The human skin blanching assay for in vivo topical corticosteroid assessment II. Subject- and observer-dependent variation in blanching responses

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Abstract

The human skin blanching (vasoconstriction) assay for the assessment of topical corticosteroids uses the skin pallor induced at the site of application as an indicator of the potency of the drug or efficacy of the delivery vehicle. Usually several volunteers and several visual observers are used in the bioassay to counteract the subjectiveness of the methodology. Given the inherent biological variability of the population, the minimum number of volunteers and observers required to give valid results in this assay has not been firmly established. This investigation consisted of three identical trials performed at 8 week intervals, utilising the same 18 volunteers and the same three observers in an attempt to address the question of intra- and inter-volunteer and observer reproducibility of the results from three replicate experiments. The results indicate that for any assay utilising this methodology, the number of application sites for a topical corticosteroid formulation should be at least 48, the number of volunteers (of either sex) should be not less than nine and the number of observers not less than two.

1. Introduction

It is well established in the natural, medical and pharmaceutical sciences that, due to inherent bio- logical variability, repeated measurements of the same response do not necessarily produce the same results (Larsen et al., 1984). The human skin blanching assay makes use of the response of whitening in human skin produced on topical application of corticosteroids. The intensity of blanching is measured since this is directly related It is well established in the natural, medical and pharmaceutical sciences that, due to inherent bio-logical variability, repeated measurements of the same response do not necessarily produce the same results (Larsen et al., 1984). The human skin blanching assay makes use of the response of whitening in human skin produced on topical application of corticosteroids. The intensity of blanching is measured since this is directly related to the clinical efficacy of the formulation (Cornell and Stoughton, 1985). The application of corticosteroids to human skin does not induce pallor in all individuals (Harding et al., 1985). It is known that the thickness of the stratum corneum from the same region of the body differs between individuals, with the number of cell layers ranging from 12 to 30 (Kligman, 1984).

Since the human skin blanching assay measures the rate and extent to which a topical corticosteroid diffuses from the formulation and passes through the stratum corneum to the vasculature, these differences in the physiological properties of human skin must produce different results in different individuals after topical application of the same corticosteroid. Given this known variability in the permeability of the stratum corneum, it would clearly be invalid to perform an assay of this type on a single individual. The question then arises of how many individuals are necessary to produce meaningful results. A large number of blanching studies have been reported in the literature and the numbers of subjects utilised in each study varies from five (Montenegro et al., 1996) to 50 (Stewart et al., 1973)

A compounding problem is the number of sites utilised on anyone individual. Some published conclusions are derived from single applications on a number of individuals, some from a number of applications on a single individual and some from a number of applications

on a number of individuals. Other volunteer variables are the ability to produce strong or weak blanching, the sex of the volunteer and the fact that the left and right arm may react differently. All three of these variables were addressed in this study.

Whilst the methodology of the human skin blanching assay is generally well accepted by the scientific community at large, the subjective nature of the visual assessment of the intensity of pallor has been criticised (Stoughton, 1987; Shah et al., 1989). Many reports have appeared in the literature concerning objective assessment techniques as alternative methods of assessing skin blanching. Reflectance (Ryatt et al., 1982), thermographic (Aiache et al., 1980) and laser Doppler velocimetry (Stevenson et al., 1987) techniques have been developed in an attempt to record the degree of skin blanching at each application site in a more quantitative manner. The chromameter, which measures skin colour by reflectance, has been in use for about 5 years and it has been reported (Clarys et al., 1995; Pershing, 1995; Montenegro et al., 1996) that objective measurements of skin colour can be made using this instrument.

Subjective assessments should be performed with care; training of observers is essential. In most reports, observations of pallor appear to be made by the researchers themselves, although there have been reports in which some observations were made by the volunteers (Gruvstad and Bengteson, 1980). Since this method is subjective, it seems reasonable to suggest that there must be some kind of internal monitoring of the ability of an observer to provide consistent evaluations of blanching. The most commonly reported method for providing this check is the inclusion of more than one observer in anyone trial, but the number of observers required to ensure accuracy of readings seems to be undecided. The number of observers employed in reported studies ranges from one (Stoughton and Cornell, 1987) to five (Clanachan et al., 1980). The results reported here are an attempt to assess the number of observers, volunteers and application sites required for the human skin blanching assay to produce meaningful results.

