value obtained for Γ_w, 0.37 nm^2/molecule, is in good agreement with the estimated cross-sectional area of the surfactant ion (13).

There is no similar region in the present system. The explanation of this difference appears to concern the ionic nature of the choline group in comparison with the nonionic nature of the alcohol. With octadecanol monolayers, the surfactant ion is being adsorbed into holes in an uncharged matrix of octadecanol molecules; with the lecithin monolayers, the matrix consists of the zwitterionic phosphatidyl choline. The positively charged quaternary ammonium part of the lecithin molecule apparently dominates the penetration process so that, even when the adsorption of surfactant ions is small, the ionic environment is roughly similar to that found with the higher adsorption at a monolayer-free surface. Hence, in both situations, the adsorption is limited by electrostatic effects and not by the size of the surfactant ion.

The shape of the surface pressure–area isotherms of the penetrated lecithin monolayers is interesting (Fig. 1). At very low areas, all surfactant molecules are squeezed out of the surface, as indicated by the tendency of the isotherms of penetrated monolayers to join up with the surface pressure–area isotherm for the lecithin monolayer on water. Unfortunately, there was too much scatter in the data to allow adsorptions to be calculated at areas per molecule lower than 0.45 nm^2/molecule, but qualitatively the ejection of surfactant is clearly shown by the isotherms in Fig. 1.

CONCLUSION

Analysis of the equilibrium penetration of dipalmitoyllecithin monolayers by cetrimonium bromide according to Eq. 1, derived from the accessible area theory (12), shows that: (*a*) good straight lines are obtained, as required by the theory; and (*b*) the values of the parameters a_M and Γ_w obtained from these analyses are physically reasonable in that they correspond to the effective molecular areas of the lecithin in the various monolayer states and to the adsorption of the surfactant at a monolayer-free surface, respectively.

It is concluded that the accessible area theory provides a satisfactory description of equilibrium penetration in the present system. Alternatively, the theory could have been used to predict equilibrium penetrations from the surface pressure–area isotherm of dipalmitoyllecithin and the adsorption of cetrimonium bromide at a monolayer-free surface.

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Effects of Paper on Performance of Antibiotic-Impregnated Disks

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Received February 7, 1977, from the *Antibiotic Residue Branch, National Center for Antibiotics Analysis, Food and Drug Administration, Washington, DC 20204.* Accepted for publication June 23, 1977.

Abstract □ Grades of paper used in the manufacture and assay of antibiotic susceptibility disks have significant effects on the diffusion of antibiotics from the paper when compared to a control grade of paper. The papers also evoke different microbiological responses to changing concentrations of some antibiotics. Regulatory implications and the need for further standardization of assays among control laboratories are explored. Grades of paper generally used for assay and control of susceptibility disks, on the other hand, appear to be comparable to each other in both respects.

Keyphrases □ Antibiotic susceptibility disks—effect of various grades of paper on performance □ Paper, various grades—effect on performance of antibiotic susceptibility disks □ Disks, paper—effect of various grades on performance in antibiotic susceptibility tests

That different grades of paper affect diameters of zones of inhibition produced by known antibiotic concentrations has been recognized for some time (1, 2). One study (1) compared two grades of paper[1] to a control grade. One grade produced inhibition zones somewhat larger than the standard disk for each of four antibiotics; the other grade produced either significantly smaller zones or none at all.

Marth *et al.* (2) determined that use of 6.35- and 12.7-mm disks of the same grade of paper permitted detection of the same low levels of penicillin in milk but that 6.35-mm disks of another grade were generally able to detect only higher concentrations of antibiotic under the same conditions.

[1] Whatman.

Three grades of paper from the same manufacturer were specified for use in assays and in collaborative studies of methods designed to detect penicillin in milk (3–6). The three were found to be comparable in absorptivity, purity, and performance.

The Code of Federal Regulations, §460.6(d) (7), requires that control disks be 6.35 mm in diameter with a basis weight of 26–34 mg/cm^2. Control disks must also be able to absorb 2.5 times their weight of distilled water. In addition, the paper used must neither buffer nor affect the pH of any solution placed on it. The paper can neither enhance nor diminish the activity of any antibiotic solution incorporated into it.

The Code of Federal Regulations, §460.1(a) (7), also requires that the paper used to produce susceptibility disks be capable of complete absorption of 20 μl of solution/31.67 mm^2 of paper (0.25-in. diameter round disk). In addition, the absorbent paper and the dye or ink used must not influence either bacterial growth or activity of the antibiotic.

