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Analysis of chromameter results obtained from corticosteroid-induced skin blanching assay: comparison of visual and chromameter data

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Abstract

In a Guidance document, the American FDA recommends the use of a Minolta chromameter rather than the human eye for the quantitative assessment of the pharmacodynamic blanching response produced by topical application of corticosteroids. The purpose of this study was to compare the appropriateness of the human eye and two models of chromameter for the estimation of skin blanching, in terms of the quality of the data generated by each method. The corticosteroid-induced skin blanching from four different betamethasone 17-valerate cream formulations was compared in a typical human skin blanching trial. The optimized assay methodology routinely practised in our laboratories was utilized. The blanching responses were assessed visually by three trained, independent observers and recorded by two chromameters (Minolta model CR-200 and model CR-300). The topical availability of the four creams was determined using visual scoring and chromameter measurements. All data were manipulated in such a manner as to produce a blanching response versus time profile from which AUBC analysis could be performed. Good correlation was observed between the visual assessments made by three independent observers. In contrast, moderate correlation was determined between visual, CR-200 and CR-300 measurements. Surprisingly, no direct linear relationship between the AUBCs produced by the two chromameters was observed indicating that the quality of the data obtained from the two instruments may not be equal. This investigation also indicated that the use of the chromameter is not completely objective. Visual scoring and chromameter measurement produce data sets that differ in quality. Each procedure needs to be validated and investigators have to be trained for both visual assessment and the operation of the chromameter, particularly with regard to the manipulation of the measuring head of the instrument.

Keywords: Topical corticosteroids; topical bioavailability; vasoconstriction; human skin blanching assay; chromameter; betamethasone 17-valerate

1. Introduction

The human skin blanching assay has been shown to be a useful tool for the comparison of the potency of different topical corticosteroid formulations, since a direct correlation has been demonstrated between the intensity of corticosteroid-induced skin blanching and clinical efficacy [1, 2, 3]. The assay is also useful for the comparison of the topical availability of

corticosteroid formulations containing the same concentration of the same corticosteroid [4, 5] and has, thus, been used extensively in topical corticosteroid bioequivalence testing for regulatory purposes.

Until recently, the assessment of the intensity of corticosteroid-induced skin blanching has been performed visually by one or more trained investigators, using an ordinal data scale. This method has been criticized as the visual scoring results were reported to lack reproducibility, due to the inherent subjectivity of the observers [6]. Recently, it has been shown by Haigh et al. [7, 8] that it is possible to perform the visually assessed skin blanching assay, in such a way that reproducible and consistent results are obtained.

In 1995, the American Food and Drug Administration (FDA) released a Guidance document [6], which attempted to standardize the technique of in vivo topical corticosteroid formulation bioequivalence testing. In this directive it is suggested that, with the availability of increasingly sophisticated and precise analytical methods for the detection of pharmacodynamic responses, the subjective visual assessment of corticosteroid-induced skin blanching is no longer appropriate. As an alternative, the Guidance suggests the use of the Minolta chromameter for the quantification of the intensity of induced vasoconstriction.

Chromameters are compact portable instruments used for the assessment of surface colour based on the tristimulus analysis of a reflected xenon light pulse. In this investigation skin colours were measured using the CIE 1976 ($L*a*b$) system [9], which simulates the sensitivity of the human eye. The L-value is the lightness variable and the a- and b-values are the chromaticity co-ordinates (red-green and blue-yellow, respectively). These three values can be used to define a point in three-dimensional space that characterizes a colour in absolute terms. The magnitude of the difference between two colours, as perceived by the human eye, is proportional to the distance between two points defining those colours in the three-dimensional space. The difference between the two colours, the Euclidean distance (ΔE), is defined by Eq. 1 [9]:

$$\Delta E*_{ab} = \sqrt{(\Delta L*)^2 + (\Delta a*)^2 + (\Delta b*)^2} \quad (1)$$

The aim of the present study was to compare the visual results obtained by three independent observers, with the skin colour measurements performed with two chromameters. Four corticosteroid cream formulations with very similar blanching profiles were chosen in order to investigate the sensitivity of the different assessment methods. According to a previous interlaboratory comparison of the Minolta CR-200 and CR-300 chromameters performed by Fullerton et al. [10], both chromameters showed very good reproducibility, with low coefficients of variation determined on standard colour plates, but precision on human forearm was significantly lower.

