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An Animal Model for Estimating the Relative Sting Potential of Shampoos

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Synopsis—Comparative studies of various experimental and commercially available SHAMPOOS, utilizing the MOUSE WRITHING TEST as an assessment of PAIN, DISCOMFORT, or STINGING, showed a reasonable rank-order CORRELATION of MILDNESS with that found through controlled EYE STING studies in MAN. In view of the poor predictive value of conventional animal primary irritancy studies in determining discomfort or eye stinging properties of shampoos and other cosmetic materials, the mouse writhing test can provide a valuable adjunct in predicting the potential for discomfort and stinging in man. The pHs of 29 shampoos studied were between 5.5 and 7.7. This narrow range did not appear to influence the potential for discomfort in the mouse, eye irritancy in the rabbit, or eye stinging in humans.

Introduction

There is an important need for screening methods which can detect and distinguish the relative discomfort or sting potential of cosmetic formulations early in their developmental stages.

Laden (1) has reported that despite extensive animal and human testing, it was sometimes observed that prototype formulations, which had low primary irritancy, still caused discomfort to the user in terms of low levels of stinging. It appeared to him that the stinging potential of a formulation was often totally unrelated to its primary irritancy.

Van Abbe (2) has also reported problems with standard rabbit and monkey eye tests in predicting potential eye irritancy and discomfort of a hair dressing product, which when test-marketed, caused an unusually high incidence of stinging, itching, and pain of the eyes following the complainants exposure to rain.

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Thus, conventional animal eye and skin irritancy tests have shown little correlation with that of the human perception of discomfort or stinging. Not only can there be a lack of evidence of irritation in rabbit eyes by substances that sting human eyes, there can also be little or no evidence of irritation in human eyes by substances that can still be perceived to be severely stinging.

In an attempt to devise an animal model, which would better predict the stinging potential of materials intended for use on humans, Laden (1) devised a rat-tail test, which measured the time for the animal to "flick" its abraded tail from acidic solutions of varying concentrations. Although a promising approach, the method suffered from large animal-to-animal and experiment-to-experiment variations. Flicking reactions by the animals for no particular reasons were often observed, and the effects of time following abrasion, along with difficulties in producing consistent degrees of abrasion, appeared to influence and limit the effectiveness of the procedure as a means for reliably estimating relative differences in stinging between materials.

The purpose of this report is to present another method which can provide an early screening procedure for the detection of the relative discomfort potential of cosmetic products that may enter the eye. The preliminary work herein will focus on shampoo products for which special mildness properties are desired (e.g., "no sting," "no-tear," "baby" shampoos).

Method

The method is based on the mouse writhing test which has been previously used primarily as a pharmacologic screening procedure for the detection of analgesic drug activity (3-8).

Mouse Writhing Criteria

The writhing syndrome has been described as a stretching or squirming response and is considered an overt manifestation of the perception of pain by the animal. The test was originally developed by eliciting pain responses in mice following the intraperitoneal injection of irritant materials such as iodinated contrast agents (3), bradykinin (4) and paraphenylquinone (5-8).

In the present study, the response was characterized by contraction of the abdominal musculature and was often accompanied by torsion and flexion of the trunk and extension of one or both of the hind limbs. It usually occurred within seconds or at most 1 or 2 min following the injection of dilute shampoo solutions into the test mouse. The syndrome was observed to be either intermittent and repetitive in nature or exhibited as a relatively steady and prolonged contraction of the middorsal abdominal wall. In either case, the end point was easily recognizable and a characteristic response to discomfort or pain by the animal. Other overt symptomatology following the intraperitoneal injection of the shampoos included ataxia, high-stepping gait, reduced mo-

tor and exploratory behavior, and excessive preening or licking of the injection site. It was the combination of these symptoms, with or without those of the actual writhing syndrome itself, which was utilized in assessing whether or not the animal was perceiving pain/discomfort.

Initial investigations of a single reference control shampoo involved quantitative assessments of the number of writhing episodes within a 15-min time period. These were eventually discontinued due to the variability observed in both the sensitivity of individual mice to pain and the range of symptomatology indicative of pain other than the presence or absence of writhing. In addition, this method of observation was tedious, time-consuming, and did not lend itself to a rapid and reproducible screening procedure for the early evaluation of discomfort potential.

