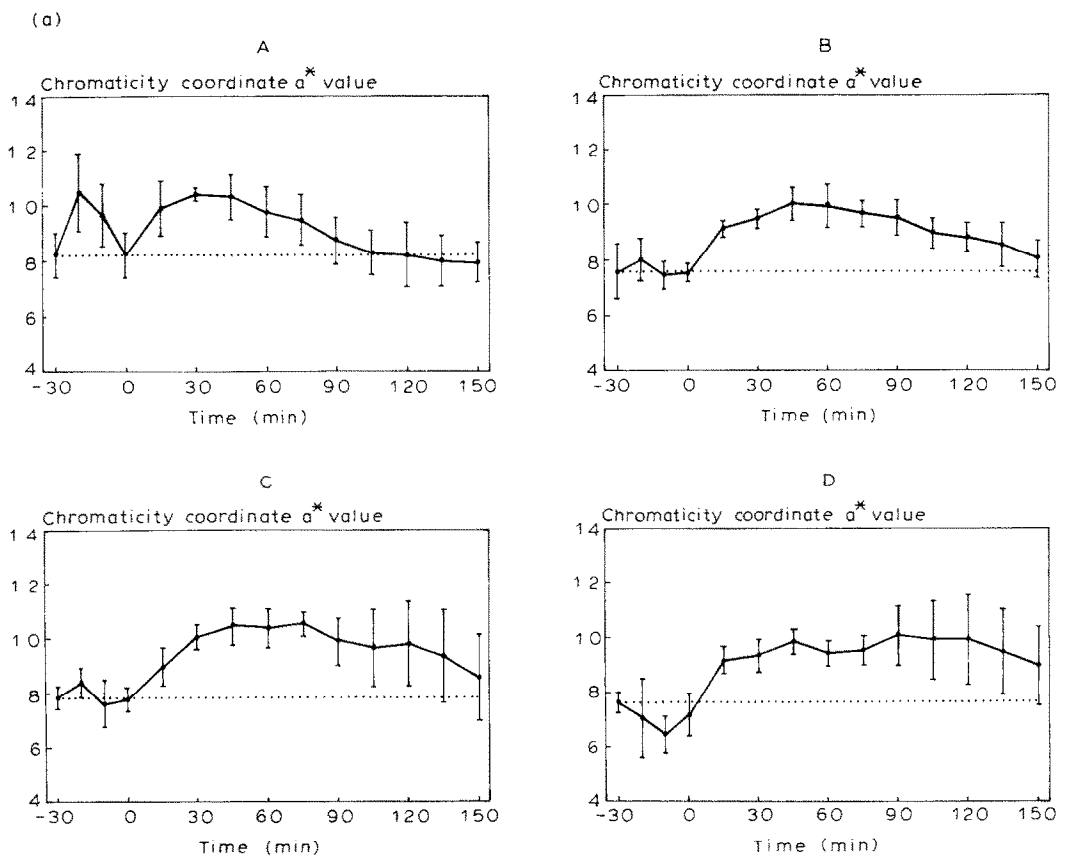

Fig. 2. (a, b) Time-response curves of chromaticity a^* coordinate in the single-subject trial. Error bars represent the standard deviations of measurements on four application sites.

Results and Discussion

The effect of topical application of non-steroidal anti-inflammatory drugs on nicotinate-induced erythema was assessed. The efficacy of these products was gauged from their inhibitory activities on the induced erythema. Therefore, the a^* chromaticity coordinate above the baseline value, the AUC and Slope parameters are inversely related to their effectiveness. The time courses of nicotinate-induced erythema on skin areas pretreated with non-steroidal anti-inflammatory topical products are illustrated in Fig. 2a and b for the single-subject study. Several anti-inflammatory preparations caused obvious erythema immediately after removal of the occlusive layer, the most prominent being associated with Traxam® gel, Feldene® gel and methyl salicylate ointment 50% w/w BP. The redness normally resolved after 30 min. Skin blanching was observed with nicotinate application on methyl salicylate ointment pretreated sites in the single-subject study. In the multi-subject trial, an interval of 30 min between the removal of the anti-inflammatory products and the application of the nicotinate solution allowed for any redness caused by the non-steroidal anti-inflammatories to subside (Fig. 3). The summary measures derived from the a^* chromaticity coordinate are graphically presented in Figs 4 and 5. They are indicative of the variable effects of the non-steroidal anti-inflammatory drugs on nicotinate-induced inflammation.