2. Materials and methods

Four cream formulations, each containing 0.12% betamethasone 17-valerate were chosen for this investigation: Adco-Betamethasone (Adcock Ingram, South Africa), Betnovate (Glaxo-Wellcome, South Africa), Celestoderm-V (Schering-Plough, South Africa) and Lenovate (Pharmacare-Lennon, South Africa). Two models of Minolta chromameter were used: the

CR-200 (Minolta, USA), and the CR-300 which underwent a standard inspection procedure (Minolta, Switzerland) prior to this investigation.

The methodology of the human skin blanching assay routinely practised in our laboratories [7] was modified to comply with the aims of this study. Twelve healthy male and female, light-skinned volunteers (aged from 18–25 years) who had been pre-screened for positive blanching response were selected. Ethical approval was obtained from the Rhodes University Ethical Standards Committee in compliance with the 1964 Declaration of Helsinki and its subsequent amendments. Written informed consent was obtained from each subject.

2.1. Application of formulation

All volunteers were processed sequentially at 5-min intervals in order to minimize any possible effects of environmental variables, such as temperature and humidity. Six adhesive labels, from which two 7×7 mm squares had been punched, were applied to the flexor aspect of both forearms to demarcate a total of 12 application sites per arm of each volunteer. Each formulation was applied to three of the twelve demarcated sites in a random manner [5] and four adjacent sites were utilized for the unmedicated control. Four different, random application patterns were used such that, in total, each formulation was represented at all sites along the entire length of the forearm; this being essential because of the forearm gradient in the pharmacodynamic response [11]. The formulations were applied by extrusion of four stripes (equivalent to approximately 3.2 mg) from a 1-ml syringe to each designated site in a double blind, randomized manner. The extruded formulations were spread evenly over the application sites using a glass rod, and were covered with a porous Perspex frame to prevent accidental removal of the applied formulations. After a contact time of six h, the protective covers and adhesive labels were removed and each application site was separately washed using cotton-tipped buds and distilled water, and patted dry.

2.2. Visual assessment of blanching response

Visual response assessments were made independently by three experienced observers [5] at 7, 8, 9, 10, 12, 13, 14, 15, 16, 18, 26, 28 and 32 h after product application. Standard overhead fluorescent lighting was used to illuminate the horizontally-placed arms of the volunteers. Responses were graded subjectively by each observer using an ordinal scale where 0= no blanching, 1= slight blanching, 2= more intense blanching, 3= general, even and distinct blanching and 4= marked and very intense blanching with distinct margins on all sides. The percentage of the total possible score (%TPS) was calculated [12] for each formulation and plotted against time in hours after product application, to produce blanching profiles for each individual observer and averaged for the whole data set. The trapezoidal rule was used to calculate the 0–32 h area under the pooled observer blanching curve (AUBC) for each formulation and arm.

2.3. Chromameter assessment of blanching response

The instruments were calibrated using the white calibration plate (CR-A43) immediately before the study. Baseline readings (zero time) were taken at all sites (including the untreated control sites) prior to the application of the formulations. Thereafter, blanching responses at all application sites and at four untreated control sites were assessed at the same times as the visual assessments. Simultaneous readings were taken with both the CR-200 and the CR-300 chromameters (*L*-, *a*-, and *b*-scale parameters) in the same laboratory by two different investigators. The arms of the volunteers were placed horizontally for these measurements,

and special care was taken with the perpendicular placement of the chromameter probe on the forearm. The manipulation of the probe required special attention so as to prevent changes in skin colour due to pressure of the head on the skin [10, 13], and inaccurate alignment of the centre of the reading head over the formulation application site.