2. Materials and methods

This investigation consisted of three identical trials performed at 8 week intervals, utilising the same 18 volunteers, the same three observers and two commercially available betamethasone 17- valerate (0.12%) creams (Betnovate cream, Glaxo, South Africa and Celestoderm- V cream, Schering- Plough, South Africa). Each preparation was applied to six sites on each arm of the volunteers: Both arms were left unoccluded and the formulations were left on the arms for 6 h. The degree of blanching was estimated at 7, 8, 9, 10, 12, 14, 16, 18, 28 and 32 h after application of the corticosteroid formulations. The complete experimental protocol has previously been reported (Haigh et al., 1997)

Blanching profiles of both formulations for each trial were produced as follows:

1. For each volunteer assessed by three observers.

2. For each observer incorporating 18 volunteers.

3. For the nine male and the nine female volunteers assessed by three observers.

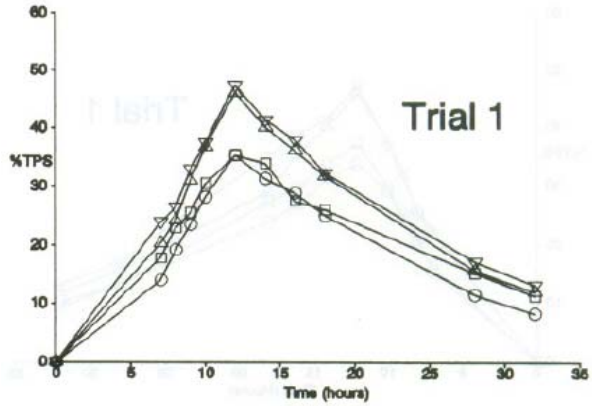
4. For strong and weak arms of 18 volunteers assessed by three observers.

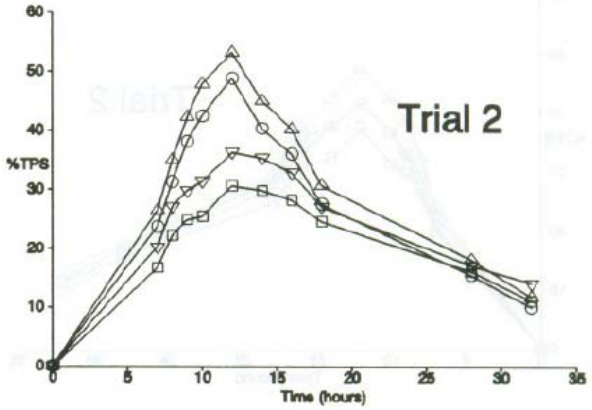
3. Results and discussion

Fig. 1 depicts the blanching curves obtained for the group of nine males and nine females and Fig. 2 shows the blanching curves for strong and weak arms. Table 1 lists the area under the blanching curve (AUBC) values for all 18 volunteers for all three trials, Table 2 for males and females and for strong and weak arms.

It is clear that there are considerable inter- and intra-individual variations in the intensity of topical corticosteroid induced skin blanching. Intraindividual precision is best for volunteer 5, Celestoderm-V formulation (AUBC mean value of 849.0 with a S.D. 14.4) and worst for volunteer 13, Betnovate formulation (AUBC mean value of 426.7 with a S.D.141.5). It might be expected that the same person would display the same degree of blanching on application of the same amount of corticosteroid on different occasions. It is evident from the above results that this is the case only with some individuals, others show large differences on replicate applications.

Why individuals should react differently to the same corticosteroid on replicate application is uncertain. A medical history was obtained from each volunteer before the commencement of the study. Three of the female volunteers were taking oral contraceptives during all three trials but the shapes of the blanching curves constructed for them were normal. Female volunteers were required to provide details of their menstrual cycles. Only one irregular cycle was reported, that of volunteer 17 who displayed the lowest degree of blanching. It was not possible to correlate the results obtained with individual medical histories.





