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Accuracy and Reproducibility of the Multiple-Reading Skin Blanching Assay

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The human skin blanching (or vasoconstrictor) assay has been used for almost 30 years [1–4] to compare the topical availability (and, by inference, the clinical efficacy) of corticosteroids in various delivery vehicles. The detailed methodology of the assay has been reported and reviewed in a number of communications [3, 4]. The basis of the assay depends on the skin-blanching side effect following corticosteroid application to the skin, the intensity of which is a measure of both the inherent potency of the drug and its capacity to diffuse through the stratum corneum from the application environment. In the vast majority of published skin blanching assay results, measurement of the intensity of pallor has been performed visually using an arbitrary, subjective scale. A number of instrumental measurement techniques have been investigated as potential replacements for the subjective visual determinations [5–9]. These investigations have shown that several instrumental methods could adequately replace visual determinations, however most of them are cumbersome, time-consuming and expensive while offering no improvements in the quality of the data over subjective visual observations. This is supported by the lack of recently published data utilizing instrumental methods (the original investigations having been conducted several years ago) and by the continued reporting, and international acceptance, of experiments conducted visually [10, 11].

Visually monitored skin blanching assays have been conducted in our laboratories for over 13 years since 1977 during which time great confidence in the integrity of the methodology has developed. To verify the

Table 1. Parameters of blanching trial data used in the retrospective assessment of reproducibility

Parameter	Application mode	
	occluded	unoccluded
Number of trials reanalyzed	44	36
Total number of volunteers used	528	432
Total number of application sites	1,989	1,953
Total number of observations made	59,670	58,590

reproducibility of this subjective bioassay, a retrospective analysis of past results was performed along the lines of that presented by Barry and Woodford [2]. Betnovate cream (0.12% betamethasone 17-valerate; Glaxo, South Africa) is included in every experiment utilizing this methodology performed in our laboratories. The inclusion of this standard preparation allows the monitoring of the assay to verify that no anomalies arise under the variable ambient test conditions, a procedure adopted by other researchers in this field [2]. In contrast to the nature of the analysis reported by Barry and Woodford [2] where 16 volunteers and one observer were used for ten trials over a 3-year period in the occluded application mode only, our retrospective analysis of blanching results for Betnovate cream cover an 11-year period, 8 trained observers (3 of whom were randomly used in each trial), several hundred volunteers and many thousands of observations in both the occluded and unoccluded application modes (see table 1).

It is important to note that these assays were initially designed to test other premises, and have subsequently been reanalyzed in such a way as to test the reproducibility of the methodology. The results of this analysis are depicted in figures 1 and 2 and demonstrate the clear distinction between the occluded and unoccluded blanching profiles. The relatively small standard deviations of the means obtained from this vast mass of data, collected from several trials utilizing different participants, lends credence to the methodology employed and, moreover, adequately justifies the continued use of the visual assessment of skin pallor.

We have calculated the percentage relative discrepancy (d) between the area of each trial curve included in this retrospective analysis and the mean curve for all the trials. This statistical parameter is an indicator of

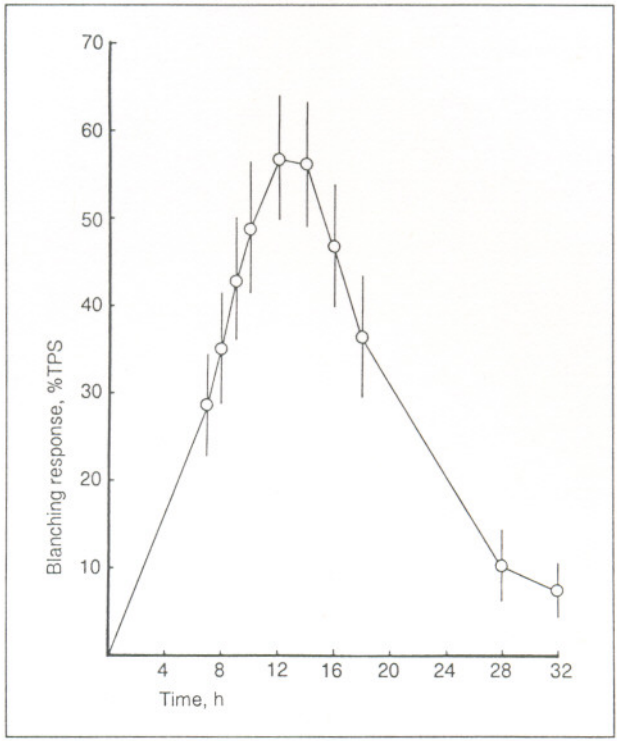

Fig. 1. Mean skin blanching responses (% total possible score, % TPS) ± standard deviations for Betnovate cream calculated from 59,670 observations in the occluded formulation application mode.