At each observation time, the recorded *L*-, *a*- and *b*-values for each medicated and unmedicated site were corrected, by subtracting the baseline (time zero) values to yield baseline-corrected values. In addition, the means of the four (baseline-corrected), unmedicated control site values for each arm at each reading time were further subtracted from the baseline-corrected values for the medicated sites at each observation time, to yield the *Δ*L, *Δ*a and *Δ*b values (Eq. 2) [6]:

$$\text{Delta values}=(M_{t=x}-M_{t=0})-(U_{t=x}-U_{t=0}) \qquad (2)$$

where *M* is the *L*-, *a*-, or *b*-value at a medicated site, *U* is the corresponding average value of four unmedicated sites for that arm and *t* is the observation time (0= premedication, *x* = reading time). This double-correction procedure is only suggested for the *a*-scale data in the FDA Guidance as the *L*- and *b*-scale data are not advocated for use in the bioequivalence assessment procedure.

The mean *ΔL*-, *Δa*- and *Δb*-values of all formulations were calculated and plotted against time. The areas under the *ΔL* and *Δa* curves (AUBC) were calculated for each preparation using the trapezoidal rule from 0 to 32 h. The *b*-scale data are not presented since there is negligible change in the *b*-scale values with increased blanching. From the mean *ΔL*, *Δa* and *Δb* values, the absolute colour differences (*ΔE*) were calculated for each formulation according to Eq. 1 and compared with the %TPS values from the visual assessment.

2.4. Statistical analysis of AUBC data

The AUBC values obtained from the left and right arms were averaged to obtain one value for each of the 12 volunteers. Locke's method was used to calculate confidence intervals from visual and chromameter AUBC data according to the directive of the FDA Guidance [6, 14]. AUBC data sets (*n*=12) for two formulations (reference (*R*) and test (*T*) formulation) were compared using Eq. 3 to obtain an exact confidence interval:

$$CI = \frac{\left(\frac{\overline{X}_T}{\overline{X}_R} - G\frac{\hat{\sigma}_{TR}}{\hat{\sigma}_{RR}}\right) \mp \frac{t}{\overline{X}_R}\sqrt{\frac{\hat{\sigma}_{RR}}{n}K}}{1-G} \qquad (3)$$

where *t* =2.2010 for the 97.5th percentile (95% confidence interval) of the *t*-distribution for 11 ($n-1$) degrees of freedom [15], and *G* is required to be <1. $\overline{X}_T$, $\overline{X}_R$ are sample means and $\hat{\sigma}_{RR}$, $\hat{\sigma}_{TT}$ are sample variances of Test and Reference Product, respectively, $\hat{\sigma}_{TR}$ is the sample covariance.

$$G = \frac{t^2\hat{\sigma}_{RR}}{n\overline{X}_R^2} \qquad (3a)$$

$$K = \left(\frac{\overline{X}_T}{\overline{X}_R}\right)^2 + \frac{\hat{\sigma}_{TT}}{\hat{\sigma}_{RR}}(1-G) + \frac{\hat{\sigma}_{TR}}{\hat{\sigma}_{RR}}\left(G\frac{\hat{\sigma}_{TR}}{\hat{\sigma}_{RR}} - 2\frac{\overline{X}_T}{\overline{X}_R}\right) \qquad (3b)$$

To investigate the relationship between visual and chromameter data, all the 48 AUBC values from all the formulations in all volunteers (4 formulations×12 volunteers) obtained by one of the assessment methods (visual, CR-200, or CR-300) were related to another AUBC value data set obtained by a different assessment method. The correlation coefficients of the regression lines were further transformed according to Eq. 4, to obtain normalized data [16].

$$z = \frac{1}{2}\ln\frac{1+r}{1-r} \quad (4)$$

significant correlation was found and the null hypothesis ρ=0 was rejected when z was outside the 95% confidence interval of 0±0.29217 [16]. Theoretically, there should be a linear relationship between the results obtained using the two different chromameters, i.e. the regression line should have a slope of unity and an intercept at the origin. A regression analysis was performed to investigate the linear association of the Δa-scale AUBCs produced from the CR-200 and CR-300 data.