For these reasons, a more rapid and fruitful approach was developed, which involved a simpler all-or-none observation. The mice either displayed overt symptoms of discomfort or they did not. By this method of observation the writhing test more completely fulfilled the criteria for an early screening procedure, as it was rapid, inexpensive, quantifiable, and reproducible.

Materials and Methods

Mouse Writhing Studies

Crl: COBS CD-1 (1 CR) BR* male mice weighing between 15 and 25 g were used throughout. All injections were made intraperitoneally (I.P.) utilizing a ⅝ in., 26-gauge needle attached to a 1-ml disposable tuberculin syringe. Graded shampoo concentrations were made on a v/v basis with distilled water. The volume injected was 0.2 ml throughout the study.

During the initial investigations, for the purpose of determining onset, duration, and characteristics of the writhing syndrome, the mice were observed in pairs for a period of 15 min. A single commercially available baby shampoo (designated "J") was used for these studies.

Subsequent all-or-none observations were made on groups of 5 mice for a period of 6 min. Immediately following injection, the mice were placed in a 12 in. length x 6 in. width x 5 in. depth opaque plastic box containing approximately ½ in. wood shaving bedding, for observation. One writhe per mouse or other overt symptoms indicative of pain or discomfort were considered a positive response. A total of 10 mice (two groups of 5) was used for each shampoo dilution tested. From 3 to 7 dilutions were required to obtain adequate dose/response effects. When a pain response was observed, the mouse was removed from the observation box and a positive reaction was recorded.

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The percentage of mice showing pain reponses at each shampoo dilution was plotted on logarithmic probability paper* and the concentration (writhing dose) necessary to elicit discomfort in 50 per cent of the population (WD_{50}) calculated. WD_{50} levels, 95 per cent confidence limits, and potency ratios were determined by the method of Litchfield and Wilcoxon (9). The potency ratios between each test shampoo and the control reference product were utilized to determine if the two materials being compared differed significantly in mildness.

Determinations of a starting dose for each of the shampoos studied indicated 1 or 2 per cent concentrations were reasonable first-observation levels. To obtain dose/response relationships, further concentrations were subsequently increased or decreased depending on the number of animals exhibiting a positive response at the starting dose level.

To determine if hydrogen ion concentration had any influence on eliciting discomfort responses, the pH of each shampoo was determined with a Beckman Zeromatic® pH Meter† using 2 per cent solutions in distilled water.

Commercially available shampoos (designated by alphabetical code) were purchased in local stores during the period January 1974 through June 1974 and represent formulations marketed and distributed during the time.

Human Eye Sting Studies‡

The eye sting characteristics of selected experimental shampoos were tested under the supervision of an ophthalmologist. Testing started with a 1 per cent dilution employing 5 subjects per shampoo. Subsequent dilutions of 5, 10, 15, and 20 per cent were tested depending on the tolerance to each previous dilution. Formulations tolerable at 20 per cent were then compared directly to a reference control. The desired concentrations were prepared by dilution with Sterile Water for Injection.** Each dilution was placed in a coded plastic bottle equipped with a dropper plug designed to deliver a drop of approximately 0.04 ml. All subjects received the indicated test shampoo dilution in the conjunctival sac of 1 eye and a control shampoo ("J") of the same dilution in the other eye. Assignment of test and control dilutions in the right or left eye was done on a random basis. One eye was treated first by placing a drop of the predetermined treatment in the conjunctival sac. The volunteer was then requested to score the intensity of the sting using the following scale: (0) no sting; (1) very slight sting; (2) slight sting; (3) mild sting; (4) moderate sting; (5) severe sting.

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The procedure was then repeated for the opposite eye. Each eye was examined by the ophthalmologist approximately 1 hr after treatment to determine the presence or absence of observable irritation.

Rabbit Eye Irritancy

A modified Draize test (10) was utilized for determining the irritancy potential of 8 experimental and 3 selected ("B," "J," and "P") commercially available shampoos. Although normally evaluated separately, Draize scores for cornea, iris, conjunctivae, and chemosis were combined for the specific purpose of presenting overall relative eye irritation caused by the shampoos utilized in this study. Since the specifics of this test are widely known, they will not be reproduced in the present paper.

