Value of Repeated Tests in a Percutaneous Absorption Study

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Abstract □ Percutaneous absorption of salicylic acid was measured by blood levels in 20 rabbits, each given four runs at 1-week intervals. Dimethyl sulfoxide was added to the ointment in two runs (Runs 1 and 3 for half the animals, Runs 2 and 4 for the others), thus forming a crossover design with one repetition of each condition. The four successive runs caused a progressive and statistically, highly reliable decrease in percutaneous absorption. This effect of prior exposure increased during successive 1.5-hr. time intervals, from 1.5 to 7.5 hr., and showed no reliable interaction with dimethyl sulfoxide and with pH. A large effect of prior exposure was the increased stability of the salicylate absorption scores and, thereby, greater reliability of the experimental effects, shown by much lower residual terms in the analysis of variance for Runs 3 and 4 than for 1 and 2. Skin dehydration and decreased emotional arousal are possible explanations for the slower and more consistent rate of percutaneous absorption after prior exposure to the test situation.

Keyphrases □ Absorption, percutaneous—salicylate in hydrophilic ointment, effect of pH, dimethyl sulfoxide, prior exposure □ pH effect—salicylate absorption from hydrophilic ointment □ Dimethyl sulfoxide effect—salicylate absorption from hydrophilic ointment □ Salicylate absorption—hydrophilic ointment

Experimenters often may choose between two types of procedure: a single measurement or repeated measurements for each subject. The single measurement is a simpler design and a briefer procedure, but it requires a separate group of subjects for each experimental condition. Repeated measurements of each subject under the same condition may provide more stable, accurate data; exposing each subject to different conditions (crossover design) can yield more reliable results with the use of a smaller number of subjects than when a different group of subjects is used for each experimental condition. A balanced crossover procedure, equalizing the number of subjects tested first with each experimental condition, permits analysis of any possible effects of the preceding tests in the subsequent measurements.

In percutaneous absorption studies using rabbits, Stolar *et al.* (1) tested each animal only once, whereas Stelzer *et al.* (2) and Marcus *et al.* (3) tested each animal four times, twice under each of two experimental conditions. In accordance with the purpose of the study, they reported the average data for all the tests, without evaluating the effects of the test sequence on percutaneous absorption. The present paper reports analysis of the data reported by Marcus *et al.* (3) in order to determine effects of the series of four tests and to evaluate the procedure of repeated tests in this type of study.

METHODS

As described in a previous article (3), percutaneous absorption of salicylic acid in a modified hydrophilic ointment was evaluated in each test run by determining salicylic acid blood levels at five time intervals (1.5, 3.0, 4.5, 6.0, and 7.5 hr.). Four rabbits, tested simultaneously, were each given four runs at intervals of 1 week. Dimethyl sulfoxide was incorporated into the ointment applied to two rabbits in Runs 1 and 3 and into the ointment applied to the other two rabbits in Runs 2 and 4. Therefore, each rabbit was given two tests under both conditions, with two rabbits having the identical sequence of conditions. A separate group of four rabbits was used for testing each of five pH levels of the ointment (2.97, 4.48, 6.80, 9.23, and 10.78).

In the previous article (3), the data were evaluated by analysis of variance in which the four runs for each rabbit and the different sequences of dimethyl sulfoxide treatment (Runs 1 and 3 or 2 and 4) were averaged together. The present paper presents additional analyses, measuring the effects of different tests and sequences of dimethyl sulfoxide treatment. As in the previous article (3), the residual term for testing effects of the experimental variables was based on the two rabbits tested under the same conditions, using appropriate interactions of this replication term with other variables (4). In tests combining the pH levels, the lowest pH of 2.97 is excluded because data on the last two runs were missing for two of the four rabbits. In comparisons among the different pH levels, the lowest pH included only the two rabbits for which data were obtained from all four runs.

RESULTS

The average level of salicylic acid in the blood at each of the five time intervals from 1.5 to 7.5 hr. is shown in Fig. 1, separately for each of the four runs. This figure combines all four pH levels and also the runs with and without dimethyl sulfoxide treatment. An analysis of variance, using as the residual term the pooled interactions of runs with replications, revealed a statistically reliable difference among the four runs ($F = 4.99$, $df = 3/24$, $p < 0.01$). The average salicylic acid level, averaging all time periods, decreased progressively (12.70 mg.% on Run 1, 11.96 on Run 2, 11.37 on Run 3, and 10.07 on Run 4). The linear function for the difference among runs was statistically highly reliable ($F = 14.52$, $df = 1/24$, $p < 0.001$). Figure 1 also indicates a differential effect of runs at the different time intervals after application of the ointment. The decrease in salicylic acid blood level from the first to the fourth run was smallest at 1.5 hr. and largest at 7.5 hr. A statistically reliable interaction was found for the linear function of runs and time intervals ($F = 5.09$, $df = 1/96$, $p < 0.05$).