3. Results and discussion

Fig. 1 depicts the mean results of the visually-assessed blanching profiles for the three observers and four cream formulations. To maintain clarity, only a few representative error bars are shown in Fig. 1, Fig. 2 and Fig. 3. Table 1 lists the areas under the visual blanching curves scored by the individual observers and the correlations between the results of different observers. The same rank order was observed for the results of all observers and the inter-individual precision of the visual blanching assessments, as determined by the correlation of the AUBC values obtained by each individual observer, was excellent.

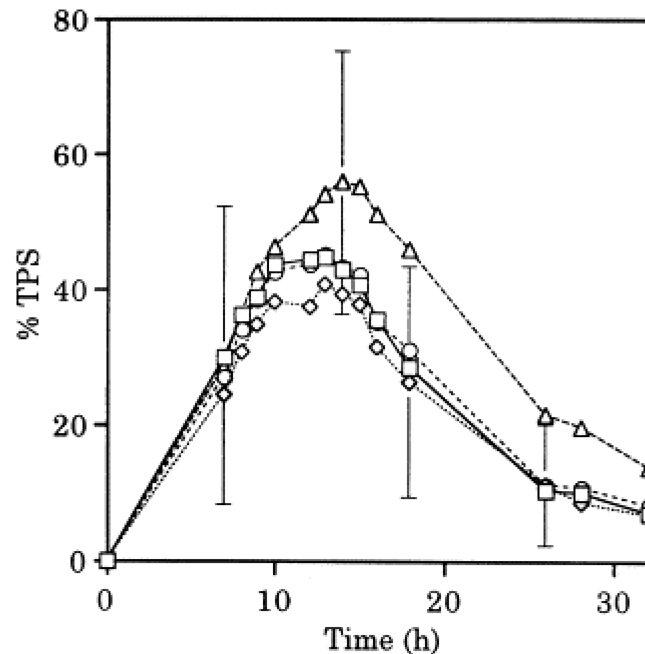
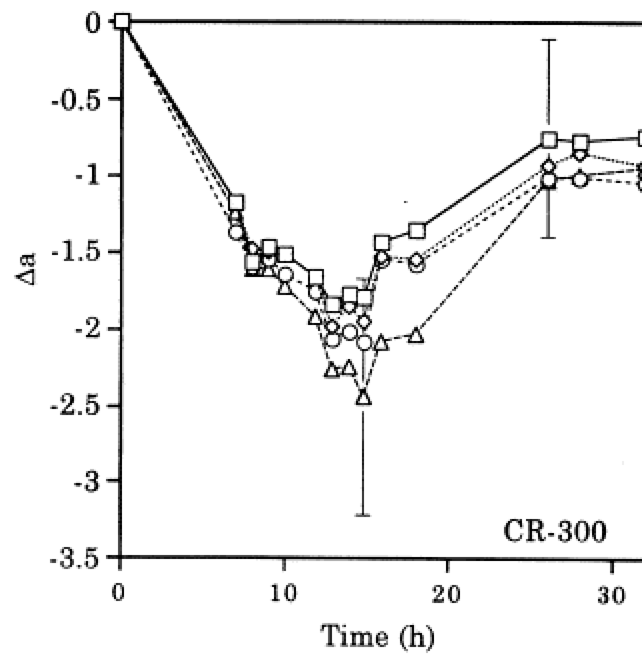


Fig. 1. Visually assessed blanching profiles of Adco-Betamethasone (circle), Betnovate (square), Celestoderm-V (rhombus) and Lenovate (triangle), obtained by the pool of three observers. Some representative error bars (standard deviation) are given.



