J. Soc. Cosmetic Chemists, **16**, 499–506(1965)

Skin Substantivity as a Criterion in the Evaluation of Antimicrobials

THOMAS F. McNAMARA, Ph.D., MARIANNE L. STEINBACH, B.S., and BENJAMIN S. SCHWARTZ, M.S.*

Presented before the New York Chapter, September 9, 1964, New York City

Synopsis—Skin substantivity is defined as the avidity of a compound for skin tissue. Compounds possessing substantivity were evaluated by two major criteria: (a) qualitatively by measuring the comparative avidity for skin tissue and (b) quantitatively by titrating the release of the compound from tissue. A method was developed to determine each of these properties for compounds having antimicrobial properties. The application of such data to the selection of a compound for a particular topical use was discussed.

Introduction

The trend toward the topical application of antimicrobial substances in cosmetic preparations and proprietary formulations has been increasing for several years. This is evidenced by the fact that the number and types of medicated cosmetics are now approaching in number those of the nonmedicated ones. A large number of chemical compounds with similar antimicrobial, physical and chemical properties and toxicity levels are available for use in topical formulations. If one is to choose the best compound for topical application, an additional criterion must be established. Such a criterion for evaluating these compounds is their avidity for tissue and their elution from it in an active form (tissue substantivity).

This report is concerned with the methodology used in the *in vitro* evaluation of four antimicrobial agents with regard to their tissue substantivity.

* Warner-Lambert Research Institute, Morris Plains, N. J. 07950.

Purchased for the exclusive use of nofirst nolast (unknown)
From: SCC Media Library & Resource Center (library.scconline.org)

Materials and Methods

Agents: For use in these studies, samples of 4-amino-1-laurylquinaldinium acetate monohydrate (Laurodin acetate),* 1,1′-hexamethylenebis[5-(2-ethylhexyl)biguanide] dihydrochloride (Sterwin #904),† alkyldimethylbenzylammonium saccharinate (Holichem HQ 3300),‡ and the 1-hexadecylpyridinium chloride (C.P.C.)§ were obtained from commercial sources.

For the microbiological studies, solutions were prepared in the following manner. Aqueous or aqueous-alcoholic stock solutions of the compounds were prepared at concentrations of 1000 μ/ml. and stored in the refrigerator until used.

In Vitro Tests: The organisms tested were obtained either from the American Type Culture Collection (AATCC) or from our own stock culture collection (WLRI).

Three basic media were used in these studies, brain heart infusion broth (Difco) for the bacteria, Sabouraud's dextrose broth (Difco) for *C. albicans*, and AATCC broth (Difco) for the determination of bactericidal activity of the compounds. (Exception: *F. polymorphum* was cultured in a mixture (1:1) of brain heart infusion broth (Difco) and fluid thioglycollate (Difco)). The determination of the minimum inhibitory concentration for each of the organisms was carried out by the twofold broth dilution method. The inoculum used, except for *F. polymorphum*, was 0.1 ml. of a 1:1000 dilution of a 50% transmission (Lumetron Colorimeter Model 402E equipped with M 465 filter) of a twenty-four-hour broth culture. For *F. polymorphum*, the inoculum used was 0.1 ml. of a 50% transmission of a twenty-four-hour broth culture. All cultures were incubated at 37°C for forty-eight hours. The minimum inhibitory concentration was recorded as the lowest concentration of the compound at which there was no visible growth of the organism.

The bactericidal activity was determined for each compound according to the U.S.F.D.A. (1) method for testing antiseptics and disinfectants. In those cases where a neutralizer was available to obviate possible bacteriostasis of the test substances, the appropriate neutralizer was added to the subculture medium. These neutralizing substances were: 0.05% sodium thioglycollate for mercurials and other

* Allen & Hanburys Ltd.
† Sterwin Chemicals, Inc.
‡ Hollichem Corp.
§ K & K Laboratories, Inc.

Purchased for the exclusive use of nofirst nolast (unknown)
From: SCC Media Library & Resource Center (library.scconline.org)

heavy metals or for oxidizing agents, letheen (0.07% azolectin and 0.5% Tween 80*) for quaternary ammonium compounds, and 1% Tween 80 for bis-phenols.

Skin substantivity studies were carried out using calf skin discs prepared from calf-skin (dehaired, untanned, and pickled) obtained from Barrett & Co., Newark, N. J. The method of preparation was a modification of that of Vinson *et al.* (2). The calf skin was immersed in a salt solution containing 31.2 g. of sodium chloride and 2.5 g. of sodium bicarbonate per 1000 ml. of distilled water. The ratio of calf skin to salt solution was 1:4 (w/v). When the calf skin reached a pH of 5.6, as ascertained by measuring pH of liquid squeezed from the skin, it was rinsed thoroughly in water to remove excess salt. It was then dehydrated by passing through two daily changes of 95% ethanol. The dehydrated skin was pinned to a board and allowed to air dry (five to six hours). Discs were cut from the dried skin using a 15 mm. diameter cork borer and discs weighing 70 mg. (±20 mg.) were sterilized by ethylene oxide prior to use in tissue substantivity determinations.