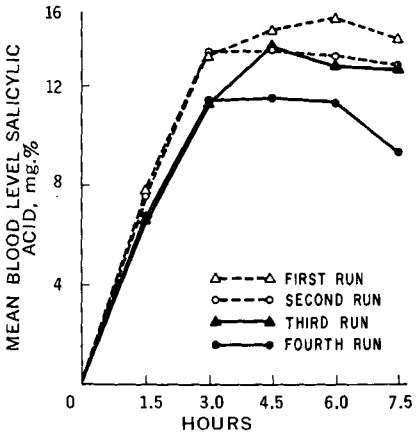

Figure 1—*Mean blood levels of salicylic acid at each time interval for 16 rabbits (four at each of four pH levels, 3.0–10.8). In each of the four weekly runs, half the animals were without dimethyl sulfoxide and the others were with dimethyl sulfoxide.*

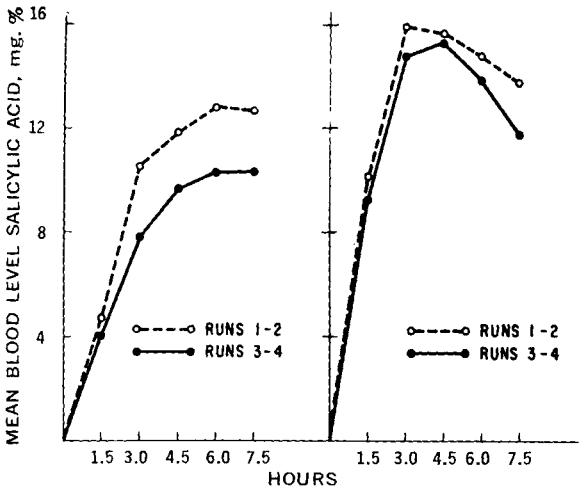

Figure 2—*Mean blood levels of salicylic acid at each time interval for 16 rabbits (four at each of four pH levels, 3.0–10.8), showing the first and second runs without dimethyl sulfoxide (left-hand graph) and with dimethyl sulfoxide (right-hand graph).*

Figure 2 shows the effect of the dimethyl sulfoxide treatment. Runs 1 and 2, and Runs 3 and 4, are combined because the first test under the specified treatment is the first run for half the animals and the second run for the other half and, likewise, the second test is the third run for half the animals and the fourth for the other half.

Table I summarizes the analysis of variance for these data. Blood salicylic acid levels were consistently lower in Runs 3 and 4 than in 1 and 2, and the difference was statistically highly reliable. The dimethyl sulfoxide treatment greatly enhanced absorption of salicylic acid, and this effect of dimethyl sulfoxide was greater at the earlier time intervals, as reported previously (3). Figure 2 shows a tendency for the difference of Runs 1 and 2 from Runs 3 and 4 to be greater in the later time intervals and without dimethyl sulfoxide, but these trends were short of statistical significance.

The effects of the different pH values are shown in Fig. 3, using for each run the time interval when the animal's blood salicylic acid level was highest. The analysis of variance (Table I) omits the lowest pH (2.97) because the data on Runs 3 and 4 were missing for two of the four animals. Increases in the four remaining pH levels resulted in a progressive increase in blood salicylic acid levels, and the linear trend was statistically reliable ($F = 11.30$, $df = 1/7$, $p < 0.05$). However, the effect of dimethyl sulfoxide in enhancing absorption tended to decrease progressively with increasing pH values, as reported previously (3). Table I shows that the interaction between these two variables, omitting the lowest pH of 2.97, was statistically significant. The dimethyl sulfoxide treatment did not reliably alter the effect of runs, but Fig. 2 suggests a tendency for the decrease in salicylic acid levels in Runs 3 and 4 to be larger without the dimethyl sulfoxide treatment.