Analysis of variance for the intra-subject study and the multi-subject trial (Table 2) reveals that the topical anti-inflammatory products used were

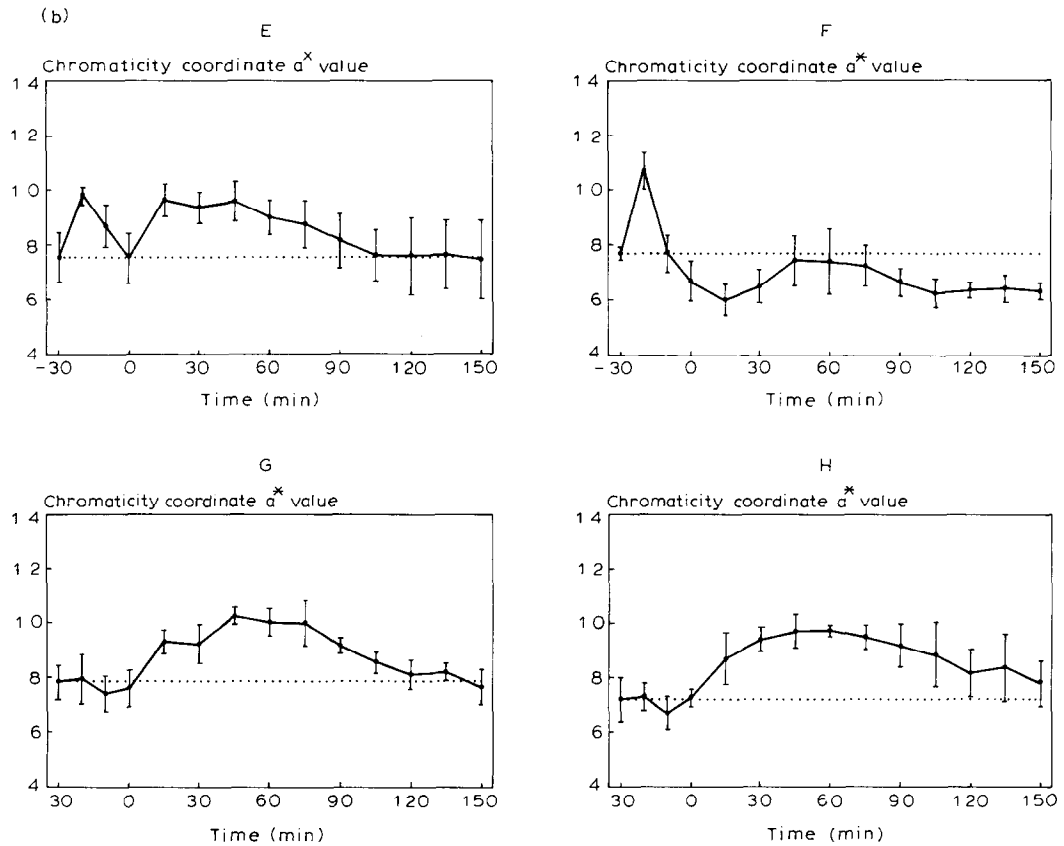

Fig. 2 (continued).

not all equal in their inhibitory activities of drug-induced inflammation ($\alpha < 0.05$). Using Tukey's multiple comparison test to rank the group means (Table 3), it was shown that methyl salicylate ointment 50% w/w was the most effective product evaluated in both trials. The ranking of the other non-steroidal anti-inflammatory topical products was not consistent with the single-subject trial. In the multi-subject trial, three products were compared with the methyl salicylate ointment and the rank orders were methyl salicylate > Proflex = Voltarol > Feldene using the AUC measure and methyl salicylate > Feldene = Proflex = Voltarol using the Slope [120–180 min] at the 0.05 confidence level. Hence, it would appear that the effects of the latter three anti-inflammatory preparations on nicotinate-induced erythema were very similar.

Nicotinate-induced erythema is an attractive in vivo model for the vascular-type acute inflammation, since it can be readily and reproducibly provoked. NSAIDs given before the application of nicotinates attenuated the usual erythematous reaction which is induced. For example, topical Trafuril® erythema was attenuated by oral aspirin (Truelove and Duthie, 1959; Winklemann and Wilhelmj, 1963; Winklemann et al., 1965; Plummer et al., 1977); topical methyl nicotinate erythema by topical bufexamac, diclofenac, ibuprofen, indomethacin, niflumic acid and phenylbutazone (Duteil et al., 1990) and by oral indomethacin, ibuprofen and aspirin (Wilkin et al., 1985); oral nicotinic acid erythema by oral indomethacin and/or aspirin (Åberg, 1973; Andersson et al., 1977; Svedmyr et al., 1977; Illingworth et al., 1981; Kane et al., 1981; Wilkin et al.,