Results

Determination of Minimum Inhibitory Concentration. The inhibitory concentrations of the four test compounds for each of the test organisms is presented in Table I. Repeated tests using the same strains on rare occasions showed slight but insignificant variations in these reported concentrations. Each of the compounds was more effective against the gram-positive organisms than it was against the gram-negative organisms and all of the compounds were equally effective against the yeast, *C. albicans*. The addition of protein, in the form of horse serum, reduced the antimicrobial activity of each of the compounds. While all of the compounds displayed excellent antimicrobial properties, none of the four evaluated compounds possessed significantly greater activity against all of the test organisms (see Table I).

Bactericidal Activity. The results of the evaluations of each of the compounds for bactericidal activity against *S. aureus*-209 are presented in Table II. Each of the compounds is bactericidal at the lowest level tested. The differences in killing times vary slightly from compound to compound but these differences are not significant. Furthermore, in routine use, the minimum concentration in a topical preparation would not be less than 1000 γ/ml. and for this reason no one of the com-

* Atlas Chemical Industries, Inc., Wilmington, Del.

Purchased for the exclusive use of nofirst nolast (unknown)
From: SCC Media Library & Resource Center (library.scconline.org)

TABLE I
Minimum Inhibitory Concentration Against Selected Microörganisms
(γ/ml.)

Organism and Strain #	Hollichem Hq 3300		Sterwin #904		Laurodin Acetate		C.P.C.	
	BHI[a]	BHI & HS[b]	BHI	BHI & HS	BHI	BHI & HS	BHI	BHI & HS
S. aureus WLRI 296	0.4	0.9	0.2	0.9	0.9	3.9	<0.2	...
L. buccalis WLRI 297	<0.2	1.9	<0.2	1.9	0.2	7.8	0.2	...
Str. mitis WLRI 298	0.4	7.8	<0.2	1.9	0.2	15.6	...	...
Str. faecalis WLRI 299	...	...	...	...	...	...	0.2	...
K. pneumoniae WLRI 300	>50.0	>100.0	...	>100.0	50.0	>100.0	31.2	...
L. acidophilus WLRI 301	3.9	15.6	0.9	3.9	3.9	15.6	3.9	...
F. polymorphum ATCC 10953	31.2	...	31.2	...	31.2	...	31.2	...
C. albicans WLRI 045	1.9	>31.2	0.2	>31.2	1.9	>31.2	1.9	...

[a] Brain heart infusion broth (Difco).
[b] Brain heart infusion broth (Difco) with horse serum added.

TABLE II
Bactericidal Activity of the Four Test Compounds Against *S. aureus*-209

Compound (γ/ml.)	Killing Time (Minutes)
Hollichen HQ 3300	
1000	<0.5
200	0.5–1.0
50	1.0–1.5
Sterwin #904	
1000	<0.5
250	<0.5
50	0.5–1.0
Laurodin acetate	
1000	<0.5
250	<0.5
50	4.0–5.0
C.P.C.	
1000	<0.5
250	<0.5
50	1.0–1.5

Purchased for the exclusive use of nofirst nolast (unknown)
From: SCC Media Library & Resource Center (library.scconline.org)

pounds could be considered to be significantly better than any of the others (see Table II).

Qualitative Measure of Substantivity. Broth dilutions of the compounds were prepared in duplicate. (A modified Rammelkamp (3) broth dilution was used in which the minimum inhibitory concentration M.I.C. was determined to within 0.1 γ/ml.). To each tube in one series of dilutions a sterile skin disc was added. Both sets of dilutions were incubated in a water bath at 37°C for four hours. The discs were then removed from the tubes and discarded. Each of the tubes received the same inoculum of *S. aureus* and all tubes were incubated at 37°C for forty-eight hours. (Inoculum: A twenty-four hour brain heart infusion broth culture of *S. aureus*-209 was standardized to 50% transmission (±2%) using the Lumetron Colorimeter. A 10^{-3} dilution in broth was made, and 0.1 ml. of this dilution was used to inoculate each tube.) After incubation the tubes were read macroscopically for growth and the lowest concentration of test compound inhibiting growth was reported as the end point (M.I.C.).

If the test compound was substantive to the skin tissue, the dilution series, which contained the skin discs, had its end point (M.I.C.) shifted to a higher concentration of the test compound. The greater this shift relative to the M.I.C., determined in tubes without addition of skin discs, the greater was the substantivity of the compound. The binding of the compound to the discs accounted for this difference in end point. This can be expressed mathematically as a substantivity potency ratio (Sp) for a given compound by the following formula:

$$\frac{D - M}{M} \times 100 = Sp$$

D = inhibitory concentration in series that had skin discs
M = inhibitory concentration in series without skin discs
Sp = substantivity-potency ratio

Comparison of the Sp for several compounds can be used for the selection of a compound based on skin substantivity (see Table III).