Table II summarizes analyses of variance separately for Runs 1 and 2 and Runs 3 and 4. The main difference between these pairs of runs is that the residual values are consistently lower in Runs 3 and 4 than in 1 and 2, indicating greater stability and consistency of the blood salicylate levels in the later runs. A statistical comparison between these residual terms shows reliable differences for the same animals in different runs ($F = 3.77$, $df = 8/8$, $p < 0.05$) and for the same runs in different time intervals ($F = 2.32$, $df = 64/64$, $p < 0.001$). The effect of runs for different groups of animals is only slightly short of statistical significance ($F = 2.94$, $df = 7/7$, $0.05 < p < 0.10$). Most of the variables included in Table II show higher F values for Runs 3 and 4 than for 1 and 2 as a result of the much lower residual terms for Runs 3 and 4.

The replications term of Table II shows that there was no reliable difference between the first and second rabbit of each pair. The sequence term likewise shows that the animals given dimethyl sulfoxide on Runs 2 and 4 did not differ reliably from those given the dimethyl sulfoxide on Runs 1 and 3. However, salicylic acid blood levels tended to be higher for the animals given dimethyl sulfoxide on Runs 1 and 3, and Table II shows that this difference approached statistical reliability in Runs 3 and 4 ($F = 3.65$, $df = 1/7$, $0.05 < p < 0.10$). Table II shows a reliable interaction between the effects of sequence and dimethyl sulfoxide in Runs 3 and 4. This result was due to a greater enhancement of absorption by dimethyl sulfoxide in the sequence group given this treatment in Runs 1 and 3 than in the other sequence group given dimethyl sulfoxide in Runs 2 and 4. The same trend occurred but was short of statistical significance in Runs 1 and 2.

Table I—Analysis of Variance for Differences among the 16 Animals (pH Levels, Sequence of Treatments, and Replications), among the Four Runs for Each Animal (First Two and Last Two Runs, with and without Dimethyl Sulfoxide Treatment), and among the Five Time Periods in Each Run

Source	*df*	*MS*	*F*
Different animals:			
pH	3	392.33	3.81
Sequence	1	266.74	2.59
Replications	1	213.01	2.07
Residual	7	103.03	—
Different runs:			
Runs	1	207.72	10.44[a]
Dimethyl sulfoxide	1	1321.29	66.40[b]
(Dimethyl sulfoxide) (pH)	3	88.80	4.46[c]
Residual	24	19.90	—
Different times:			
Times	4	414.72	34.33[b]
(Times) (dimethyl sulfoxide)	4	63.36	5.25[b]
Residual	128	12.08	—

[a] $p < 0.01$. [b] $p < 0.001$. [c] $p < 0.05$.

The greater sensitivity of the experimental measurement, obtained by testing each animal under the different experimental conditions, is evident in the much smaller residual terms for different runs of the same animal than for different animals (Tables I and II). The difference in size of these terms is statistically reliable for the analysis of variance which included all four runs, shown in Table I ($F = 5.18$, $df = 7/24$, $p < 0.01$), and for Runs 3 and 4, shown in Table II ($F = 4.17$, $df = 7/8$, $p < 0.05$). The residual term is likewise smaller for different time intervals of the same run than for different runs, and this difference is statistically significant for the analysis of variance shown in Table I ($F = 1.65$, $df = 24/128$, $p < 0.05$).

DISCUSSION

Irritation of the skin during the test procedures might have caused dehydration and formation of scar tissue, thereby decreasing

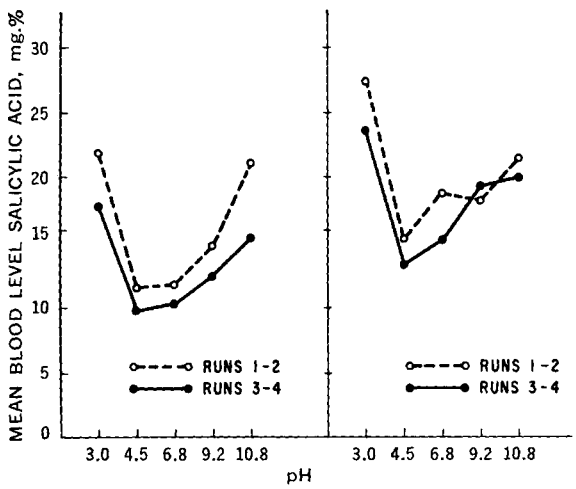

Figure 3—*Mean blood levels of salicylic acid at the time interval of each run when the animal's blood level was highest, separately for the rabbits at each pH level (two at 3.0, four each at 4.5, 6.8, 9.2, and 10.8), showing the first and second runs without dimethyl sulfoxide (left-hand graph) and with dimethyl sulfoxide (right-hand graph).*