Results

Preliminary studies to determine the onset and duration of the writhing syndrome were conducted with baby shampoo "J." Fig. 1 shows the results obtained using 4 aqueous dilutions over a 15-min observation period. These data indicated that the peak time for writhing in paired mice occurred be-

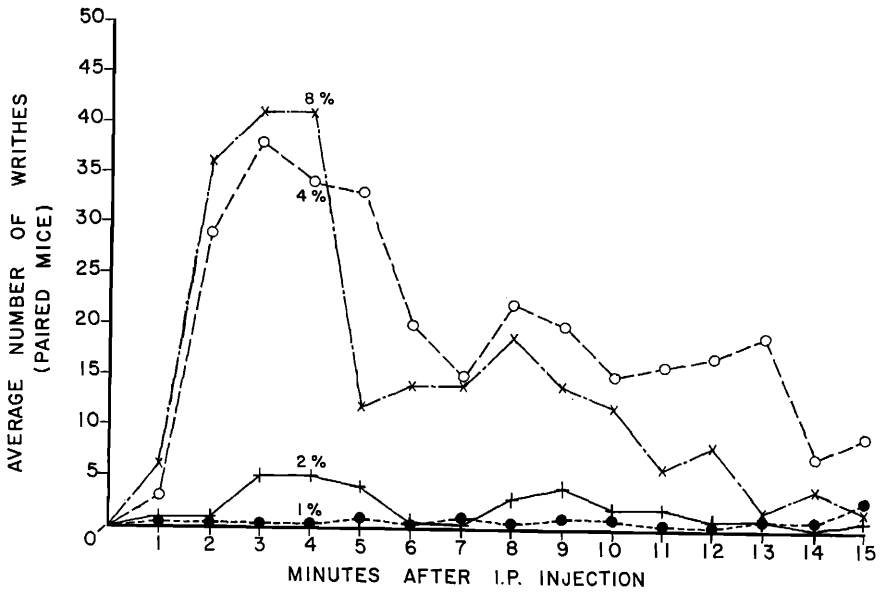

Figure 1. Outset and duration of the writhing syndrome. Data based on average number of writhes exhibited by 10 pairs of mice at each of 4 aqueous concentrations of a reference control shampoo (J)

tween 1 and 6 min following injection and, thereafter, steadily decreased during the remainder of the 15-min observation period. Peak incidence of writhing occurred at the 3-min observation time. From these studies, it was judged that a 6-min observation time would be sufficient for future experiments where an all-or-none observation was to be used.

Distilled water, used as the shampoo vehicle during these studies, produced no incidences of writhing or other overt symptoms of pain or discomfort in any of 10 separate experiments using a total of 20 mice.

Table I shows the dose/response effects of 3 commercially available and 8 experimental shampoos using the all-or-none method of observation.

The data in Table II represent comparative studies of 3 selected commercially available products ("B," "J," and "P") and a series of 8 experimental shampoos (08B, 14F, 14G, 08A, 14E, 25S, 25M, 101) being developed specifically for special mildness properties.* They are ranked in order of the highest (mildest) to lowest (least mild) WD_{50} values. The results obtained following human eye-sting evaluation and rabbit eye irritation studies are included for comparison purposes.

These data show that the mouse writhing test was successful in identifying four shampoos ("P," 25S, 25M, and 101), which were significantly less mild ($P \leq 95$ per cent) than the reference control (J). All four of these shampoos were also considered by human testers to produce the highest degree of eye-sting (moderate to severe). Five of the experimental shampoos (08B, 14F, 14G, 08A, 14E) and a single commercial "baby" shampoo ("B"), were equivalent in mildness to the reference control ("J") via the mouse writhing procedure. These six were also the mildest as judged by human eye-sting evaluations and were considered to have a sting potential ranging from absent to slight. None of the shampoos caused observable irritation in human testers at the concentrations studied.