Quantitative Titration of Skin Substantivity. Each sterile skin disc was rehydrated, placed in 10.0 ml. of a known concentration (0.1%) of the test compound; mixed for one minute at 37°C and finally pressed with a 50 g. weight between two Microfiber Glass Prefilter pads* (one minute) to remove excess fluid. The disc was serially washed in each of

* Millipore Filter Corp., Bedford, Mass.

Purchased for the exclusive use of nofirst nolast (unknown)
From: SCC Media Library & Resource Center (library.scconline.org)

TABLE III

Determination of Substantivity-Potency Ratio (*Sp*) for each of Test Compounds

Compound	*D* (mcg./ml.)	*M* (mcg./ml.)	*Sp*
Hollichem HQ 3300	0.90	0.70	28.6
Sterwin #904	0.60	0.45	33.3
C.P.C.	0.55	0.35	57.1
Laurodin acetate	0.65	0.40	62.5

TABLE IV

Quantitative Substantivity Measurement of Test Compounds Titrated Against *S. aureus*-209

Compound	Concentration (γ/ml.)	Test Rinse Showing Inhibition
Hollichem HQ 3300	500.0	0
	250.0	0
	125.0	0
	62.5	0
Sterwin #904	500.0	4
	250.0	0
	125.0	0
	62.5	0
Laurodin acetate	500.0	8
	250.0	2
	125.0	0
	62.5	0
C.P.C.	500.0	7
	250.0	2
	125.0	1
	62.5	1

15 broth tubes containing 5.0 ml. of broth. All tubes were inoculated with *S. aureus*, incubated at 37°C for seventy-two hours and read macroscopically for growth. (Inoculum: A twenty-four-hour brain heart infusion broth culture of *S. aureus*-209 was standardized to 50% transmission (±2%) using the Lumetron Colorimeter. A 1:10 dilution was prepared, and 0.2 ml. was used to inoculate each tube.) The rate of elution was correlated to the number of consecutive rinse tubes in which visible growth is inhibited at the concentration used. If the compound was not eluted from the disc, inhibition of bacterial growth did not occur in any of the rinse tubes. If the compound was eluted slowly, bacterial growth was inhibited in a large number of tubes. If the compound was eluted rapidly, bacterial growth was inhibited in a smaller number of rinse tubes.

Purchased for the exclusive use of nofirst nolast (unknown)
From: SCC Media Library & Resource Center (library.scconline.org)

Discussion

From the data obtained in the bacteriostatic and bactericidal tests (Tables I and II), it was evident that there was no significant difference in the antimicrobial activity of the four test compounds. Each of the four compounds was effective against a spectrum of microorganisms in the absence or presence of horse serum, and low concentrations of all the compounds were bactericidal. Hence, these criteria did not offer a means of judiciously selecting any one compound from the group for topical use. However, the skin substantivity results offered a means not only of selecting compounds for topical use but of selecting the compound best suited for a specific need.

The data presented in Table III indicated that all four compounds were substantive to the skin tissue and that C.P.C. and Laurodin acetate had a greater avidity for the skin than either of the other two compounds. If the only criterion for selecting a compound for use in a topical preparation were the property of skin substantivity then these data (Table III) would be sufficient for selection of the best compound for topical use. If the criteria for selection of a compound are extended beyond the property of skin substantivity to include the elution characteristics of active material, additional data would be required. Such data were obtained by titration of the active material released from the skin discs. The data in Table IV indicate that if the selection of a compound were to be based on the elution of active material for an extended period of time then selection of Laurodin acetate as the compound of choice would be indicated. If, however, the criterion for selection were the retention of the compound at the site of application then the selection of Hollichem HQ 3300 would be indicated. If rapid release of all or nearly all of the compound were desired, then the selection of Sterwin #904 would be indicated.

Summary

Skin substantivity of quaternary antimicrobial agents, which may have utility for topical application, was studied by several methods. By use of the described methods, it was possible to determine whether an antimicrobial agent had calf skin substantivity. Once this fact was ascertained, it was possible to determine whether the compound was bound irreversibly to the skin; if not, it could be shown whether the active material was released rapidly or slowly from the skin. From such

Purchased for the exclusive use of nofirst nolast (unknown)
From: SCC Media Library & Resource Center (library.scconline.org)

information and from knowledge of the intended usage the selection of the compound best suited for a particular type of topical formulation can be made.

(Received September 3, 1964)

References

(1) Circular 198, *United States Food and Drug Administration Methods of Testing Antiseptics and Disinfectants*, U. S. Dept. Agriculture, 1931.
(2) Vinson, L. J., Ambye, E. L., Bennett, A. G., Schneider, W. C., and Travers, J. J., *J. Pharm. Sci.*, **50,** 827 (1961).
(3) Rammelkamp, C. H., *Proc. Soc. Exptl. Biol. Med.*, **51,** 95 (1942).

Purchased for the exclusive use of nofirst nolast (unknown)
From: SCC Media Library & Resource Center (library.scconline.org)