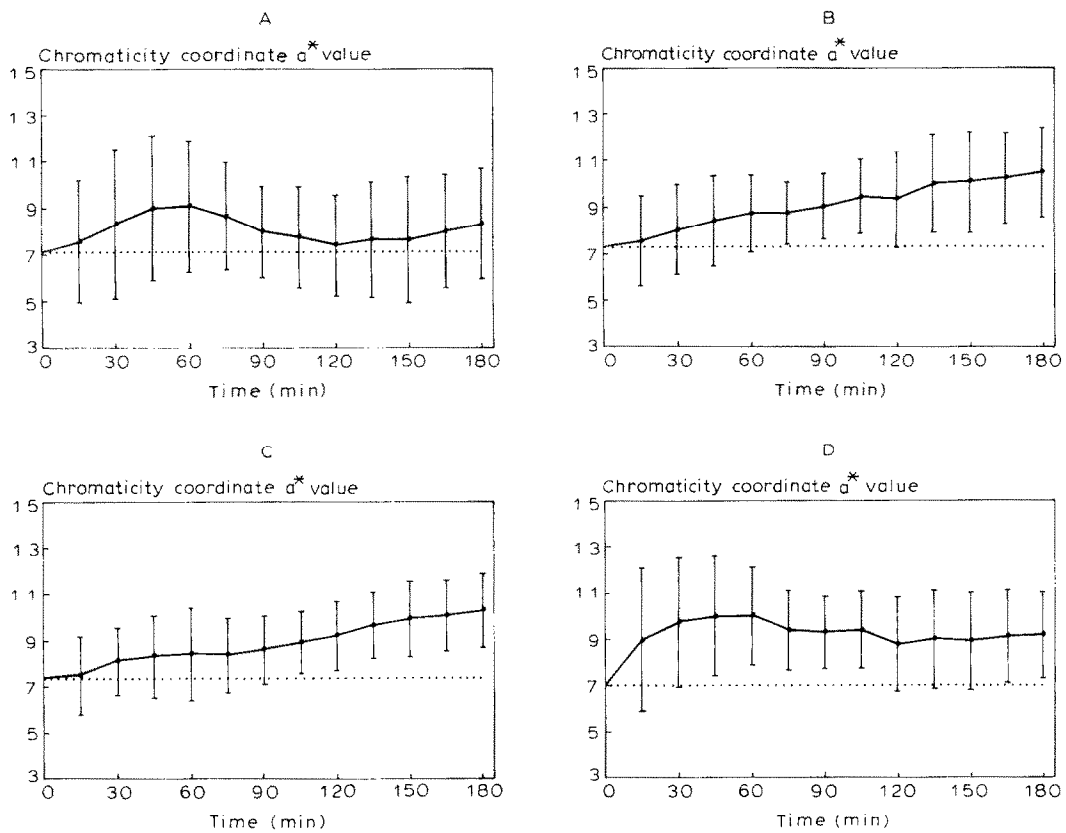

Fig. 3. Time-response curves of chromaticity a^* coordinate in the multi-subject trial. Error bars represent the standard deviation of measurements on 12 application sites among six individuals.

1982); oral pentaerythritol tetranicotinate erythema by oral aspirin (Hamazaki et al., 1985; Jay et al., 1990) and intravenous nicotinic acid erythema by indomethacin and benorylate (Phillips and Lightman, 1981).

The levels of various mediators of inflammation were measured and it was concluded that prior administration of an oral dose of aspirin in human volunteers diminished the Trafuril-induced erythema by reduction of prostaglandin activity without affecting the concentrations of histamine, kinins and protein exudates (Plummer et al., 1977). Andersson et al. (1977) showed that aspirin and indomethacin blocked the increase in ear temperature of guinea-pigs and the rise in cyclic AMP levels associated with the administration of nicotinic acid. Phillips and Lightman (1981) devised an arbitrary scale based on changes in facial temperature to measure the anti-prostaglandin action of non-steroidal anti-inflammatory drugs. Wilkin et al. (1982) proposed a more accurate method called 'the change in malar thermal circulation index' as it took into account the non-linear relationship between skin temperature and blood flow. These were straightforward in vivo assessments of non-steroidal anti-inflammatory potency in humans. Laser-Doppler velocimetry was employed to compare the activity of oral and topical anti-inflammatory drugs in the same model by Wilkin et al. (1985) and Duteil et al. (1990), respectively. Summary measures used by Duteil et al. (1990) were the maximum response (F_{max}) and the areas under the curve calculated at 15 and 30 min (AUC_{15}, AUC_{30}). Subsequent global comparison of these parameters differentiated the effect of these topical