Kramer and Kirshbaum (1) concluded that control disks in all laboratories should be prepared from the same specified grade of paper to eliminate interlaboratory variation due to papers. The laboratories that control the disks sold in the United States have used various grades to prepare standard disks. Each disk manufacturer punches antibiotic disks from its own proprietary grade of paper. These proprietary grades are represented by the paper manufacturer[2] to be of essentially the same composition and to perform similarly.

The present study compared the performances of disks of all four grades of paper currently used for manufacture or control of disks to the performance of a lot of control disks. In addition, the performance of a second lot of control disks is discussed.

EXPERIMENTAL

Antibiotics selected for the study generally were those listed in the "Standardized Disc Susceptibility Test" described in §460.1 (7), with the exception of clindamycin and oleandomycin. For each antibiotic tested, one or more weighings of the appropriate Food and Drug Administration (FDA) working standard were dissolved in the solvent indicated in §460.6(d) (7). In each instance, the concentration of the initial stock solution was the highest concentration of the standard curve. Two dilutions in the same solvent were made from each stock solution to provide three doses that were equally spaced logarithmically. Interdose ratios varied from 2:1 to 4:1.

Blank disks of various grades of paper from three suppliers[3] and two lots of blank A-740-E[4] disks were used. All disks were 6.35 mm in diameter.

Disks of grades B-126[5], B-225[5], B-676[5], A-470[6], and lots 7810 and 9996 of A-740-E were placed on a 10-mesh stainless steel screen mounted on a wooden frame. Aliquots (20 μl) of each solution were dispensed to all disks being tested at one time with a syringe[7] mounted in a repeating dispenser[8]. Disks were then dried in circulating air at ambient temperature (24–28°) for a minimum of 3 hr.

Except as specified below, 150 × 15-mm assay dishes were prepared according to directions in §460.6(c) (7). Gentamicin and tobramycin plates contained a 42-ml base layer of neomycin assay agar (Antibiotic Medium 11) overlaid with 8 ml of neomycin assay agar inoculated with 0.1% of a suspension of *Staphylococcus aureus* (ATCC 13150), which had been grown for 24 hr at 37° on a Roux bottle containing 250 ml of seed agar (Antibiotic Medium 1) and washed off with 50 ml of sterile USP saline TS. Carbenicillin plates contained a 42-ml base layer of Antibiotic Medium 1 overlaid with 8 ml of 1.5% agar, which had been inoculated with 5% of a suspension of *Pseudomonas aeruginosa* (ATCC 25619) prepared according to §460.6(b) (12) (7).

Assays were performed, and the results were calculated according to the 3 × 3 factorial design of USP XIX (8). Lot 7810 of A-740-E paper was designated as the standard. All other papers were considered as unknowns. Each dose was replicated nine times on each lot of paper for each assay. Twelve disks, two of each content of antibiotic on both the standard and one test lot of paper, were placed in a circle in each plate. Five plates thus were used for each assay. All plates were incubated for 16–18 hr at 34 or 37°.

After incubation, zone diameters were estimated to the nearest 0.1 mm by projecting images onto a calibrated screen with an overhead projector[9] (12.5× magnification). Assay potencies and validity and slope values were calculated with a programmable calculator[10].

Table I—Relative Potencies of Antibiotic Solutions Obtained from Various Absorbent Papers Using 3 × 3 Assay Design [a]

Antibiotic	A-740-E, Lot 9996	A-470	B-126	B-225	B-676
Ampicillin	—[b]	106	108	123	96
	95	105	110	—	89
Bacitracin	100	97	113	109	92
Carbenicillin	98	104	100	109	99
Cephalothin	—	106	113	141	89
	95	106	117	—	87
Chloramphenicol	97	105	109	115	95
Colistin	—	93	98	80	55
	—	—	—	76	54
	95	92	103	—	—
Erythromycin	101	100	99	101	92
Gentamicin	99	99	97	107	60
Kanamycin	—	97	93	94	69
	99	98	90	—	70
Methicillin	104	101	99	111	98
Neomycin	105	99	80	86	53
Novobiocin	98	99	102	113	93
Penicillin G	100	102	103	129	82
Polymyxin B	94	98	115	120	69
Rifampin	—	107	96	122	71
	98	106	99	120	—
Streptomycin	96	99	92	105	69
Tetracycline	—	91	—	100	85
	—	97	138	—	94
	100	93	119	102	89
Tobramycin	—	106	—	96	52
	101	—	—	108	59
	110	107	91	—	—
Vancomycin	98	96	97	118	84

[a] Expressed as percentages of the standard, which is A-740-E, lot 7810. [b] Each line for an antibiotic represents assays done on a single day; dashes indicate that no assay was performed with the particular paper on that day.