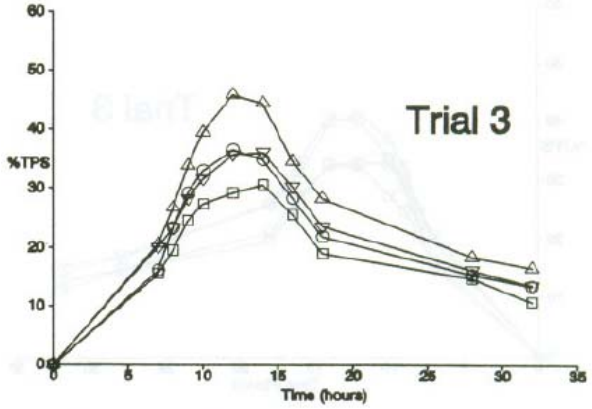


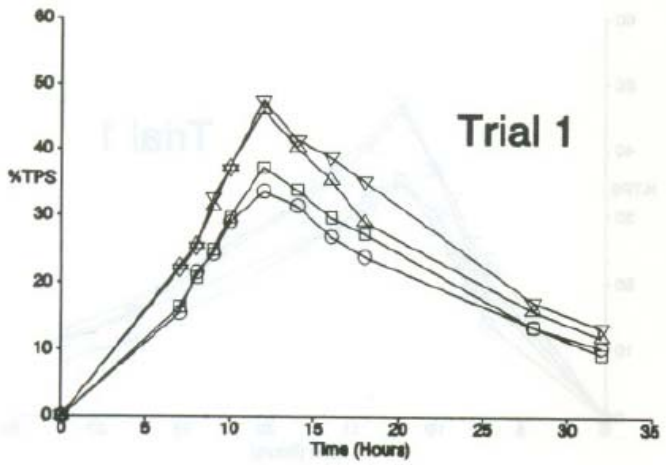
Fig. 1. Blanching profiles for males and females for trials 1, 2 and 3, pooled results of all volunteers and observers. △, Celestoderm-V (males); ▽, Celestoderm-V (females); ○, Betnovate (males); □, Betnovate (females).

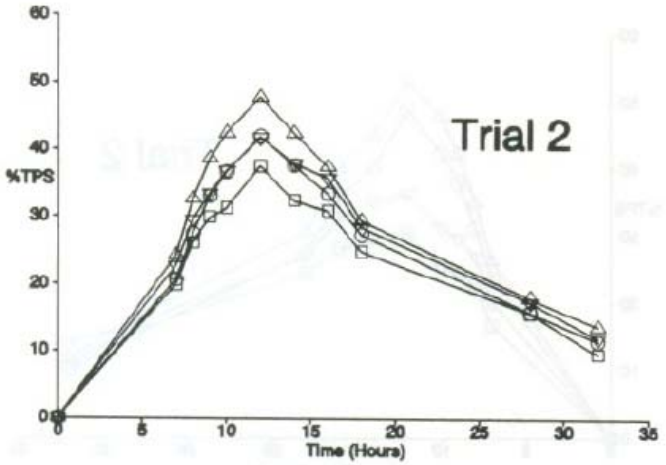
Inter-individual variations are remarkably constant. Calculated as a percentage of the lowest AUBC to the highest AUBC for each preparation in each trial, inter-individual variability ranges from 20-30%. Biological variability is the obvious explanation for these differences which are commonly observed in trials of this nature.

Since one of the reasons for performing this assay is to differentiate between formulations on the basis of the intensity of blanching, and given the large degree of variability of blanching between individuals, it is necessary to determine whether this individual variability will effect the final result. As can be seen from Table 1, of the 54 AUBC values reported, 47 indicate that Celestoderm- V produces a higher degree of blanching than Betnovate. All of the seven cases of reversal show marginally greater blanching in favour of Betnovate but only occur in certain trials for any one individual, the other trials showing the expected rank order.

The number of volunteers reported to be required in a study of this type has varied considerably. Clearly it is important to have enough people to produce a meaningful result and yet to have a small enough panel to make the trial reasonably easy to perform. Coupled with this is the number of applications of an individual formulation. In this investigation, each formulation was applied to six sites per arm, i.e. 216 sites were evaluated by three observers at each reading time and the results pooled for final analysis. In an attempt to ascertain the minimum number of volunteers required to produce profiles which mirrored those obtained from all 18, different groups of volunteers were analyzed and blanching profiles produced.

