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In vitro and *in vivo* study of the substantivity of p-amino benzoic acid and two of its esters

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Synopsis

The substantivity of p-amino benzoic acid (PABA) and two of its esters (Escalol 507® and Amerscreen "P"®), in an alcoholic vehicle, has been studied both *in vitro* and *in vivo* in order to establish the possible correlation between the two methods.

Eight healthy volunteers participated in the *in vivo* method. Solutions of the sunscreen substances were prepared in isopropanol (IPA): PABA at 5%, and Escalol 507® and Amerscreen "P"®, both at 2%. In order to study the substantivity of each of the sunscreen substances to the skin, the subjects placed their hands in the solutions. The hands were then rinsed with water and extracted with IPA at 50°C to quantitate the amount of substance retained by the skin. A crossover design was used; all the subjects participated in experiments with each of the substances at one week intervals.

In the *in vitro* experiment, keratin powder obtained from hard, insensible tissues (human calluses of the feet) was used. The keratin was kept in an ethanol solution of the sunscreen for 72 hr, then treated with water for 48 hr, and finally extracted with ethanol at 50°C for 24 hr in order to determine the amount of sunscreen retained by the keratin.

The sunscreen concentrations in the different solutions in both *in vitro* and *in vivo* methods were determined by spectrophotometry.

The *in vivo* percent substantivities (i.e. percentage of sunscreen deposited which was not rinsed off by water) obtained in the study were: PABA = 0.30 ± 0.05; Escalol 507® = 57.96 ± 0.63; Amerscreen "P"® = 0.46 ± 0.08. In the *in vitro* method the following values were obtained: PABA = 0.30 ± 0.02; Escalol 507® = 50.84 ± 0.66; Amerscreen "P"® = 0.47 ± 0.03. The differences are statistically significant ($p < 0.01$) and Escalol 507® is the substance that shows this property in the highest degree.

The results obtained by the *in vitro* and *in vivo* methods were linearly correlated.

INTRODUCTION

The function of a sunscreen preparation is to absorb as completely as possible the erythemic ultraviolet radiation of sunlight while transmitting a maximum of the other wavelengths. During the last few years, several researchers have focused their attention on the loss of efficacy of sunscreens due to their removal from the skin by perspiration and/or swimming (1,14). The term substantivity is currently used to express the ca-

pacity of a compound to adhere or combine with a keratinized substrate (2). In general it is accepted that a sunscreen preparation must have some degree of substantivity.

Our literature search has failed to reveal studies oriented toward establishing standard methods of evaluating in the laboratory the ability of a U.V. absorber to resist being removed by water. Some studies of affinities of sunscreens for human skin *in vivo* and *in vitro* (3,4) or animal skin *in vitro* (5) have been reported. Nevertheless, very little is known about the significance of the *in vitro* methods and their capability to predict the behaviors of the compounds in human skin. An *in vitro* method that correlates with actual *in vivo* application on human skin is desirable to avoid the inconveniences and limitations of human experimentation.

In our work we have studied the substantivities of p-amino benzoic acid (PABA) and two of its esters (Escalol 507® and Amerscreen "P"®), using both an *in vivo* and *in vitro* method in order to find a correlation between the two methods.

Healthy volunteers participated in the *in vivo* method, thus studying the substantivity of the sunscreen compounds under conditions which were near to actual use conditions. Human keratin in the form of excised human callus tissue was employed in the *in vitro* method. The results in terms of percentage of substantivity obtained by the two methods were compared and a very strong linear correlation was found.

EXPERIMENTAL DESIGN

MATERIALS

The test materials were: p-amino benzoic acid, analytical reagent grade (USP); octyl dimethyl (Escalol 507®, Van Dyk & Co., Inc.); and ethyl dihydroxypropyl (Amerscreen "P"®, Amerchol Corporation) p-aminobenzoate; and ethanol 95% and 2-propanol (E. Merck, Darmstadt, W. Germany). The human keratin was obtained from human foot callus tissue which had not been treated with chemical agents prior to removal. The callus tissue was pulvarized while suspended in acetone refrigerated below 0°C with solid CO_2 using a Waring Blender®. After the comminution procedure, the material was washed with ether and allowed to dry (6). After pulverization the keratin was sieved before use, utilizing the fraction that passed through a 70-mesh sieve but which was retained by a 100-mesh sieve. The mean particle size of the powder was 150 μ.

DETERMINATION OF SUBSTANTIVITY *IN VIVO*

Eight healthy volunteers, one male and seven females, ranging in age from 20 to 30 yr (mean 24 yr) and in weight from 45 to 70 kg (mean = 60 kg), with no external signs of allergy or other skin disease, participated in this study.