Table II—Analysis of Variance Separately for the First Two and Last Two Runs, Showing Differences among the 16 Animals (pH, Sequence of Treatments, and Replications), between the Two Runs for Each Animal (with and without Dimethyl Sulfoxide Treatment), and among the Five Time Periods in Each Run

Source	*df*	—Runs 1 and 2— *MS*	*F*	—Runs 3 and 4— *MS*	*F*
Different animals:					
pH	3	188.34	2.01	213.57	6.72[a]
Sequence	1	151.96	1.62	115.99	3.65
Replications	1	199.83	2.14	42.30	1.33
Residual	7	93.55	—	31.78	—
Different runs:					
Dimethyl sulfoxide	1	504.28	17.52[b]	838.09	109.84[c]
(Dimethyl sulfoxide) (pH)	3	64.79	2.25	33.34	4.37[a]
(Dimethyl sulfoxide) (sequence)	1	22.09	0.77	67.69	8.87[a]
Residual	8	28.78	—	7.63	—
Different times:					
Times	4	243.02	14.39[c]	177.29	24.39[c]
(Times) (dimethyl sulfoxide)	4	28.60	1.69	38.16	5.25[b]
Residual	64	16.89	—	7.27	—

[a] $p < 0.05$. [b] $p < 0.01$. [c] $p < 0.001$.

cutaneous permeability in subsequent runs. Long-standing clinical experience indicates that increasing hydration of the skin by a watertight occlusive covering definitely promotes the percutaneous absorption of drugs. For example, Wurster and Kramer (5) demonstrated that moisture conditioning greatly enhanced absorption of three salicylate esters. Shelmire (6) stated that hydration of the stratum corneum is one of the most important factors in penetration of the skin by a medicament. The stratum corneum is hygroscopic; in humans, it requires at least 10% moisture to maintain its softness and pliability (7). This hygroscopicity is due at least partially to the water-retention capacity of keratin (8). Salicylic acid has been widely used as a keratolytic agent, promoting the exfoliation of the keratin-containing layer. The loss of the keratin layer after one or more salicylic acid–hydrophilic ointment treatments could lead to a decrease in moisture content and hydration of the skin, and this could account for the lower blood levels in Runs 3 and 4 than in 1 and 2.

An additional possible explanation for the lower salicylic acid blood levels in later runs is that the repeated tests would be expected to result in progressively diminished emotional arousal to the stressful pretreatment and treatment procedures. Decreasing emotional arousal might be expected to diminish blood flow and hormonal secretion, thereby retarding absorption.

Whatever may be the mechanism for the decreased cutaneous permeability in later runs, a general, long-lasting effect is indicated by its persistence during the 1-week interval between tests and its enhancement at the end of the test period. The tendency for the effect to be smaller under dimethyl sulfoxide suggests that this treatment may enhance absorption partly by counteracting the same mechanism that causes increased resistance to absorption after previous tests. The similar effect with a wide range of pH values indicates that the differential action of pH on the skin is not responsible for the decrease in absorption after prior tests.

The decrease in percutaneous drug absorption after previous exposures has potential applications for therapy. It may be necessary to increase the amount or duration of drug application to maintain a desired effect. A diminished response to the drug, due to decreased percutaneous absorption, may be erroneously attributed to the development of tolerance.

The blood salicylic acid levels, obtained by the present procedure for measuring percutaneous absorption, showed considerable variation among animals, together with a high degree of consistency for the same animal in different tests. The crossover design of testing each animal under different conditions thereby yielded much smaller residual scores for different runs of the same animal than for different animals. This procedure either increases the statistical reliability of the results or allows the same degree of reliability to be obtained with much fewer animals. An important practical advantage of prior exposure to the test conditions is indicated by the greater consistency and stability of the data in the second exposure to each condition (Runs 3 and 4) than in the first exposure (Runs 1 and 2). Emotional arousal may have increased variability in the initial runs; familiarization of animals to the test procedures is a method used in many situations to maximize consistency and predictability of behavior. An additional possible explanation is that the physical reactions of the skin to previous treatments resulted in greater uniformity of percutaneous permeability among the different animals and a more uniform response to the experimental treatments.

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