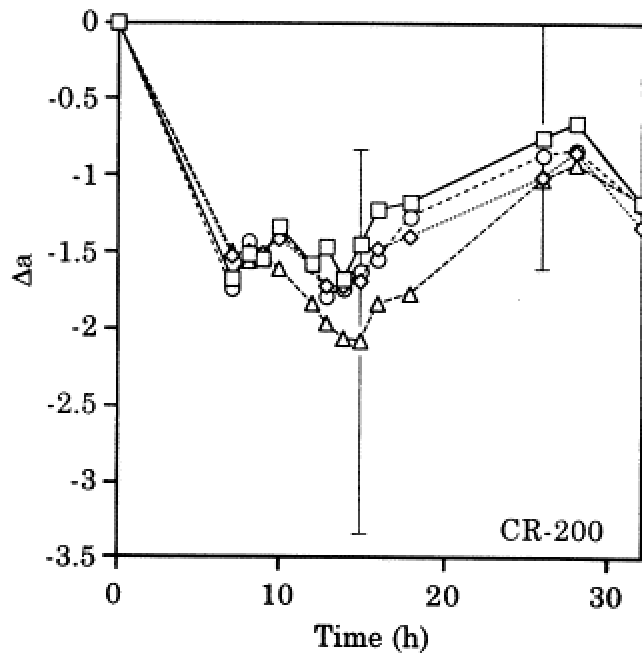
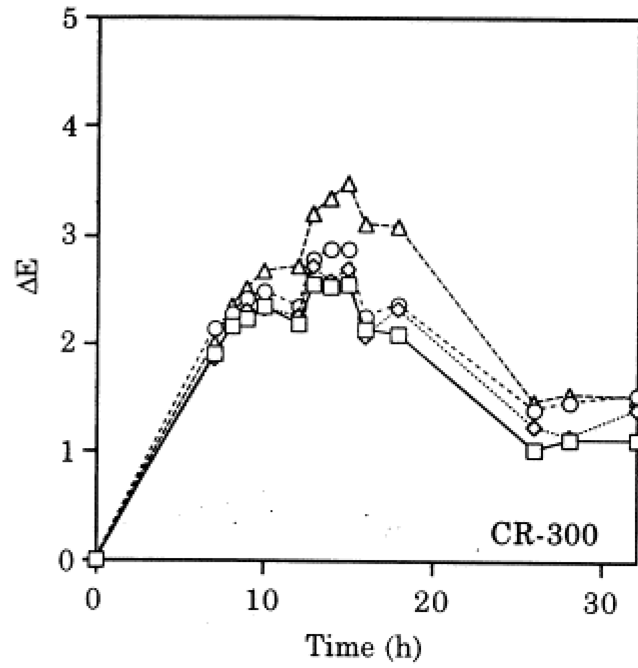


Fig. 2. Blanching profiles of chromameter results: *Δa*-scale values for Adco-Betamethasone (circle), Betnovate (square), Celestoderm-V (rhombus) and Lenovate (triangle). Some representative error bars (standard deviation) are given.