The method previously described by Cumpelik (4) was employed, utilizing 2% w/v solutions of Escalol 507® and Amerscreen "P"® in isopropanol (IPA). A 5% PABA solution in IPA was used for comparison. Hands and arms of all subjects were washed up to the elbows with soap and tapwater, dried, and then washed in IPA at 50°C repeatedly until 100% transmittance of the washings was obtained between 240 and 400 nm, using a Carl Zeiss spectrophotometer. Hands were air dried.

Following the cleaning procedure, all the subjects had their right hand dipped into the PABA solution for 2 min. For comparison the left hand of each subject was dipped

into a comparison sunscreen solution. The amount of each sunscreen deposited on each subject's skin was obtained by weighting the solutions before and after the introduction of the hands. Hands were allowed to air dry for 35 min and then submerged in distilled water at 25°C for 30 min. Hands were again air dried and, finally, dipped into IPA at 50°C for 2 min. Aliquots of the IPA solution were read by spectrophotometer, thus obtaining the amount of screen remaining on the skin after the water treatment. The substantivity of the screen was estimated using the following equation:

$$S\% = \frac{\text{amount of screen (mg) recovered} \times 100}{\text{amount of screen (mg) deposited initially on the skin}}$$

In the spectrophotometric analysis PABA was read at 289 nm and both Escalol 507® and Amerscreen "P"® at 310 nm using a Carl Zeiss M4 Q III spectrophotometer.

DETERMINATION OF SUBSTANTIVITY *IN VITRO*

Experiments were performed utilizing human keratin and ethanol as solvent because preliminary experiments proved that there were no differences in the results when using either IPA or ethanol.

The concentration ranges for each sunscreen were established between those at which the affinity of the material for the skin remains constant, determining in each case the sorption isotherm. The values obtained for the three substances were: PABA, 80-115 mg%; Escalol 507®, 80-120 mg%; and Amerscreen "P"®, 80-120 mg%.

Once the concentration range was selected, the substantivity was determined using the procedure described by Bottari *et al.* (1), modified by us as follows:

A 100-mesh stainless steel basket containing 0.1 g of human keratin powder was placed in a flask with 20 ml of water and held 40 hr in a thermostatically controlled shaker bath, to allow hydration of the keratin. Hydration of the keratin previous to the treatment with the sunscreen was necessary. Experiments performed without previous hydration failed. The material was then allowed to dry at 20°C for 30 min.

The following sunscreen solutions in ethanol were prepared: PABA, 85-90-105 mg%; Escalol 507®, 100-110-120 mg%; and Amerscreen "P"®, 80-110-120 mg%.

Twenty ml of sunscreen solution were placed in a glass flask and the stainless steel basket containing the previously hydrated keratin was then added. The flask was stoppered, sealed, and held for 72 hr in a thermostatically controlled shaker bath at 37°C. The solution was then filtered using a millipore H.A.-type filter and and assayed by spectrophotometer. The basket containing the keratin was then allowed to dry at 30°C for 30 min.

After drying, the basket with the keratin was placed in a glass flask containing twenty ml of distilled water. The stoppered and sealed flask was held for 48 hr in a thermostatically controlled (25 ± 1°C) bath. At the end of this time the basket was taken from the solution and allowed to dry for 60 min at 30°C.

Finally, the basket containing the keratin was placed into a glass flask with twenty ml of ethanol. The flask was stoppered, sealed, and shaken in a constant temperature bath at 50 ± 1°C for 24 hr. The resultant solution was filtered and assayed by spectrophotometer.

The K values for the sorption isotherms at 37°C were calculated according to Bottari (1) using the following equation:

$$K = \frac{Ck}{Cs}$$

where Cs is the equilibrium concentration of the sunscreen in the solvent and Ck is the concentration of the sunscreen in the keratin substrate.

The percentage of substantivity was calculated as indicated for the *in vivo* method.

STATISTICAL ANALYSIS

The differences in substantivity obtained in the *in vivo* method were examined using an analysis of variance (ANOVA) (7) and the test of Dunnett (8).

RESULTS

Table I shows the percentages of substantivity obtained for the three sunscreens in the *in vivo* studies. Tables II and III contain the ANOVA of the data and application of the Dunnett's significant differences test, respectively. Table IV shows the mean K values for different concentrations of PABA, Escalol 507®, and Amerscreen "P"®. In Table V a comparison of the solubilities in water and percentages of substantivity of PABA and its esters determined by the *in vivo* method can be seen. Table VI shows the percentages of substantivity obtained for the three sunscreens by the *in vitro* method.

It can be seen that there are very significant differences between the substantivity found for Escalol 507® and those obtained for the other two substances but that there are no significant differences between PABA and Amerscreen "P"®.