Table III shows the comparative WD_{50} and 95 per cent confidence limits of 21 commercially available shampoos. When the highest to lowest WD_{50} values were ranked, nearly all of the leading "baby" shampoos tested appeared in the mildest class of shampoo products. Seven of 9 baby shampoos (B, N, U, S, K, G, and V), which make claims or inference of special mildness properties, had WD_{50} values that were not statistically different from the reference control, "J." On the other hand, two baby shampoos (G and H) and all eleven of the "adult" variety of shampoos tested were significantly less mild than the reference baby shampoo.

Rabbit eye irritancy scores indicated all eight of the experimental and both commercially available "baby" shampoos were relatively innocuous in terms of significant, visible eye irritation potential. A popular "adult" variety

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Table I
Dose Response Curves Obtained With 8 Experimental and 3 Commercially Available Shampoos

Shampoo	Per Cent Aqueous Concentration	Number of Mice Writhing/Dosed[a]	Per Cent Writhing	WD_{50} (95 Per Cent C.L.)[b]
"B"	2.0	3/10	30	
	3.0	5/10	50	
	4.0	8/15	53	
	5.0	13/15	87	3.0 (1.7-5.2)
08B	1.0	0/10	0	
	2.0	2/10	20	
	4.0	10/10	100	2.5 (2.0-3.2)
14F	1.0	0/10	0	
	2.0	3/10	30	
	4.0	10/10	100	2.2 (1.8-2.7)
14G	1.0	1/10	10	
	2.0	4/10	40	
	4.0	9/10	90	2.2 (1.4-3.4)
"J"	0.5	0/10	0	
	1.0	1/10	10	
	2.0	5/10	50	
	4.0	8/10	80	2.1 (1.5-3.1)
08A	1.0	0/10	0	
	2.0	8/10	80	
	4.0	9/10	90	1.7 (1.1-2.5)
14E	1.0	0/10	0	
	2.0	8/10	80	
	4.0	10/10	100	1.6 (1.3-2.0)
"P"	0.25	0/10	0	
	0.5	8/10	80	
	1.0	9/10	90	
	2.0	10/10	100	0.42 (0.26-0.67)
25S	0.125	0/10	0	
	0.25	6/10	60	
	0.5	10/10	100	
	1.0	5/5	100	
	2.0	5/5	100	
	4.0	5/5	100	0.24 (0.20-0.28)
25M	0.125	1/10	10	
	0.25	6/10	60	
	0.5	9/10	90	
	1.0	10/10	100	
	2.0	5/5	100	
	4.0	5/5	100	0.23 (0.15-0.36)
101	0.125	1/10	10	
	0.25	8/10	80	
	0.5	10/10	100	
	1.0	5/5	100	
	2.0	5/5	100	
	4.0	5/5	100	0.19 (0.14-0.25)

[a]Dose: intraperitoneal injection with 0.2 ml of each shampoo concentration.
[b]WD_{50}: Per cent aqueous concentration required to produce writhing syndrome (discomfort) in 50 per cent of the test mice.

Table II
Comparative Mouse Writhing, Human Eye-Sting, and Draize Rabbit Eye Test Data
(8 Experimental and 3 Selected Commercial Shampoos)

	Mouse Writhing				Human Eye-Sting			Rabbit Eye Irritation					
									Average Daily Draize Score[g]				
Shampoo	Type	pH[a]	WD_{50}[b]	(95 Per Cent C.L.)	Number of Subjects	Test[d] Concentration	Average Sting Score	Number of Animals	1	2	3	4	5
"B"	Baby	6.6	3.0	(1.7-5.2)	5	20 per cent	2.4 (slight)	6	6	0	0	0	0
08B	Baby	6.6	2.5	(2.0-3.2)	5	20 " "	1.0 (very slight)	6	5	1	0	0	0[h]
14F	Baby	6.5	2.2	(1.8-2.7)	15	20 " "	1.5 (very slight)	6	10	4	6	4	0
14G	Baby	6.8	2.2	(1.4-3.4)	10	20 " "	2.1 (slight)	6	8	3	5	4	0
"J"	Baby	6.6	2.1	(1.5-3.1)	95	20 " "	1.0 (very slight)	48	8	6	2	1	2[i]
08A	Baby	6.9	1.7	(1.1-2.5)	15	20 " "	0.5 (almost absent)	6	5	2	0	0	0[h]
14E	Baby	6.6	1.6	(1.3-2.0)	10	20 " "	1.1 (very slight)	6	8	4	5	4	0
"P"	Adult	7.0	0.4	(0.3-0.7)[c]	—[f]	1 " "	4+ (severe)[e]	6	40	37	26	20	20
25S	Baby	6.2	0.2	(0.2-0.3)[c]	5	10 " "	3.6 (moderate)[e]	6	2	3	2	—	—
25M	Baby	6.5	0.2	(0.1-0.4)[c]	5	5 " "	4.0 (moderate)[e]	6	2	2	2	—	—
101	Baby	6.7	0.2	(0.1-0.2)[c]	2	5 " "	4+ (severe)[e]	6	7	2	1	0	0