Random selections of nine (108 application sites), six (72 application sites) and four (48 application sites) volunteers produced blanching profiles which were almost identical to those produced by all 18 volunteers in terms of the differences between the two formulations. The results from random selections of three volunteers showed the same results, but the differences between formulations were smaller. A group of six selected volunteers (which included the five volunteers who showed some reversals of the expected differences between preparations for some trials, the worst possible case) produced profiles which showed the expected differences between formulations, but these differences were small. This would indicate that nine is the smallest volunteer panel that would produce valid conclusions.





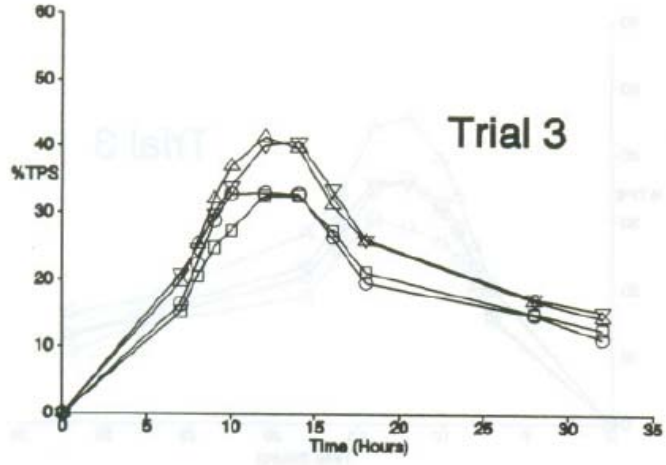


Fig. 2. Blanching profiles for strong and weak arms for trials 1, 2 and 3, pooled results of all volunteers and observers. △, Celestoderm-V (strong arm); ▽, Celestoderm-V (weak arm); ○, Betnovate (strong arm); □, Betnovate (weak arm).

Table 1
AUBC values for Celestoderm-V (Cel-V) and Betnovate (Bet) for individual volunteers (Vol)

Vol	Trial 1		Trial 2		Trial 3	
	Cel-V	Bet	Cel-V	Bet	Cel-V	Bet
1	927	992	1676	1549	1097	867
2	1041	640	783	774	814	706
3	1256	989	765	549	1145	804
4	685	590	752	653	996	784
5	865	508	845	552	837	604
6	644	377	825	788	552	498
7	687	480	1193	1096	693	572
8	327	298	379	432	379	415
9	417	336	577	552	536	424
10	1117	782	896	693	1120	716
11	834	814	972	867	794	829
12	772	706	692	655	438	373
13	887	570	571	423	466	287
14	843	597	753	627	773	533
15	1240	972	912	860	1111	898
16	782	671	604	534	762	756
17	260	192	523	408	239	269
18	517	489	410	432	273	339

Pooled results of all three observers.

The results were then recalculated in terms of the intensity of induced blanching as measured in the group of nine females and in the group of nine males. As can be seen from Fig. 1 and Table 2, the results obtained for trials 2 and 3 were very similar, indicating quite clearly that the nine males produced considerably more blanching than the nine females. Trial 1, however, produced different results, indicating that the degree of blanching produced was very similar for both males and females. It can be therefore be concluded that, on average, males will produce more intense blanching than females. The similarities observed in Trial 1 may well be related to the fact that an individual often produces different intensities of blanching on repeated applications of the same corticosteroid. The important conclusion which must be drawn from this particular recalculation is that both males and females show exactly the same ability to discriminate between different formulations on the basis of the assessment of the induced skin blanching.

The results were recalculated to produce blanching profiles and AUBC values for the different arms of all volunteers. Arms were divided into strong (writing) and weak arms as opposed to right or left arms. Since only two of the 18 volunteers were left-handed, comparisons are essentially between right and left arms. It can be seen from Fig. 2 and Table 2 that the arms of individuals are essentially identical in terms of the assessed induced blanching and the ability to discriminate between products. In this trial, both arms were

treated similarly, i.e. they were both unoccluded. In many trials of this type, one arm is occluded and one left unoccluded. Since the arms are equivalent, it is obvious that it is unimportant which arm is occluded if the trial demands such a protocol.

Table 2
AUBC values for Celestoderm-V and Betnovate for nine male and nine female volunteers and strong and weak arms of all volunteers for three trials

	Trial 1		Trial 2		Trial 3	
	Cel-V	Bet	Cel-V	Bet	Cel-V	Bet
Males	761	597	866	772	783	631
Females	806	644	704	611	664	556
Strong arm	753	592	815	721	720	596
Weak arm	813	631	754	662	727	590

Pooled results of three observers.

