In Vitro Evaluation of the Tissue Substantivity of Selected Antibacterial Agents

T. F. McNAMARA and M. STEINBACH,

Warner Lambert Research Institute, Morris Plains, New Jersey

Abstract

Selected compounds and selected synergistic combinations have been evaluated for activity against *S. aureus* and for tissue substantivity. No direct correlation could be found between antibacterial activity and tissue substantivity. It was also apparent that the mode of action by which a compound or mixtures of compounds inhibit the growth of organisms is not directly related to the mechanism of tissue substantivity. Preliminary data indicate that no simple explanation of tissue substantivity can be made on the basis of chemical structure. Until more is known about the mechanism of tissue substantivity, each compound will have to be evaluated separately.

Introduction

SELECTED COMPOUNDS have been evaluated for antimicrobial activity and tissue substantivity. The compounds were selected because they were currently being used in topical applications and were reported to be substantive to tissue. The objective of the present study was to accumulate data on such compounds and to obtain some idea as to the extent of substantivity as well as the relationship, if any, of the antibacterial activity and/or chemical structure to tissue substantivity. Several synergistic combinations have also been included to obtain information on the effect that the combination of antimicrobial agents may have on the substantivity of the resulting mixture.

Experimental Section

Materials

For use in these studies, samples of 3,4,4′-trichlorocarbanilide (Monsanto Chemical Corporation), 4′,5-dibromosalicylanilide (Fine Organics Inc.), 3,4′5-tribromosalicylanilide (Fine Organics Inc.), 1-hexadecyl pyridinium chloride (K & K Laboratories Inc.), decamethylenebis (4-aminoquinaldinium acetate) (Allen and Hanbury Ltd.), alkyl (C12-C16) dimethylbenzyl ammonium chloride (Sterwin Chemicals Inc.), 2,2′-methylenebis (3,4,6-trichlorophenol) (Givaudan Corporation), and dodecyldimethyl (2-phenoxyethyl) ammonium bromide (Ciba Pharmaceutical Company) were obtained from commercial sources.

For the microbiological studies, aqueous or aqueous-alcoholic stock solutions of the compounds were prepared at concentrations of 100γ/ml and stored in the dark until used.

Methods

The *S. aureus*-209 used throughout these studies was obtained from the American Type Culture Collection.

The medium used in these studies was brain heart infusion broth (Difco). The determination of the minimum inhibitory concentration for each of the test compounds was carried out by a modified Rammelkamp (1) broth dilution technique. The inoculum used was 0.01 ml of a 1:1000 dilution of a 50% transmission (Lumetron Colorimeter Model 402E, equipped with M 465 filter) of a 24-hour broth culture. All tests were incubated at 37C for 48 hours. The minimum inhibitory concentration for each test compound was recorded as the lowest concentration of the compound at which there was no visible growth of the organisms.

Preparation of Skin Discs

Skin substantivity studies were carried out by using calf skin discs prepared from calf skin (dehaired, untanned, and pickled) which was obtained from Barrett and Company, 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 1,000 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 the 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 before being placed in acetone to complete the drying process. The dehydrated skin was pinned to a board and allowed to air-dry (five to six hours). Discs were cut from the dried skin by 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.

Determination of Substantivity

Duplicate broth dilutions of the compounds were prepared by a modified Rammelkamp procedure. (The minimum inhibitory concentration was determined to within 0.06 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 37C for four hours. The discs were then removed from the tubes and discarded. Each of the tubes in each dilution series received the same inoculum of *S. aureus*, and all tubes were incubated at 37C for 48 hours. 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.).

Evidence of the substantivity of the test compounds to tissue would be obtained from the shift of the end-point as a result of the incubation with skin discs. If the test compound was substantive to the skin tissue, the dilution series, in which the tissue discs had been incubated for four hours, would have its end-point shifted to a higher concentration of the test compound. The greater this shift relative to the end-point in tubes without skin discs, the greater was the substantivity of the compound. The adherence of the compound to the discs would account for this difference in end-point. This can be expressed mathemati-

cally as a substantivity constant (S−P) for a given compound by the following formula:

$$(D - M)/M\ 100 = S - P$$

D = inhibitory concentration in series with skin discs

M = inhibitory concentration in series without skin discs

S − P = substantivity-potency ratio

The greater the value (S−P), the greater is the substantivity of the compound.