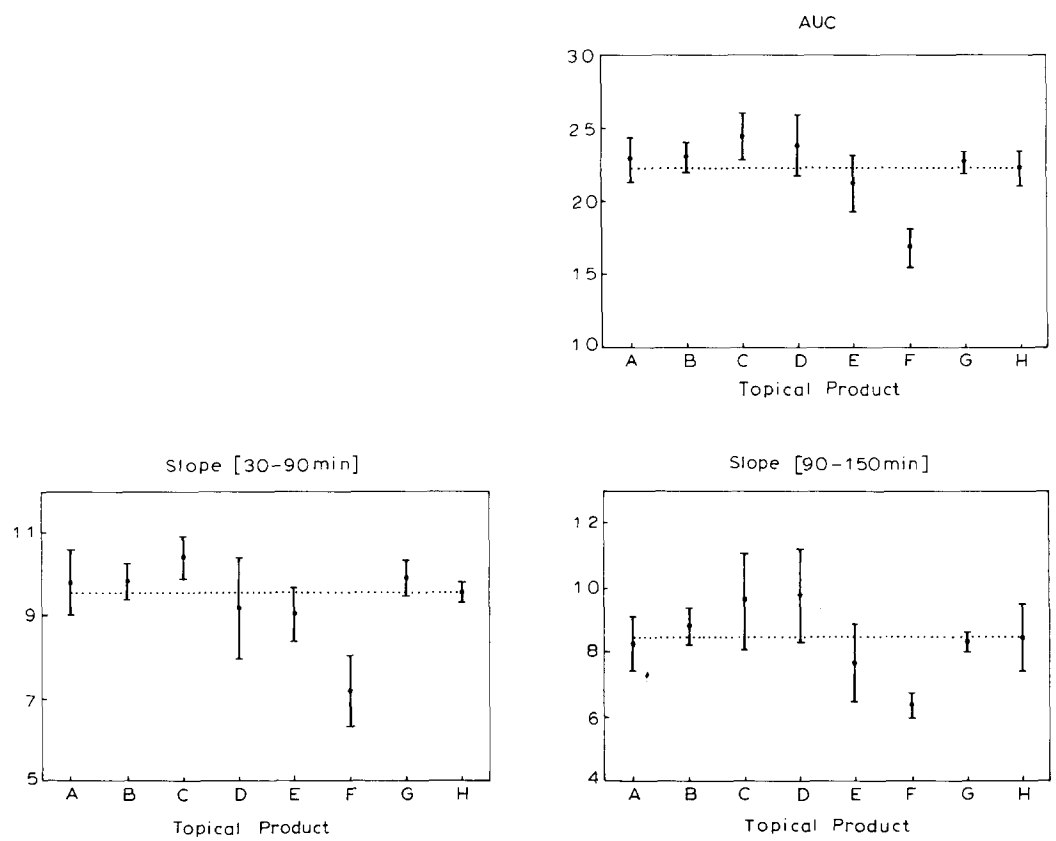

Fig. 4. Effect of topical non-steroidal anti-inflammatory preparations on nicotinate-induced erythema in the single-subject trial. Error bar represents the standard deviation of measurements on four application sites.

TABLE 2

Analysis of variance of the effect of non-steroidal anti-inflammatory preparations on methyl nicotinate-induced erythema