Figs. 3-5 represent the blanching profiles as calculated for each observer for each of the three trials. As can be seen from these figures and the area under the blanching curve (AUBC) values reported in Table 3, it is clear that individual observers assign different scores to corticosteroid induced blanching in human skin. Observers I and 2 showed remarkable consistency, however the results produced by observer 3 are substantially different from those recorded by observers I and 2, in the main due to lower assessments of the induced blanching.

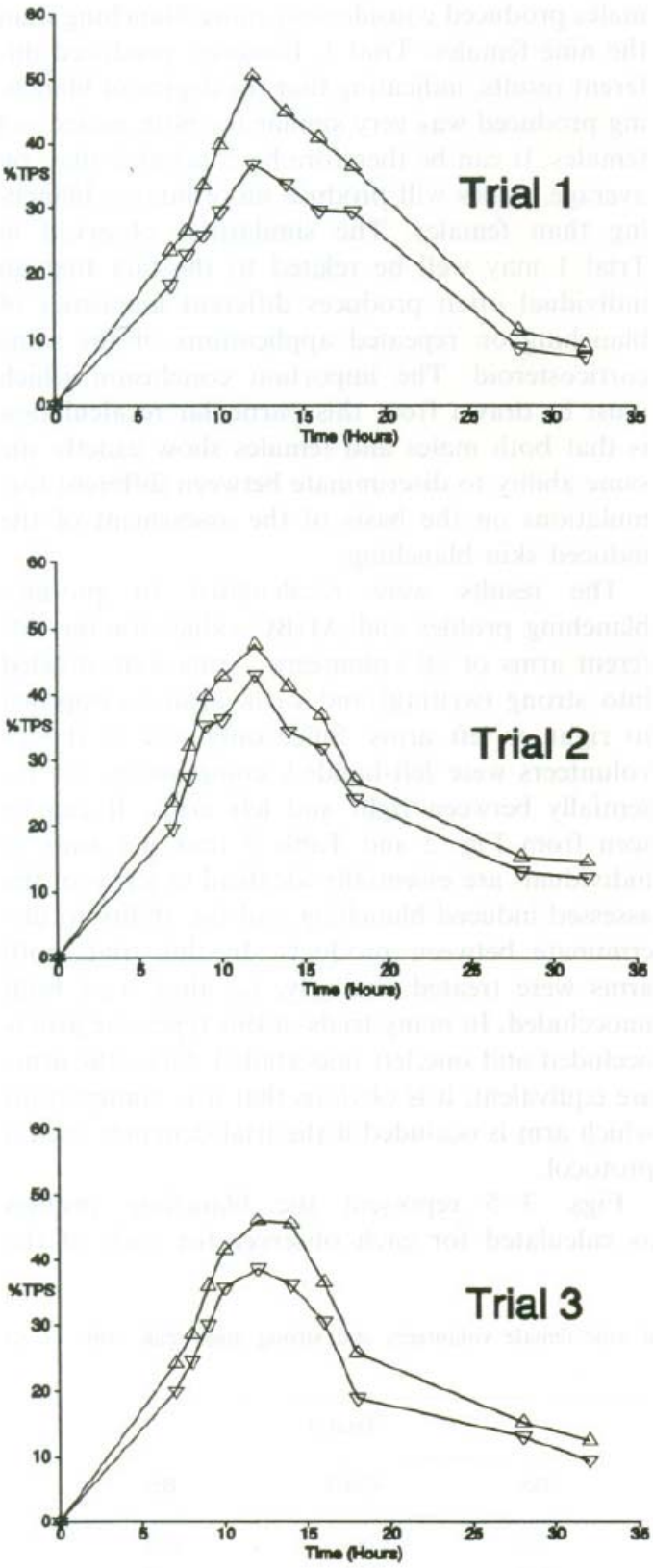


Fig. 3. Blanching profiles for all volunteers for each trial recorded by observer 1. △, Celestoderm-V; ▽, Betnovate.