[a]pH: determined on 2 per cent aqueous solutions.
[b]WD_{50}—Per cent aqueous concentration required to produce writhing syndrome (discomfort) in 50 per cent of the test mice.
[c]Significantly less mild ($P \leq 95$ per cent) than reference control ("J").
[d]Human eye-sting tests started with a 1 per cent dilution employing 5 subjects per shampoo. Subsequent dilutions of 5, 10, 15, and 20 per cent were tested depending on the tolerance to each dilution. Formulations tolerable at 20 per cent were then compared directly to the reference control ("J").
[e]Sting potential considered too high for claims of "special mildness."
[f]Verbal communication.
[g]Shampoos instilled full strength (100 per cent), no rinse.
[h]Draize tests conducted on prototype formulations, which differed very slightly in composition from 08A and 08B.
[i]Averaged daily scores of 8 separate studies.

Table III
Mouse Writhing Test Ranking of Commercially Available Shampoos[a]

Shampoo	Type	pH	WD_{50}[b] (95 Per Cent Confidence Limits)
"B"	Baby	6.6	3.0 (1.7-5.2)
"J"	Baby (reference control)	6.6	2.1 (1.5-3.1)
"N"	Baby	7.3	2.0 (1.2-3.2)
"U"	Baby	7.6	2.0 (1.2-3.2)
"S"	Baby	7.4	1.9 (1.3-2.9)
"K"	Baby	7.3	1.7 (1.1-2.9)
"C"	Baby	6.6	1.6 (1.0-2.3)
"V"	Baby	7.4	1.3 (0.8-2.1)
"G"	Baby	7.7	1.0(0.7-1.5)[c]
"L"	Adult	7.0	0.9(0.6-1.3)[c]
"A"	Adult ((hypoallergenic)	6.8	0.7(0.5-1.0)[c]
"V"	Adult	6.5	0.6(0.4-1.0)[c]
"I"	Adult	6.9	0.6(0.4-0.9)[c]
"B"	Adult	7.6	0.5(0.3-0.7)[c]
"H"	Baby	7.0	0.4(0.3-0.7)[c]
"P"	Adult	7.0	0.4(0.3-0.7)[c]
"E"	Adult (antidandruff)	5.5	0.4(0.3-0.6)[c]
"G"	Adult	7.6	0.3(0.3-0.5)[c]
"O"	Adult	7.1	0.3(0.3-0.5)[c]
"D"	Adult (antidandruff)	7.3	0.1(0.1-0.2)[c]
"B"	Adult (antidandruff)	7.2	0.09(0.06-0.13)[c]

[a]Listed from most to least mild.
[b]WD_{50}: per cent aqueous concentration required to produce writhing syndrome (discomfort) in 50 per cent of the test mice.
[c]Significantly less mild ($P \leq 95$ per cent) than the reference control ("J").

shampoo ("P") caused comparatively high and persistent eye damage follow-the Draize procedure.

The pH of all shampoos studied ranged between 5.5 and 7.7. Correlation of product mildness in terms of WD_{50}, the human perception of eye-sting, and rabbit eye irritancy appeared to be unrelated to this limited pH range.