Source	DF	SS	MS	*F*	*P*
Single subject					
(1) AUC [0–150 min]					
Treatment	7	156.03	22.29	20.65	0.0001
Site	7	37.20	5.31	4.92	0.0055
Replicate	3	0.59	0.20	0.18	NS
Error	14	15.11	1.08		
Total	31	208.93			
$R^2 = 0.928$; root MSE = 0.039; mean 22.10					
(2) Slope [30–90 min]					
Treatment	7	26.61	3.80	10.61	0.0001
Site	7	6.44	0.92	2.56	NS
Replicate	3	0.46	0.15	0.43	NS
Error	14	5.02	0.36		
Total	31	38.53			
$R^2 = 0.870$; root MSE = 0.599; mean = 9.35					
(3) Slope [90–150 min]					
Treatment	7	32.33	4.62	7.24	0.0009
Site	7	13.93	1.99	3.12	0.0333
Replicate	3	1.19	0.40	0.62	NS
Error	14	8.93	0.64		
Total	31	56.39			
$R^2 = 0.842$; root MSE = 0.799; mean = 8.38					
Multi-subject					
(4) AUC [0–180 min]					
Treatment	3	87.28	29.09	3.24	0.0336
Site	3	15.12	5.04	0.56	NS
Subject	5	1046.33	209.27	23.31	0.0001
Arm	1	6.16	6.16	0.69	NS
Error	35	314.18	8.98		
Total	47	1469.07			
$R^2 = 0.786$; root MSE = 2.996; mean = 26.05					
(5) Slope [120–180 min]					
Treatment	3	39.09	13.03	10.42	0.0001
Site	3	1.82	0.61	0.48	NS
Subject	5	133.85	26.77	21.41	0.0001
Arm	1	0.50	0.50	0.40	NS
Error	35	43.77	1.25		
Total	47	219.03			
$R^2 = 0.800$; root MSE = 1.118; mean = 9.18					
(ANOVA of slope [30–90 min] with treatment was not significant)					

NS, not significant ($p > 0.05$); MSE, mean square error; DF, degrees of freedom.

non-steroidal anti-inflammatory drugs and topical corticosteroids on the nicotinate-provoked inflammation.

A rational approach to the evaluation of anti-inflammatory agents would be to establish the pharmacological profile of each drug using a battery of assays which mimic activity against different aspects of inflammatory disease, for example, erythema, oedema, infiltration and proliferation of inflammatory cells. Ideally, a drug showing

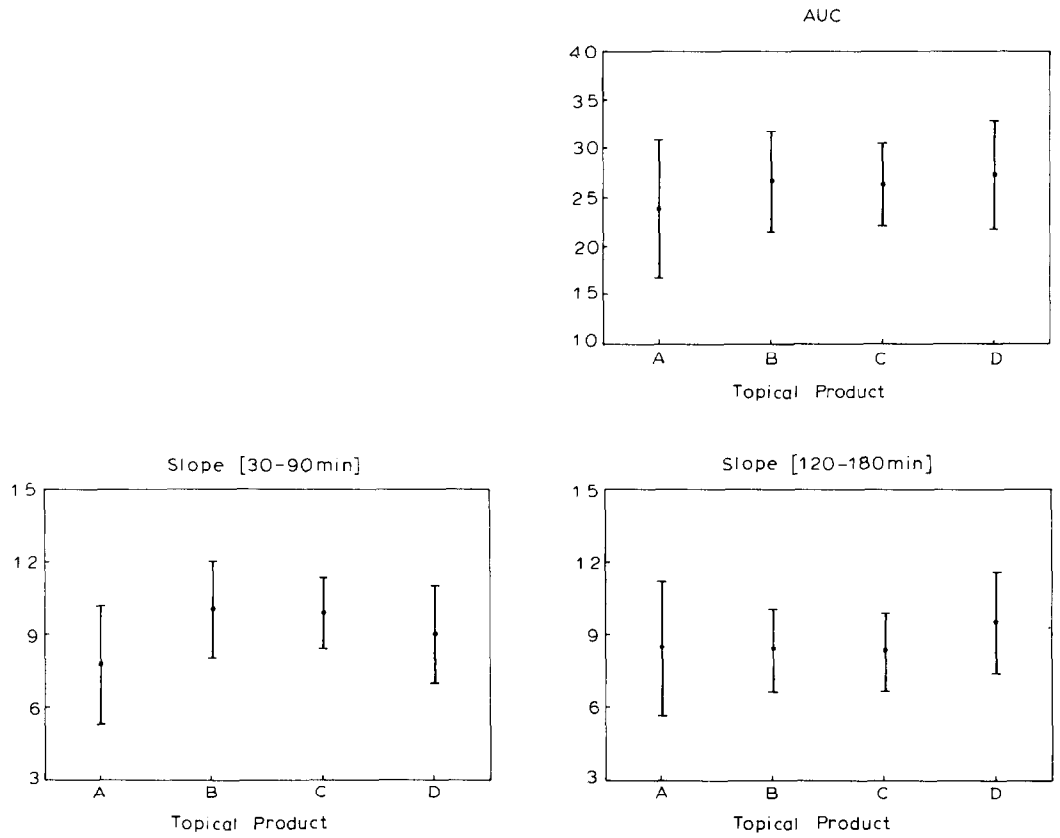

Fig. 5. Effect of topical non-steroidal anti-inflammatory preparations on nicotinate-induced erythema in the multi-subject trial. Error bar represents the standard deviation of measurements on six individuals (two sites per person).

significant activity against the different features of inflammation would have a greater chance of therapeutic success than would a more selective drug. The model described in the present article may be a useful addition to the screening and potency-ranking tests available for anti-inflammatory compounds and their formulations.