RESULTS AND DISCUSSION

Relative potency results obtained from 3 × 3 assays for each grade of paper are presented in Table I. Each value represents one assay of 27 disks of the test lot of paper compared to 27 disks of the standard. All values given on a single line in Table I were derived from disks of the various papers prepared consecutively by one analyst from the three solutions of antibiotic.

It is apparent from the data that the performances of A-470 and lot 9996 of A-740-E papers were comparable to the performance of the standard. Of 47 assays performed with the two papers, only one test of lot 9996 yielded a result differing from the standard by >10%. Under the conditions followed in this study, an assay result that deviates by >±10% from the standard of comparison is considered significant.

Papers used for commercial production of disks generally do not release antibiotics comparably either to another manufacturer's papers or to each other. Grade B-676 released less antibiotic than any other paper tested.

[2] Eaton-Dikeman, Mount Holly Springs, PA 17065, personal communication.
[3] BBL Division of BioQuest, Cockeysville, MD 21030; Difco Laboratories, Detroit, MI 48232; and Pfizer Disks, Inc., Barceloneta, PR 00617.
[4] Grade 740-E paper, Schleicher & Schuell, Keene, NH 03431.
[5] Paper grades 126, 225, and 676, Eaton-Dikeman, Mount Holly Springs, PA 17065.
[6] Grade 470 paper, Schleicher & Schuell, Keene, NH 03431.
[7] Model 1001, Hamilton Co., Reno, NV 89502.
[8] Model PB 600-1, Hamilton Co.
[9] Balopticon, Bausch & Lomb, Rochester, NY 14602.
[10] Model 9830-A, Hewlett-Packard, Loveland, CO 80537.

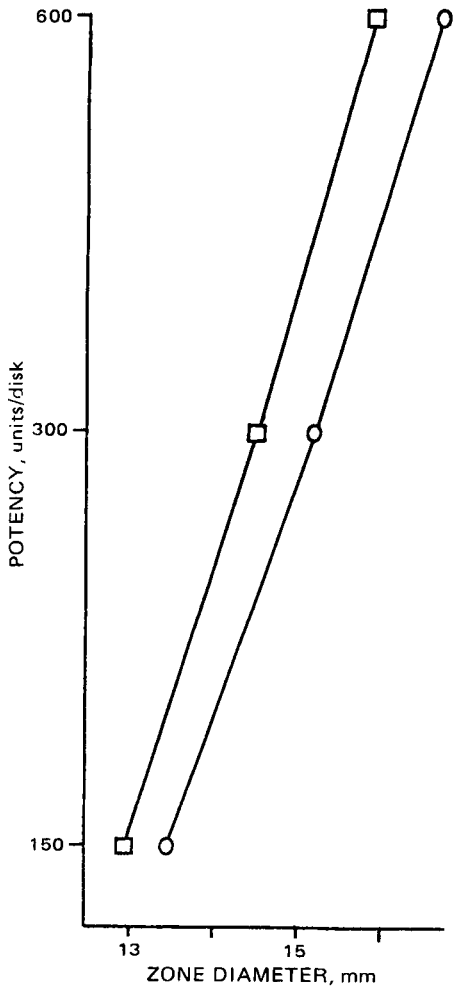

Figure 1—*Graph showing the parallel and valid response lines promoted by B-676 (□) and A-740-E (○) paper disks impregnated with polymyxin B. Grade B-676 paper appeared to adsorb or bind a significant quantity of several antibiotic solutions applied to it. In most instances, the loss of activity was proportional to the initial concentration of solution so that the slope of the dose response was not affected.*

Of all drugs studied, B-676 performed similarly to A-740-E only for bacitracin, carbenicillin, chloramphenicol, erythromycin, methicillin, and novobiocin. Grade B-676 paper appeared to bind, either physically or chemically, a particularly high proportion of the oligosaccharide and polypeptide antibiotics. This binding effect tied up 30–50% of the antibiotic loaded onto the disk compared to the standard paper.