Discussion

Conventional animal tests for determining primary skin irritancy and ocular damage such as those proposed by Draize et al (10) and others (11, 12, 13), extensively reviewed by Lansdown (14), lack the sensitivity to detect milder adverse reactions such as discomfort or eye-sting. Apparently satisfactory irritancy scores obtained through these standard procedures will not necessarily ensure a lack of discomfort or stinging potential. This is particularly empha-

sized by the results obtained in the present study (Table II), which showed all of the experimental "baby" shampoos tested to have equally low Draize scores for visible eye irritancy and yet marked differences were subsequently obtained following human eye-sting and mouse writhing studies.

Additionally, a popular adult-variety shampoo ("P"), which caused relatively high ocular irritation following the Draize test along with a higher potential to produce discomfort and stinging, was ranked among other shampoos which were relatively innocuous in the Draize procedure and still caused significantly higher discomfort and stinging potential.

These apparent paradoxes can lead to considerable difficulty in the development on low eye-sting shampoos, especially when a formulation successfully passes conventional Draize eye tests only to fail later tests involving the human evaluations of eye-sting.

Consequently, of particular significance in the present study, is the correlation observed between the human perception of eye-sting and the results of the mouse writhing test. In every instance, the writhing test was capable of identifying those shampoos considered to have milder eye-sting properties from those considered more severe following human testing.

Although the relationship between the pain receptors in the viscera of the mouse and those of the highly innervated human eye is open to question, the results obtained in the present study, when the rank order of mildness obtained via the writhing test and that of the human perception of eye-sting in a series of experimental shampoos were compared, are highly encouraging.

Based on this correlation, the mouse writhing test appears to offer a reliable, rapid, inexpensive, and reproducible screening procedure for detecting relative differences in discomfort and/or stinging potential.

A comparison of 2 shampoos by the writhing test ordinarily takes less than 2 hr and utilizes between 60 and 120 mice. Thus the cost and time factors during early stages of formula development are minimal.

The test was found to be dose-responsive and capable of detecting the relative discomfort potential of shampoo materials at concentrations far below those usually required to produce primary skin irritation or visible damage to the eyes of animals.

The method is quantitative and lends itself well to statistical analysis. It facilitates comparison between two dose-response curves for both parallelism and the computation of relative potencies with their confidence limits. In the present studies, comparison of potency ratios appeared to reliably show relative differences in discomfort which correlated well with those observed following human evaluations of stinging.

In using the method the investigator is encouraged to use control materials which serve as reference standards, materials which, by their known composition and/or history of acceptable human experience, are reasonable target

goals for the purpose of mildness. The investigator is cautioned against scoring a positive pain response unless completely convinced that the animal is feeling discomfort and responding to the injected material. An apparent stretch when the animal attempts to reach or peer over the edge of the observation box or other misleading activity should not be construed as a part of the writhing syndrome. In addition, because of the inherent curiosity and explorative nature of the mouse, the test should be conducted in a quiet area, one which provides no distraction to the animal in the form or noise, smoking, strong odors, or laboratory personnel working nearby. The low levels of pain or discomfort being elicited by mild formulations or low concentrations can often go unnoticed by an animal being distracted in its environment.

The writhing test may not be limited to a specific product type such as shampoos. Further studies should be conducted to ascertain its value in determining possible differences in "discomfort" potential of other cosmetic materials such as face and eye makeups, hair products, creams, lotions, and other product types, which can be diluted, put into aqueous solution, or suspended in other innocuous, injectable vehicle types. These vehicles by themselves should not elicit writhing-like overt symptoms.

In the present study, there was a lack of correlation between the narrow pH range of the shampoos tested and their mildness in terms of both human eye sting evaluations and mouse WD_{50} values. This lends support to the work of Laden (1), who indicated that no general conclusions could be drawn in predicting the stinging potential of solutions of acidic materials by considering only the pH, tonicity, or nature of anionic materials.

Summary

Comparative studies of various experimental and commercially available shampoos, utilizing the mouse writhing test as an assessment of pain, discomfort or stinging, showed a reasonable rank-order correlation of mildness with that found through controlled eye-sting studies in man.

In view of the poor predictive value of conventional animal primary irritancy studies in determining discomfort or eye-stinging properties of cosmetic materials, the mouse writhing test can provide a valuable adjunct in predicting the potential for discomfort and stinging.

The limited pH range (5.5 to 7.7) of the shampoos studied did not appear to influence the potential for eye-sting.

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