DISCUSSION

The mechanism of the interaction of the sunscreen substances and the skin have not been established clearly. It is known that there are several factors involved. Sayre *et al.*

Table I
Substantivities of PABA and Its Esters Determined by the *In Vivo* Method

	Substantivity (%)		
Subject #	PABA	Escalol 507®	Amerscreen "P"®
1	0.29	57.5	0.58
2	0.28	58.2	0.39
3	0.32	58.5	0.46
4	0.30	57.4	0.41
5	0.27	57.5	0.56
6	0.32	57.2	0.46
7	0.36	58.6	0.46
8	0.29	58.8	0.37
Mean ± SD	0.30 ± 0.03	57.96 ± 0.63	0.46 ± 0.08

Table II
Analysis of Variance (ANOVA) Determined From the % Substantivity of PABA and Its Esters in the *In Vivo* Method [$F = 5.78$ ($p < 0.01$)]

Sum of Squares	Degrees of Freedom	Mean Squares	F
SS Explained	k − 1	8841.24	
17682.48	2		
SS Error	N − k	0.123	65490
2.84	21		
SS Total	N − 1		
17685.32	23		

Table III
Experimental 't' Values From the Dunnett Test for the Substantivity of PABA and Its Esters in the *In Vivo* Method [$t = 0.573$ ($p < 0.01$)]

	PABA vs Escalol® 507	PABA vs Amerscreen "P"®	Escalol 507® vs Amerscreen "P"®
t	57.66	0.16	57.5

Table IV
Mean K Values for Different Concentrations of PABA, Escalol 507®, and Amerscreen "P"® in Ethanol

PABA (mg%)	K	Escalol 507® (mg%)	K	Amerscreen "P"® (mg%)	K
80	18.35 ± 5.04	80	7.38 ± 2.15	80	17.84 ± 0.62
85	19.31 ± 2.35	90	8.01 ± 1.45	90	17.14 ± 0.69
90	19.50 ± 2.76	100	7.01 ± 0.64	100	17.38 ± 0.72
105	19.50 ± 2.59	110	7.09 ± 0.55	110	18.08 ± 1.41
115	18.10 ± 3.31	120	7.14 ± 0.51	120	18.18 ± 1.67

Table V
Comparison of the Solubilities in Water and Substantivities of PABA and Its Esters

Sunscreen	Solubility (mg/ml)	Substantivity (%)
PABA	4.7	0.30
Amerscreen "P"®	3.9	0.46
Escalol 507®	1.6×10^{-3}	57.96

(9) have pointed out that both the chemical characteristics of the sunscreen and the vehicle are important.

Other researchers have made clear the influence of the duration of the contact of the sunscreen and the skin before washing. It has been proposed that 15 min of contact allow the sunscreen to penetrate the horny layer, thus increasing the resistance to wash-off (10). It has also been suggested that some sunscreens need an optimum time to penetrate the stratum corneum (4). Whether substantivity confers detrimental or ben-

Table VI
Substantivities of PABA, and Its Esters Determined by the *In Vivo* Method, for Different Concentrations of Each Sunscreen

	PABA		Escalol 507®		Amerscreen "P"®	
	Concentration (mg%)	Substantivity (%)	Concentration (mg%)	Substantivity (%)	Concentration (mg%)	Substantivity (%)
	85	0.33 ± 0.05	100	50.08 ± 2.16	80	0.44 ± 0.04
	90	0.29 ± 0.04	110	51.20 ± 3.33	110	0.49 ± 0.04
	105	0.30 ± 0.04	120	51.23 ± 2.13	120	0.49 ± 0.05
Mean ± SD		0.30 ± 0.02		50.84 ± 0.66		0.47 ± 0.03

eficial properties to a product is a question still open to discussion (1). An excessive substantivity might result in irreversible binding to constituents of living tissues, thus introducing a distinct risk of toxicity (11). On the other hand, if substantivity is accepted as arising from adsorption of the molecule on active sites of the skin barrier, a high substantivity might induce a delayed percutaneous absorption, resulting possibly in a reduced systemic toxicity (1).

The solubility in water of the sunscreen has been recognized as a major determinant in establishing its retention on the skin after sweating or swimming (4). The results of the present study support this conclusion. Inspection of the values reported in Table V reveals that the substantivities of the three agents studied increase with their decreasing the water solubility. These results are in agreement with those reported by Cumpelik (4).

The sorption isotherms of the three sunscreens under study in ethanol were in all cases linear, having the origin as intercept. The straight lines did not show a final inflection or plateau within the concentration range studied. This would indicate a constant partition of the solute between substrate and solution, analogous to the partitioning between two immiscible solvents, and corresponds to a type C isotherm. In such a linear plot, K corresponds to the slope of the isotherm. Table IV shows the K values for the three sunscreen agents at the different concentrations studied.