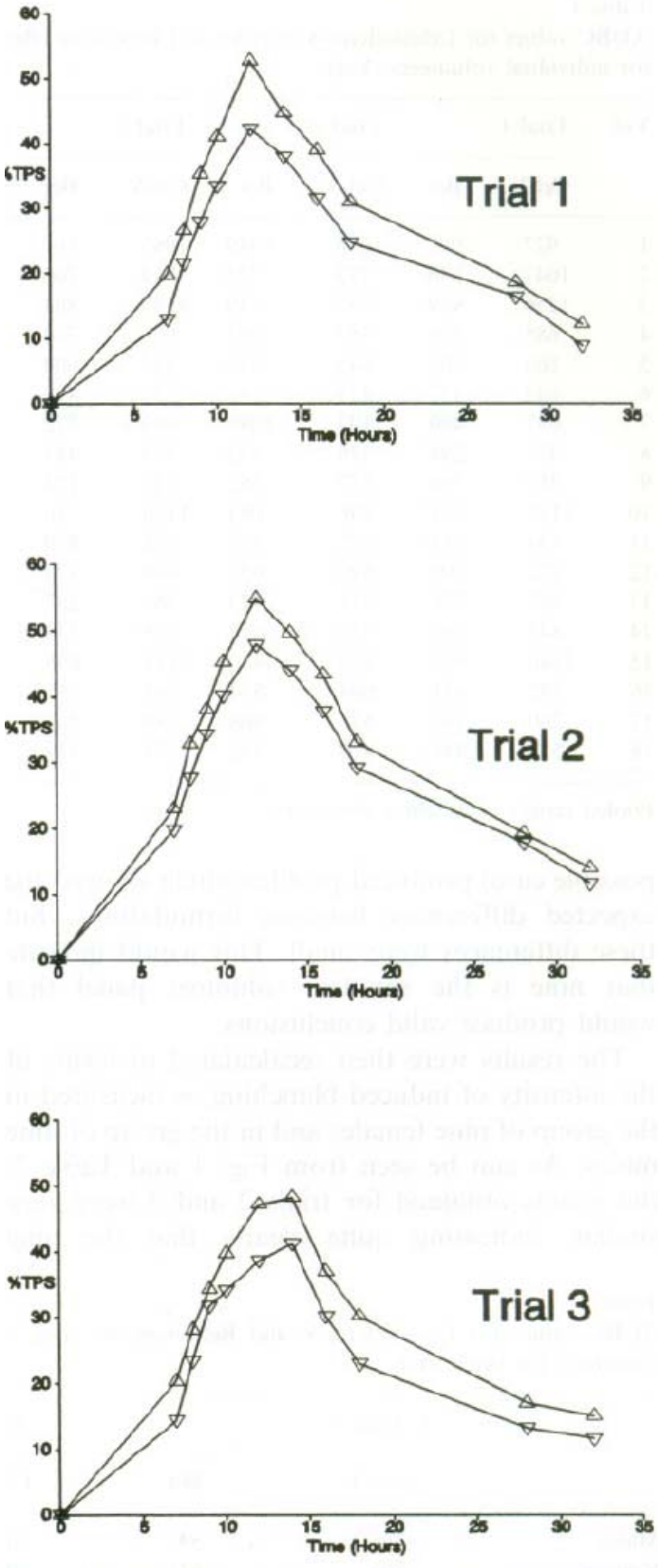


Fig. 4. Blanching profiles for all volunteers for each trial recorded by observer 2. △, Celestoderm-V; ▽, Betnovate.