Appendix: Calculation of Summary Measures

With the many sophisticated instruments available for monitoring and recording the progress of treatment effects in scientific and clinical studies, there are few limitations to the data collection process. Currently, the common problem facing many investigators instead is the capacity to analyse the abundant data generated in order to derive sound conclusions. It is recognized that multiple-point measurements over an interval provide more valid summary measures for statistical analysis in contrast to single end-point measurements. Response-time profiles are often constructed. The method of summary measures allows for the compression of a sizable number of dependent observations to a few relevant summary parameters (Matthews et al., 1990). The summary measures are then treated as raw data for the appropriate statistical analysis. By using this method, the erroneous rejection of the null hypothesis is precisely controlled by avoiding multiple pairwise comparisons using standard two-sample tests such as the t-test.

The time to peak response, the value of the peak response and the regression coefficient are summary measures which are commonly used to

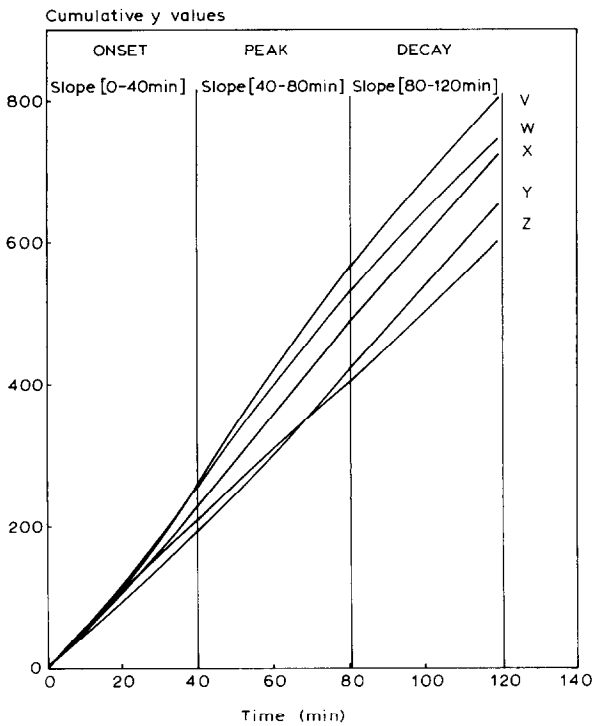

Fig. 6. Typical area under curve plot.

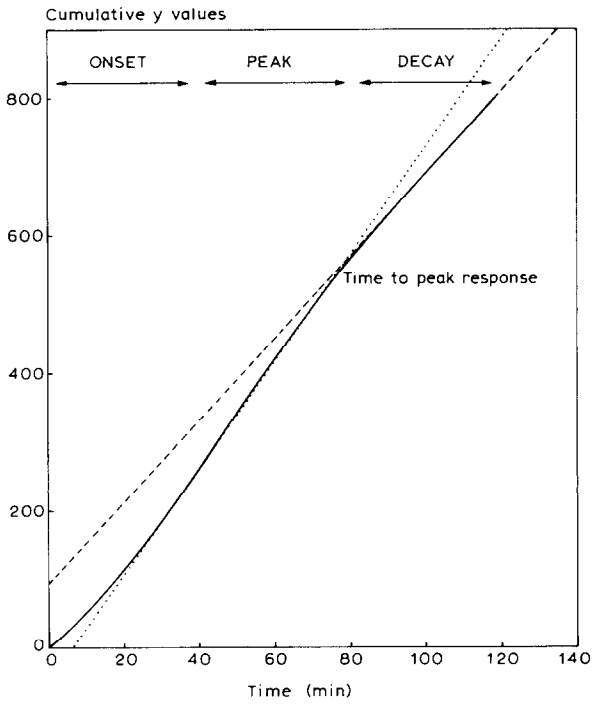

Fig. 7. Estimation of time to peak response from the AUC-time curve.