the shape difference or skewness between a specific assay profile and the mean profile for the entire assay population [12]. The limits of $\underline{d}$ lie between 0% (superimposition of profiles) and 100% (no coincidence of profiles). This descriptor is a useful indicator of bioassay reproducibility since the test and mean profiles may have similar AUC values but very different response/time relationships; such a situation would be indicated by a large $\underline{d}$ value. The generally accepted gradations for the degree of discrepancy are: 0–20%, small discrepancy between test and mean profiles; 21–35%, moderate discrepancy and $>35\%$ implies a large difference. Of the 44 blanching assays conducted in the occluded application mode, 39 have $\underline{d}$ values of $<20\%$ and 5 fall into the moderate discrepancy classification (mean $\underline{d} = 14.54$, SD = 6.25). Of the 36 blanching assays conducted

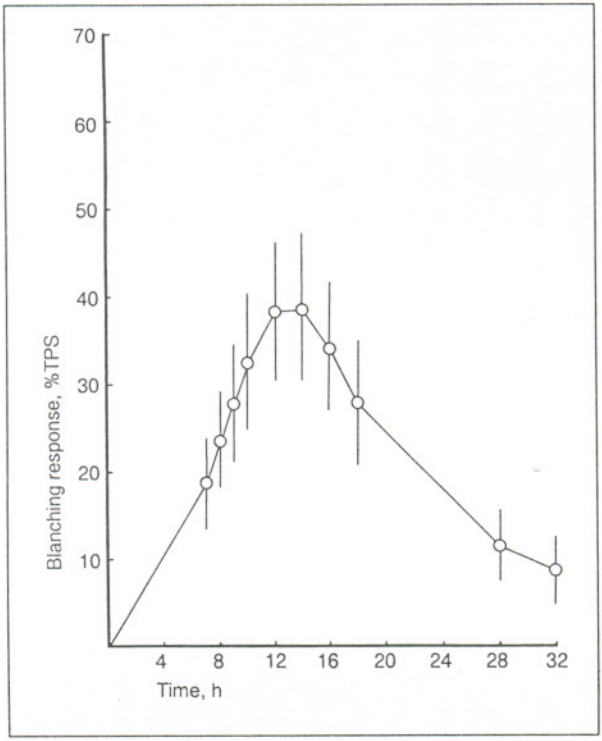

Fig. 2. Mean skin blanching responses (% total possible score, % TPS) ± standard deviations for Betnovate cream calculated from 58,590 observations in the unoccluded formulation application mode.

in the unoccluded application mode, 24 show small, 8 have moderate and 4 trials have large discrepancy values (mean $\underline{d} = 18.92$, SD = 11.69). These remarkable results clearly indicate how indicative individual trials are of the 'population' mean results, in spite of the subjectivity of the result acquisition, the normal biological variability of the volunteer and observer panels, and the nonuniform ambient test conditions. The greater variability observed in the unoccluded application mode is not unexpected since the intensity of the blanching induced in the skin is lower without occlusive covering and, subsequently, the grading of this blanching by the observers becomes more difficult.

As further evidence for our confidence in the discriminatory powers of the assay methodology, we present the results from blanching trials con-

Table 2. AUC values for Betnovate cream preparations manufactured in three different countries

Country	Application mode	
	occluded	unoccluded
Australia	1,208	798
United Kingdom	1,253	855
South Africa	1,003	542
South Africa	1,004	556

ducted to compare the corticosteroid availability from the same brand name topical formulations manufactured in three different countries (Australia, South Africa and the United Kingdom). In all trials in this series, Betnovate cream (South Africa) was included as the standard. In the trial designed to assess Betnovate cream prepared in the three different countries, therefore, the South African preparation was represented twice among the four formulations tested. The results of this double-blind investigation are shown in table 2 as AUC values of the multiple-reading blanching profiles in both application modes. It is obvious that the data for the South African preparations are essentially idential. Furthermore, the blanching profiles for these two preparations are virtually superimposable and statistical analysis shows no significant differences at any time interval during the entire period of experimentation. The calculated $\underline{d}$ values for the two South African preparation profiles each equal 1.12% in the occluded mode; and 2.47% in the unoccluded application mode. Bearing in mind the double-blind nature of the methodology, these strikingly similar results obtained for the same preparation demonstrate the unequivocal discriminative potential of this 'subjective' bioassay. Provided the observers are adequately trained and optimized methdology is employed, data generated by the eye *cannot* be held as less meaningful than data generated by instrumental methods.

We have stated on numerous occasions [4, 10, 11, 13, 14] that one of the main pitfalls of the assay methodology, which many researchers perpetuate and many authorities condone, is the practice of taking a single reading of the intensity of skin blanching, often taken as early as 7 h after corticosteroid application [15, 16]. Often, important conclusions are reached and decisions are made from this single result (for example the

registration or rejection of a generic formulation by the regulatory authorities). A single result reading does not allow the construction of topical availability profiles from which rapidity of onset of blanching, rate of decline, duration of action, and AUC values may be determined. Bioavailability/bioequivalence studies on all other pharmaceutical dosage forms are performed on full-curve statistical analyses; why, therefore, should the scientific community accept topical availability results for corticosteroid products which would be totally unacceptable for all other pharmaceutical dosage forms? The following examples observed in our laboratories show the erroneous conclusions, that would have been drawn if data acquisition had been limited to a single observation.