These values represent the partition coefficients of the sunscreen substances between the keratin and the solvents and indicate that K PABA > K Amerscreen "P"® > K Escalol 507®. Thus PABA is shown to have the highest interaction with keratin when the solvent used is alcohol and is followed by Amerscreen "P"® and Escalol 507® in decreasing order, which is in conformity with the solubility of the substances in ethanol, i.e. Escalol 507® > Amerscreen "P"® > PABA.

The substantivies of the three sunscreens were determined at three concentrations; these concentrations were selected at levels where the affinities of the three substances for the keratin (K values) were constants (Table VI).

Hydration favors the adherence of the sunscreen to the keratin, which results in higher K values. Thus, vehicles that modify the skin hydration may affect its affinity for a

compound. It has been found that an increased hydration of the keratin molecules parallels an increased affinity for various substances (12). Apparently hydration causes unfolding of the keratin molecules, thus exposing more binding sites (1,12). In addition, according to Scheuplein (13), it seems likely that the swelling and softening of the keratin filaments in water is accompanied by a partial dissolution of the cell membranes which open larger holes, which thus facilitates diffusion.

Table VI contains the percentage of substantivity obtained in the *in vitro* studies. The results are almost identical to those determined using the *in vivo* method, as can be seen in Figure 1, where a comparison is made of the substativities of the three sunscreen substances obtained by the two methods.

It seems desirable to establish a laboratory method for evaluating the relative resistance of sunscreen products to wash off, as a preliminary "screen" prior to testing on humans under actual use conditions. The static water bath immersion procedure carried out by Cumpelik (4) is one such method which, however, has all the limitations and problems

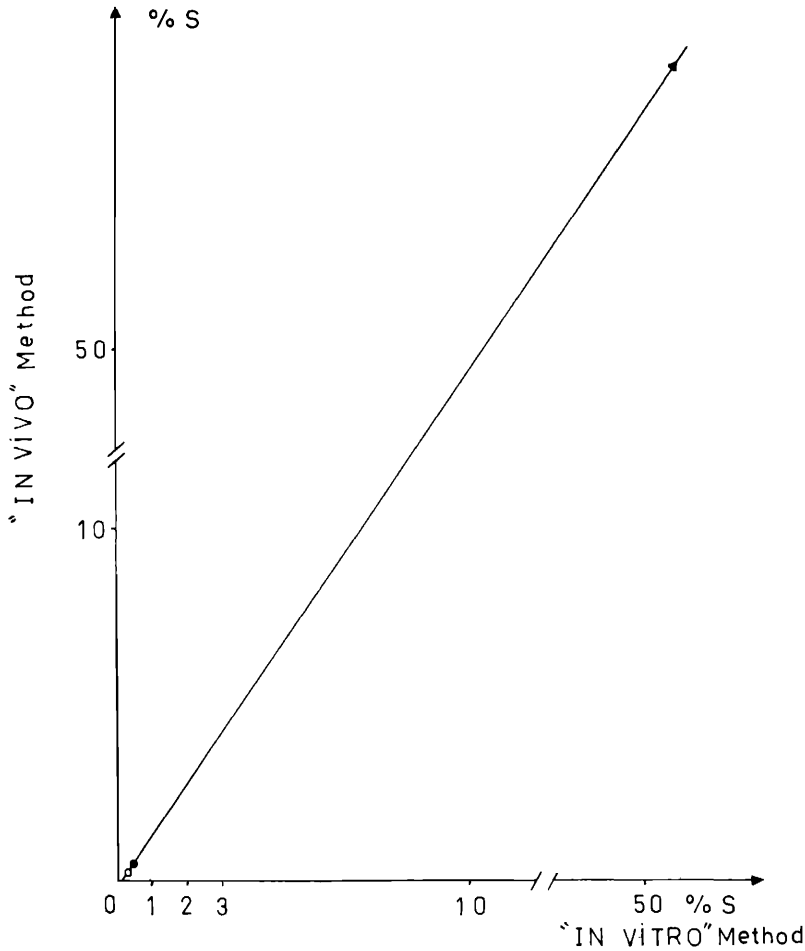

Figure 1. Comparison of substantivities of sunscreens obtained by *in vitro* and *in vivo* methods. ○ = PABA, ● = Amerscreen "P"®, ▲ = Escalol 507®.

involving experimentation with humans. Our finding that the *in vitro* method shows good correlation with the water bath immersion procedure indicates that probably a method like the one using keratin could, after further experimentation, be developed as a preliminary laboratory screen for evaluating the water resistance of sunscreen products.

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