TABLE 3

Tukey's multiple-range test for comparison [a] of the effect of NSAID preparations on methyl nicotinate-induced erythema

Single subject	NSAID preparations							
(1) AUC [0–150 min]	C	D	B	A	G	H	E	F
df = 14, MSE = 1.079								
q = 4.990, MSD = 2.59								
(2) Slope [30–90 min]	C	G	B	A	H	D	E	F
df = 14, MSE = 0.358,								
q = 4.990, MSD = 1.494								
(3) Slope [90–150 min]	D	C	B	H	G	A	E	F
df = 14, MSE = 0.638,								
q = 4.990, MSD = 1.993								

Multi-subject	NSAID preparations			
(4) AUC [0–180 min]	Feldene	Voltarol	Proflex	Methyl salicylate
df = 35, MSE = 8.977,				
q = 3.814, MSD = 3.29				
(5) Slope [120–180 min]	Voltarol	Proflex	Feldene	Methyl salicylate
df = 35, MSE = 1.251,				
q = 3.814, MSD = 1.231				

[a] A continuous line indicates that comparisons are not significant at the 0.05 level.

df, degree of freedom; MSE, mean square error; q, critical value of studentized range; MSD, minimum significance difference.

TABLE 4

Cell formulae of calculation format in Symphony 2.1 spreadsheet

Column / Row	A	B	C	D	E	F	G	H	I	J
1	x	$\Delta x/2$	y			AUC	$y_n - y_0$			CAUC
2	x_0	+A2/2	y_0	+C2	+D2*B2	+E2	+C2 − C2	+G2	+H2*B2	+I2
3	x_1	+(A3 − A2)/2	y_1	+C2 + C3	+D3*B3	+E3 + F2	+C3 − C2	+G2 + G3	+H3*B3	+I3 + J2
4	x_2	+(A4 − A3)/2	y_2	+C3 + C4	+D4*B4	+E4 + F3	+C4 − C2	+G3 + G4	+H4*B4	+I4 + J3
5	x_3	+(A5 − A4)/2	y_3	+C4 + C5	+D5*B5	+E5 + F4	+C5 − C2	+G4 + G5	+H5*B5	+I5 + J4
6	x_4	+(A6 − A5)/2	y_4	+C5 + C6	+D6*B6	+E6 + F5	+C6 − C2	+G5 + G6	+H6*B6	+I6 + J5
7	x_5	+(A7 − A6)/2	y_5	+C6 + C7	+D7*B7	+E7 + F6	+C7 − C2	+G6 + G7	+H7*B7	+I7 + J6
8	x_6	+(A8 − A7)/2	y_6	+C7 + C8	+D8*B8	+E8 + F7	+C8 − C2	+G7 + G8	+H8*B8	+I8 + J7
9	x_7	+(A9 − A8)/2	y_7	+C8 + C9	+D9*B9	+E9 + F8	+C9 − C2	+G8 + G9	+H9*B9	+I9 + J8
10	x_8	+(A10 − A9)/2	y_8	+C9 + C10	+D10*B10	+E10 + F9	+C10 − C2	+G9 + G10	+H10*B10	+I10 + J9
11	x_9	+(A11 − A10)/2	y_9	+C10 + C11	+D11*B11	+E11 + F10	+C11 − C2	+G10 + G11	+H11*B11	+I11 + J10
12	x_{10}	+(A12 − A11)/2	y_{10}	+C11 + C12	+D12*B12	+E12 + F11	+C12 − C2	+G11 + G12	+H12*B12	+I12 + J11
13	x_{11}	+(A13 − A12)/2	y_{11}	+C12 + C13	+D13*B13	+E13 + F12	+C13 − C2	+G12 + G13	+H13*B13	+I13 + J12
14	x_{12}	+(A14 − A13)/2	y_{12}	+C13 + C14	+D14*B14	+E14 + F13	+C14 − C2	+G13 + G14	+H14*B14	+I14 + J13

compare serial data between different treatments. They are straightforward to obtain. The area under the response-time curve is another useful parameter and is calculated using the linear trapezoidal rule. The corrected area under the response-time curve is computed when the baseline value is numerically much larger than zero. This is carried out by first subtracting the baseline value from each outcome data point before calculating the cumulative area under curve. A graphical plot of the incremental area under curve or corrected area under curve vs time often yields a relatively straight line (Fig. 6). The slope parameter is the gradient of that line,