The comparative blanching activities of Betnovate and Eumovate (0.05% clobetasone 17-butyrate; Glaxo, South Africa) have been previously reported for creams and ointments [10]. These results concern different corticosteroid drugs in the same formulation type and in each case clearly demonstrate that a single reading of blanching taken from 7 to 10 h after application would suggest equivalence of the formulations. However, extended observations produce AUC values of 724 and 1,267 for the ointments, and 823 and 1,157 for the creams. This is a clear indication of the different blanching potentials (and consequent clinical efficacy) between the formulations.

In addition, when considering products of the same formulation type containing the same steroid molecule, similar situations have been observed. Figure 3 shows the results of a blanching trial conducted to compare three commercially available creams containing 0.12% betametasone 17-valerate (Betnovate; Celestoderm-V, Scherag, South Africa; Persivate, Lennon, South Africa). It is obvious that from 7 to 10 h after application the response for each preparation is similar at each observation time. This is borne out by the statistical analysis which shows no significant differences between any of the three responses at the 7-, 8-, 9- and 10-hour reading times. A single reading during this interval would confirm bioequivalence of all three preparations. However, as is patently obvious from figure 3, analysis of the full blanching profiles shows a clear rank-order difference between preparations, statistical analysis confirming these differences to be significant at observation times later than 10 h.

In contrast, we have also on occasion observed instances where statistically significant blanching differences between preparations are manifest at 7 h after application when full curve analysis demonstrates almost identical AUC values.

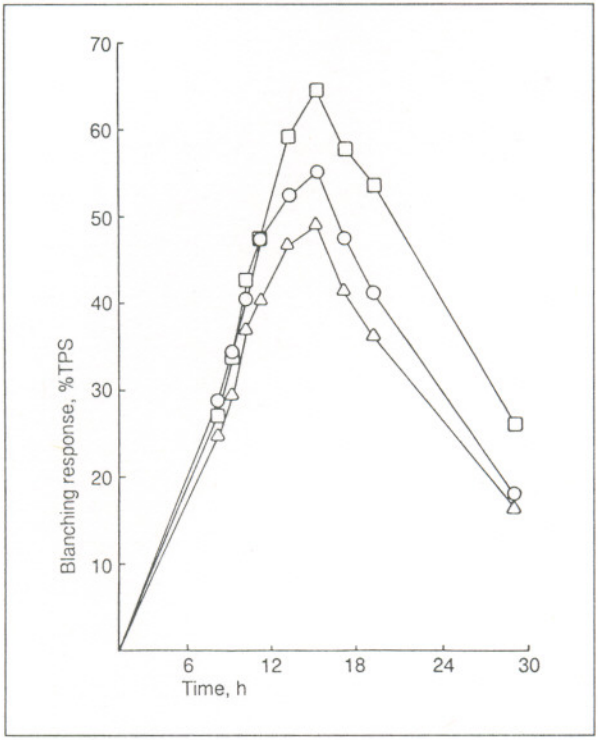

Fig. 3. Mean skin blanching responses (% total possible score, % TPS) for three commercially available betamethasone 17-valerate-containing creams assessed in the unoccluded application mode. ○ = Betnovate; △ = Celestoderm-V; □ = Persivate.

It has previously been argued [17] that a single reading of blanching taken at 7 h after formulation application is adequate for product discrimination when studying preparations containing the same steroid molecule in different concentrations (serial dilutions of a corticosteroid formulation for example), the rationale being that the same steroid should produce the same curve shape, varying only in area due to the drug concentration differences. We suggest that *any* change to the parent formulation, even dilution with the cream base, will change the microenvironment of the delivery vehicle, and this may change the rate and extent of drug passage through the skin. We have formulated under-strength creams that elicit (nonlinear) superior responses compared to full-strength products. We therefore do not regard formulation dilutions as a homologous series that qualifies for

abbreviated treatment; these are considered as formulations of the same pharmaceutical type containing the same active ingredient, a classification to which our argument stated above applies. Clearly, full curve analysis is essential for intelligent comparisons to be made between preparations.

In conclusion, therefore, as noted from the above points, the human skin vasoconstrictor or blanching assay remains a valid, reliable, reproducible and accurate indicator of topical corticosteroid availability, provided the correct methodology is applied [3, 4, 18]. In addition to the normal constraints such as double-blind conditions, random volunteer selection and uniform data acquisition that are practiced in all comparative bioavailability studies, in this methodology it is imperative that many observations are taken over a prolonged period with the consequent production of a blanching profile and subsequent valid statistical analysis. If this assay is conducted in the manner prescribed above, it may be utilized with confidence as a comparative test for topical corticosteroid formulations at the research and development stage. Furthermore, we believe that the data acquired from visual blanching trials will continue to be used to produce authoritative comparisons which will be invaluable to the pharmaceutical drug regulatory bodies for registration formalities.

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