Results and Discussion

The bacteriostatic and tissue substantivity results are tabulated in Table I. From these data it is evident that all of the test compounds are active against the organism, *S. aureus*. With the exception of two of the compounds (G and H, Table I), no significance can be attached to the difference in the antibacterial activity of the remaining compounds. (In a normal two-fold broth dilution screening test, these compounds would fall within the two-tube variation inherent in the technique.) This is not wholly unexpected since all or nearly all of the compounds tested have been selected for use in topical applications because of their known antimicrobial properties. However there is a wide variation in the tissue substantivity of these compounds. It was hoped that a clearer understanding of the mechanism of substantivity might be possible from a consideration of the antibacterial activity of the selected compounds. If a relationship existed between biological activity, i.e., antibacterial activity, it would be reasonable to assume that the variation in tissue substantivity would, within narrow limits, be similar to antibacterial activity. That this is not the case is evident from the data revealed in Table I. The compounds are tabulated in descending order with relation to their antibacterial activity. Such an arrangement does not consistently correlate with the tissue substantivity data.

Further evidence of lack of correlation between substantivity and biological activity is obtained from a similar consideration with reference to the antibacterial activity of synergistic mixtures. In Table II are listed several synergistic combinations with the corresponding antibacterial and tissue substantivity data for these mixtures. It is evident that the advantage obtained in antibacterial activity by admixing these compounds does not correspond with the results obtained for tissue substantivity, that is to say, the substantivity of the mixture does not consistently exceed the level of the more substantive component in that mixture. In fact, the components of such a mixture may actually work greatly to reduce the substantivity of the combinations.

With regard to the two quaternary ammonium mixtures this is quite evident. This combination can rightfully be termed synergistic with specific reference to the antibacterial properties of the combination. The other combinations (Table II) are not synergistic in an antibacterial reference but are superior as combinations with reference to their tissue substantivity. The data on synergistic combinations, to a greater extent than the corresponding data on separate components, provide some evidence that antibacterial activity is no criterion by which to predict the tissue substantivity. The substantivity will have to be determined separately.

It is valid to conclude from these data that the mode of action by which a compound or a mixture of compounds inhibits the growth of organisms is not directly related to the mechanism of tissue substantivity. If it were, then the more active compounds should also be expected to be the more substantive, as determined by the procedure used in the present study.

The same lack of correlation between antibacterial activity and substantivity appears to be true of the relationship of chemical structure and substantivity. However it must be pointed out that more definitive studies need to be completed before any final conclusions can be made. From the evidence obtained thus far it appears that, at best, no simple correlation between chemical structure and tissue substantivity can be made.

TABLE I

Minimum Inhibitory Concentration and Tissue Substantivity of Selected Compounds vs. *S. aureus*

	Compound	M.I.C.[1] (γ/ml.)	Substantivity[1] (S—P)
A.	3,4,4′-Trichlorocarbanilide	0.133	134
B.	4′,5-Dibromosalicylanilide	0.375	83
C.	Cetyl pyridinium chloride	0.406	116
D.	Decamethylenebis (4-aminoquinaldinium acetate)	0.541	28
E.	Alkylbenzyldimethyl ammonium chloride	0.617	72
F.	2,2′-Methylenebis (3,4,6-trichlorophenol)	0.719	56
G.	Dodecyldimethyl (2-phenoxyethyl) ammonium bromide	1.15	31
H.	3,4′,5-Tribromosalicylanilide	1.18	37

[1] Average of at least three determinations.

TABLE II

Minimum Inhibitory Concentration and Tissue Substantivity of Synergistic Combinations vs. *S. aureus*

Combinations	M.I.C. (γ/ml.)	Substantivity (S—P)
Cetyl pyridinium chloride (10/11)[1] + Decamethylenebis (4-aminoquinaldinium acetate) (1/11)[1]	0.325	64
Cetyl pyridinium chloride (10/11)[1] + Dodecyldimethyl (2-phenoxyethyl) ammonium bromide (1/11)[1]	0.355	58
3,4′,5-Tribromosalicylanilide (4/5)[1] + 4′,5-Dibromosalicylanilide (1/5)[1]	0.666	77
2,2′-Methylenebis (3,4,6-trichlorophenol) (1/2)[1] + 3,4,4′-Trichlorocarbanilide (1/2)[1]	0.147	130

[1] Ratio of the weight of each component to the weight of its combination.