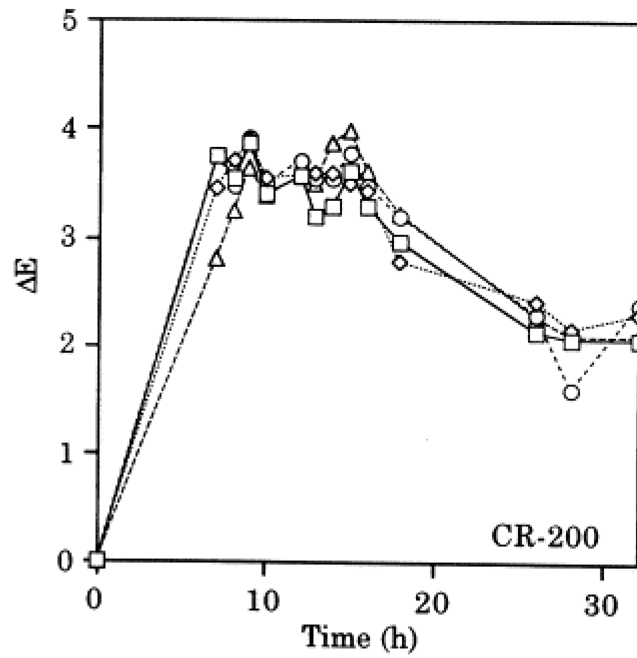


Fig. 3. Blanching profiles of chromameter results: absolute colour difference *ΔE*-values for Adco-Betamethasone (circle), Betnovate (square), Celestoderm-V (rhombus) and Lenovate (triangle).

Table 1. Areas under the visual blanching curves (AUBC) for each observer, and coefficient of AUBC-correlation between observers

AUBC values for	Observer 1	Observer 2	Observer 3
Betnovate	794	603	855
Celestoderm-V	688	560	766
Adco-Betamethasone	793	616	860
Lenovate	1064	836	1123
Coefficients of correlation			
Observer 1 vs. observer 2	r=0.991		
Observer 1 vs. observer 3	r=0.999		
Observer 2 vs. observer 3	r=0.995		

The *Δa*-scale values determined using the CR-200 and CR-300 (Fig. 2) were plotted against time as recommended by the FDA guidance [6]. The total absolute colour differences between application sites at each observation time are depicted in Fig. 3 as Euclidean distances versus time. Table 2 lists the mean areas under the visually-assessed blanching response curves and under the *ΔL*, *Δa* and *ΔE* curves.

Table 2. AUBC values from visual assessment (observer-pool) and chromameter CR-200 and CR-300 *ΔL*, *Δa*, and *ΔE*-data

	Visual	CR-300			CR-200		
		ΔL	Δa	ΔE	ΔL	Δa	ΔE
Betnovate	751	36.75	−34.47	65.54	74.95	− 34.52	83.85
Celestoderm-V	671	37.68	− 37.45	65.88	74.28	− 38.38	85.06
Adco-Betamethasone	756	42.05	− 40.51	70.34	77.01	− 37.44	86.43
Lenovate	1008	48.51	− 44.04	76.68	70.62	− 42.36	83.21

Compared with the visual graph, the curves of the *Δa* values obtained with both chromameters show similar shapes. In each of these graphs, the plots of Betnovate, Celestoderm-V and Adco-Betamethasone are almost identical, whereas the plot of Lenovate shows a larger AUBC. The areas under the *Δa* curve are comparable for both measuring devices, whereas the areas under the *ΔL* curves (data not shown) obtained by the CR-200 chromameter were larger by a factor of 1.9 compared with the CR-300, and no significant differences between the products were detected. As can be seen from Fig. 3, the graph obtained using the CR-300 measurements, which represents the Euclidean distances between the baseline-adjusted medicated and baseline-adjusted unmedicated sites, correlated with the visual data, and the comparison resulted in a correlation coefficient of 0.714. The divergent values of the CR-200 *L*-scale measurements, therefore, influenced the respective *ΔE*-curve.

Reasonable correlation was observed between visual and *Δa* AUBC values (r=−0.799 for CR-300 and r=−0.716 for CR-200) and visual and *ΔL* AUBC values (r=0.683 for CR-300 and r=0.584 for CR-200). Correlation within the same colour coordinate AUBC values determined by the two different devices were r=0.579 for *Δa* AUBC values and r=0.375 for *ΔL* AUBC values. Regression analysis showed that there were no direct linear relations between the measurements obtained by utilization of the two different chromameters. For the

Δa AUBC values, the slope of the regression line was 0.387 (0.225 − 0.549; 95% CI) and the intercept was −24.3 (−31.8 − −16.8; 95% CI). In case of ΔL AUBC values a slope of 0.278 (0.074 − 0.482; 95% CI) and an intercept of 20.6 (4.3 − 36.9; 95% CI) was obtained.