TABLE 5

Tabulation of data and macro program in Symphony 2.1 spreadsheet

Column / Row		A	B	C	D	.....Z	AA	AB	AC
25	Treatment/site	$X/1$	$Y/3$	$Z/2$		C	{M} ~ C28..C40		
26	Time (h)						~ C2..C14 ~ {GOTO}F2 ~		
27	x	y	y	y			{M}rvF2..F14 ~		
28	0.00	5.93	7.44	4.00			C44..C56 ~ {GOTO}G2 ~		
29	0.25	6.18	8.37	4.67			{M}rvG2..G14 ~		
30	0.5	5.25	8.23	3.92			C58..C70 ~ {GOTO}J2 ~		
31	0.75	5.47	7.69	5.66			{M}rvJ2..J14 ~		
32	1.00	7.10	5.39	7.78			C73..C85 ~ {M} ~		
33	1.25	3.48	5.65	6.68		D	{M} ~ D28..D40		
34	1.50	5.27	7.43	3.33			~ C2..C13 ~ {GOTO}F2 ~		
35	1.75	7.10	5.12	5.89			{M}rvF2..F14 ~		
36	2.00	3.49	7.43	4.93			D44..D56 ~ {GOTO}G2 ~		
37	2.25	4.64	6.11	4.86			{M}rvG2..G14 ~		
38	2.50	4.27	7.30	2.95			D58..D70 ~ {GOTO}J2 ~		
39	2.75	4.34	8.12	3.20			{M}rvJ2..J14 ~		
40	3.00	4.27	7.80	3.24			D73..D85 ~ {M} ~		
						E.....F.....Z.....ZZ			

obtained by linear regression of the cumulative outcome variable vs time. The slope and corrected slope parameters define the trend of the response over time and are useful when the functional form of a response is unknown. Slope values can be used to compare the total response or the response over a certain phase of the response-time plot, for example, at the onset phase, the maximal period or the decay stage.

When the response-time curve does not have an obvious peak, as in the case of a plateau, the time to peak response may not be obvious on a graphical plot. The time to peak response can be better estimated by the intersection of the two linear plots of cumulative outcome variable with time depicting the maximal/minimal phase and the decay phase (Fig. 7).

Computer program

The calculation of area under the curve is carried out on an ad hoc Symphony® (or Lotus® 123) spreadsheet macro program on a personal computer. However, it should be possible to carry out similar steps using other spreadsheet programs. Table 4 shows the set up of mathematical formulae for calculating the area under curve (AUC) in column F, the difference of outcome variable (y_n) and the baseline (y_0) in column G, and the corrected area under curve (CAUC) in column J. The experimental data are tabulated in the specified format and imported into the macro program (Table 5). The macro program stored in columns AA–AC of the spreadsheet will copy each 'y' data set onto the table of mathematical formulae, then copy the computed AUC, [$y_n - y_0$]

TABLE 6

Tabulation of results in Symphony 2.1 spreadsheet

Column Row	A	B	C	D	E	.....Z
41	Treatment/site		X/1	Y/3	Z/2	
42						
43	Time (h)		AUC	AUC	AUC	
44	0.00		0.00	0.00	0.00	
45	0.25		1.51	1.98	1.08	
46	0.5		2.94	4.05	2.16	
.....	.....		.....	.....	.....	
54	2.50		13.27	17.20	12.80	
55	2.75		14.35	19.13	13.57	
56	3.00		15.42	21.12	14.37	
57	Time (h)		$y_n - y_0$	$y_n - y_0$	$y_n - y_0$	
58	0.00		0.00	0.00	0.00	
59	0.25		0.25	0.93	0.67	
60	0.50		−0.69	0.79	−0.08	
.....	.....		.....	.....	.....	
68	2.50		−1.66	−0.14	−1.05	
69	2.75		−1.59	0.68	−0.80	
70	3.00		−1.66	0.36	−0.76	
71						
72	Time (h)		CAUC	CAUC	CAUC	
73	0.00		0.00	0.00	0.00	
74	0.25		0.03	0.12	0.08	
75	0.50		−0.02	0.33	0.16	
.....	.....		.....	.....	.....	
83	2.50		−1.56	−1.40	2.79	
84	2.75		−1.97	−1.33	2.56	
85	3.00		−2.38	−1.20	2.36	

and CAUC values from the table of formulae onto the table of results in the same spreadsheet (Table 6). This ad hoc program can be tailored to accept various types of data and its capacity is probably limited only by that of the spreadsheet program.

The slope summary measures are obtained by importing the corresponding incremental AUC values with the 'x' variable into the Minitab® Release 7.2 program for linear regression. The gradient of the best-fitting line is adopted as the slope parameter over the specified time span.

The resulting summary measures are subjected to statistical treatment to compare the outcome relative to the response to different experimental variables.

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