TABLE III

Chemical Structure and Tissue Substanti·

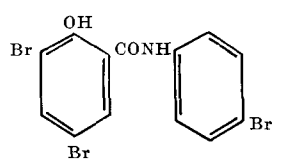
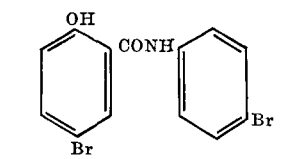
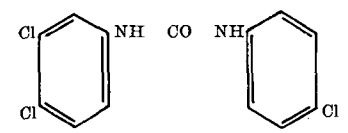
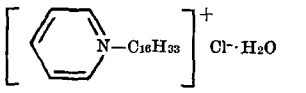
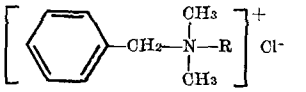
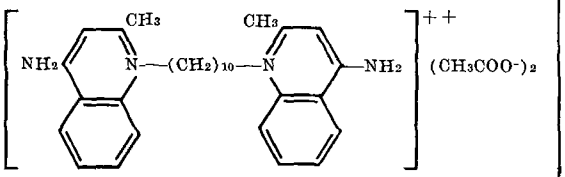
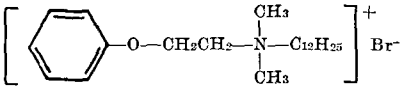
Structure	Substantivity (S—P)
	37
	83
	134

TABLE IV
Quaternary Ammonium Compounds and Tissue Substantivity

Compounds	Substantivity (S—P)
	116
 where R = $C_{12}H_{25}$ to $C_{16}H_{33}$	72
	28
	31

Evidence of this can be obtained from the wide variation given in Table III. If there were a relationship, the substantivity data for the salicylanilides should be quite similar. However, as can be seen, they are quite unlike, and the change from the dibromo- to the tribromosalicylanilide would not normally be expected to cause such a great difference in substantivity of the two compounds. The tissue substantivity of the dibromosalicylanilide might possibly be explained on the basis of lesser acidity, i.e., less acidity than the tribromosalicylanilide. However further investigations would have to be carried out to determine if this is so. This point could be answered if the substantivity of a 3,4′-dibromo-5-methylsalicylanilide were evaluated. Should this compound be more substantive than the dibromosalicylanilide, there would be some evidence for explaining substantivity on the basis of acidity. On the other hand, to determine if the presence of bulky groups around the hydroxyl in the 3,4′,5-tribromosalicylanilide hinders its phenolic function (making this a cryptophenol) and thereby lowers the substantivity of the compound, the evaluation of another compound, for example 4,4′,5-tribromosalicylanilide, would have to be studied. If this compound were more substantive than the 3,4′,5-tribromosalicylanilide, the reduction in substantivity could then be related to the presence of the elements of a cryptophenol. These are only some of the ways in which mechanisms of tissue substantivity could be studied.

An even more marked difference exists in substantivity between the tribromosalicylanilide and the trichlorocarbanilide, which are related to the degree that both are amides with substituted benzene rings. Again, there is no obvious relationship between these structures and substantivity. Other examples of the lack of relationship between chemical structure and substantivity are given in Table IV. The comparative data obtained for the quaternary ammonium compounds are even more divergent. This class of compounds, taken as a group, shows almost no correlation with reference to substantivity. However if, in the case of these compounds, substantivity is related to the charged nitrogen atom, then it may be possible to explain the results (Table IV) in terms of the effects of steric hindrance. As the charged nitrogen atom is progressively blocked by the surrounding chemical groups, one would expect a reduction in the substantivity of the compound, and this appears to be the case. Whole series of homologous compounds similar to those indicated (Table IV) would have to be studied before a final conclusion could be made. Preliminary evidence seems to indicate that there is no simple relationship between tissue substantivity and chemical structure.

ACKNOWLEDGMENTS

The authors acknowledge the cooperation and helpful suggestions obtained from discussions held with C. Fox relating to the quaternary ammonium compounds and E. Goldschmidt relating to salicylanilides and related compounds.

REFERENCES

1. Rammelkamp, C. H., Proc. Soc. Exptl. Biol. Med. *51*, 95 (1942).
2. Vinson, L. J., E. L. Ambye, A. G. Bennett, W. C. Schneider and J. J. Travers, J. Pharm. Sci. *50*, 827 (1961).