Table 3 lists the percentage ratios of the pharmacodynamic blanching responses of the superior Lenovate cream compared with the three other formulations. The visually-assessed AUBC for Lenovate was significantly higher than those of all the other products.

Table 3. Ratio of AUBCs between formulations in percent (and range of 95% CI calculated according to Locke's method is given)

Comparison	Lenovate/Betnovate (%)	Lenovate/Celestoderm (%)	Lenovate/Adco-Betamethasone (%)
Visual AUBC	134.3*	150.1*	133.2*
	(113.3 – 170.5)	(121.4 – 206.9)	(114.3 – 169.3)
Δa-AUBC CR-200	122.7*	110.4%	113.2*
	(103.8 – 160.4)	(93.2 – 160.6)	(102.1 – 134.4)
Δa-AUBC CR-300	127.8*	117.6*	108.7
	(107.5 – 160.2)	(108.4 – 131.9)	(94.6 – 126.3)
ΔL-AUBC CR-300	132.0*	128.8*	115.4
	(110.2 – 164.8)	(104.7 – 171.7)	(96.7 – 148.3)
ΔE-AUBC CR-300	117.0*	116.4*	109.0
	(105.4 – 131.6)	(106.2 – 127.5)	(97.5 – 126.0)

*Significant differences.

The results obtained with the two models of Minolta chromameter demonstrated an acceptable similarity. Whereas Lenovate cream showed superiority over Adco-Betamethasone cream in both chromameter assessments, the superiority over Celestoderm-V and Adco-Betamethasone was lesser and close to the borderline of significance in both cases. It should be stressed that in the case of the visual assessment, the blanching reaction was compared with the skin surrounding the application site, whereas with the chromameter, blanching was compared with the skin colour at adjacent unmedicated sites, although one would not expect this to make appreciable difference to the results.

These results further confirm previous observations that the chromameter is better at distinguishing between markedly different blanching intensities, and produces superior results when the blanching is intense. Discrimination is poorer when products that are only marginally different are compared and measurement sensitivity is lower compared with visual assessment. This poses a problem for bioequivalence assessments, where formulations that produce only subtle differences in skin blanching are compared.

4. Conclusion

A high interinstrumental reproducibility between both models of chromameters has been reported [10]. However, the reproducibility of readings from in vivo forearm skin was not as good as that from a standard colour plate. The lower reproducibility on human skin is considered to be evoked by physiological fluctuations of forearm skin colour and investigator related factors, such as the perpendicular alignment and the application force of the measuring head on the skin sites. The results obtained in this study with the CR-300 chromameter showed better correlation with the visually-assessed response and are more consistent than those obtained with the CR-200. This difference might be explained rather by the fact that the chromameters were operated by two individuals, than by difference of the two chromameter models. Since the measuring head alignment, contact pressure and

positioning over the reading site are techniques which are operator dependent; there is obviously some subjectivity in the results obtained. This operator dependency can be responsible for any interlaboratory variability of chromameter results, which might occur.

Our results illustrate that, even though the FDA [6] considers the chromameter as an ‘objective', quantifiable measuring device compared with the ‘subjective' visual scoring, the measurements obtained by the chromameter are operator dependent and are, therefore, also subjective. The operator requires extensive training and experience before reliable and reproducible results can be produced. In addition, personal experience has suggested that self-assessment with the chromameter may produce superior results compared with operator-assessment, since the alignment, positioning and surface pressure can be judged more precisely on one's own arm than on the arm of another subject.

Although no linear relationship was detected between the AUBC values of the CR-200 and CR-300 chromameter results, acceptable reproducibility was achieved by the two measuring devices. However, care must be taken when the chromameter is used in a human skin blanching trial as it has been observed that the results are not completely objective, and the overall precision of the instrumental method must be considered when very similar formulations are compared. It seems obvious, therefore, that before a chromameter is utilized in this way, a validation procedure should be performed to ensure the reliability of results in any bioequivalence study.

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