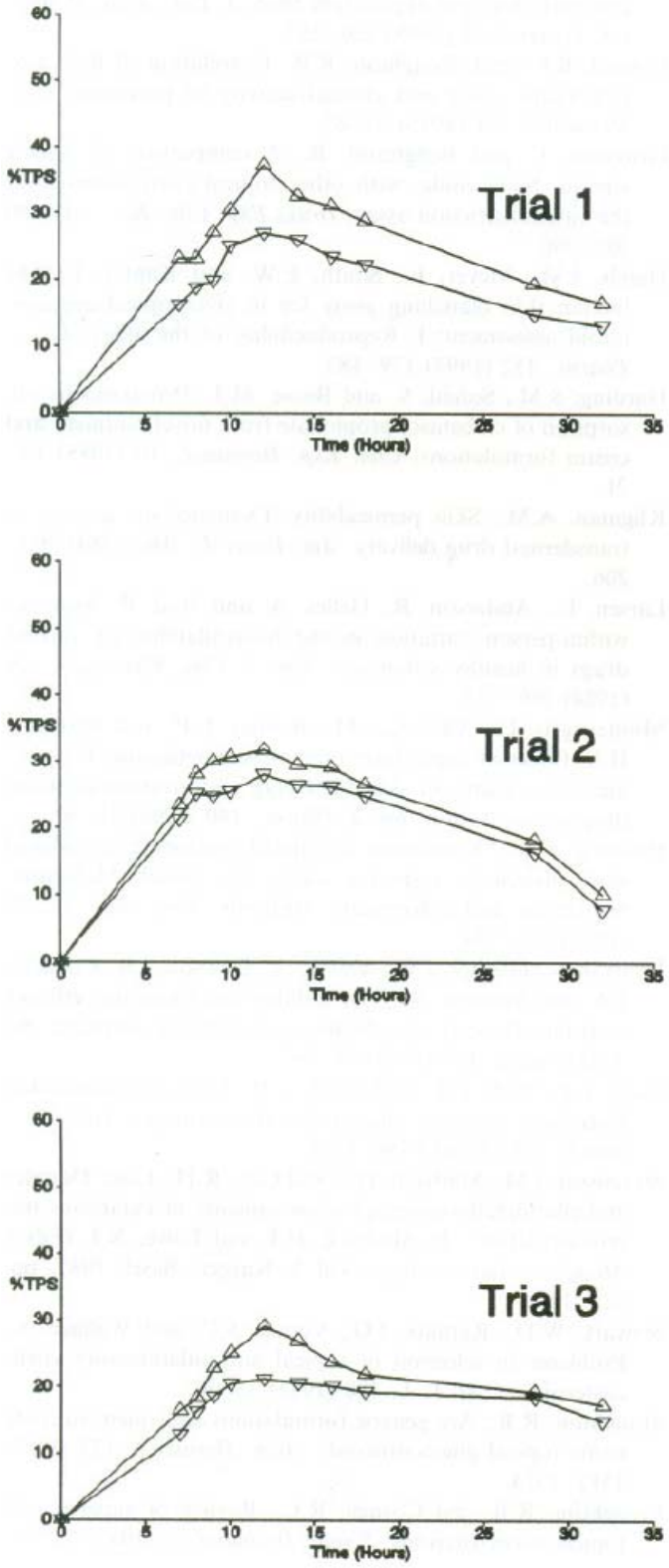


Fig. 5. Blanching profiles for all volunteers for each trial recorded by observer 3. △, Celestoderm-V; ▽, Betnovate.

It would be unreasonable to expect three observers scoring' independently to assign the same scores to the blanching response elicited by a preparation in any single trial because of the

subjective nature of the decisions required. If, however, the assay procedure and the results it produces are to be valid, then the same conclusions must be reached by each individual observer. These results show that all three observers recorded the same results in all three trials, i.e., the blanching produced by Celestoderm- V is always greater than that produced by Betnovate. Even observer 3, who scored the blanching response considerably lower than observers I and 2, obtained blanching profiles and AUBC values which were in agreement with those of observers I and 2 in terms of their ability to discriminate between formulations.

Table 3
Individual observer AUBC values for each trial

Trial no.	Celestoderm-V	Betnovate
1		
Observer 1	803	619
Observer 2	815	656
Observer 3	731	558
2		
Observer 1	781	674
Observer 2	888	784
Observer 3	686	616
3		
Observer 1	767	624
Observer 2	799	639
Observer 3	605	516

4. Conclusions

Given the large intra-individual variability of the blanching response, a number of volunteers must be utilised. From the results presented here, it seems that, for anyone assay utilising this methodology, the number of application sites for anyone topical corticosteroid formulation should be not less than 48 and the number of volunteers should be not less than nine. However, the more volunteers used and the greater the number of application sites, the more valid the results obtained from an in vivo assay such as the human skin blanching assay. The results show that the trials can be performed using male or female volunteers and the left and/or right arm of each volunteer without compromising the outcome. The question of how many observers should be utilised in anyone trial must be addressed. From the above results, it seems that a single observer would suffice, however, it is reasonable to suggest that a minimum of two observers would provide internal monitoring which would give greater confidence to the results obtained using this assay procedure. Utilisation of more observers could, however, be an advantage in that more data would be generated and this would produce results which would have a higher degree of relevance.

Acknowledgements

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