Of the production papers studied, the performance of B-126 was the most similar to that of the A-740-E disks. This paper produced results differing by more than 10% from the standard only for bacitracin, cephalothin, neomycin, polymyxin, and tetracycline. In every instance, B-126 promoted greater diffusion than did B-676 paper. Grade B-126 generally indicated lower potencies than did B-225; higher potencies were obtained from B-126 only for bacitracin, colistin, and tetracycline. The result obtained for tetracycline with B-126 was significantly higher than for B-225 and B-676.

Relative potencies obtained with B-225 paper frequently differed significantly from the standard. Assays of colistin and neomycin were 14–24% lower than the standard, while relative potencies of ampicillin, cephalothin, chloramphenicol, methicillin, novobiocin, penicillin G, polymyxin B, rifampin, and vancomycin exceeded the standard by 10–41%. Ampicillin, penicillin G, and cephalothin results were particularly high. With few exceptions, this paper resulted in higher assays than any other paper tested, but B-126 gave higher recoveries of colistin and tetracycline than did B-225.

The regulations, §460.1(a) (7), state: "The absorbent paper and dye or ink used (to produce antibiotic susceptibility discs) must not affect either bacterial growth or the antibiotic." The data presented here strongly suggest that the grades of paper used in the manufacture of commercial susceptibility disks do affect the diffusibility of all antibiotics studied except carbenicillin and erythromycin. The widely disparate relative potencies obtained from one or more of the production papers for each of the other antibiotics shown in Table I indicate that antibiotic activity is influenced by the respective papers. This influence may be either negative or positive.

All official microbiological assays of antibiotics in the United States involve use of multiple doses of standard to define a response line and a single dose of sample, the response of which is compared to the standard curve. This assay design requires three basic assumptions: the response line must be statistically linear throughout the range of contents covered by the standard curve, the standard and sample must produce parallel response lines, and a given quantity of antibiotic in a sample must cause the *same* response as a like quantity of standard.

Antibiotic disks are certified by FDA on the basis of their performance under specified conditions rather than on actual content of antibiotic in the disks. Potency assays only determine that commercial disks produce inhibition zones of similar diameter and definition as disks made from official FDA antibiotic standards. As long as standard and manufactured disks exhibit parallel dose responses, determinations of potency by this means are accurate and valid.

Dose responses evoked by the papers for each antibiotic were studied with the USP 3 × 3 factorial design (8). Since disks of all papers were prepared by hand from the same solutions of antibiotic standards, any influences of formulation and/or manufacturing processes were eliminated. As expected, most assays demonstrated parallel responses by the different papers. The A-470 and A-740-E papers performed similarly to each other in this regard. The majority of the very low relative potencies obtained from B-676 paper (Table I) were valid because of the parallelism of the dose responses (Fig. 1). As Fig. 1 clearly shows, however, a quantity of polymyxin on B-676 paper did not produce the same response as the same quantity on the standard paper. The paper caused the same effect with cephalothin, kanamycin, penicillin, rifampin, streptomycin, tetracycline, tobramycin, and vancomycin. The assays of colistin, gentamicin, and neomycin on B-676 paper, which produced exceptionally low results, were not valid because of a lack of parallelism between the respective dose responses and those of the standard.

Standard control disks are prepared for each antibiotic subject to certification by FDA from blank disks of A-740-E paper. Thus, in the certification procedure, the quantity of antibiotic loaded onto a commercial disk is tested in combination with the commercial paper against a known quantity of drug loaded onto, and acting in concert with, A-740-E paper. Within limits specified in the regulations, §460.6(f) (7), the antibiotic and paper of production disks must perform similarly to the FDA working standards and the A-740-E paper.

In some instances, the various grades of production paper appeared to influence markedly the dose response of the test organism to varying amounts of antibiotic. Grade B-126 paper seemed to diminish the dose response to tetracycline, producing a slope (defined as the change in zone diameter caused by a 10-fold increase in antibiotic content, *i.e.*, a logarithmic cycle, as determined by the zone diameters produced by the lowest and highest doses of antibiotic) of approximately 4.5 mm while the other grades, including A-470 and A-740-E papers, resulted in slopes of about 6.5 mm.

More frequently, however, these papers tended to enhance the dose response to the antibiotics. Grades B-225 and B-676 both appeared to produce a significantly greater dose response to colistin and gentamicin than did the other papers. With gentamicin, A-470, A-740-E, and B-126 produced slopes of approximately 4 mm, whereas the B-225 and B-676 values were closer to 6 mm. These two papers promoted slopes of essentially 7.5 mm with colistin, whereas the other papers each evoked about a 5.5-mm response. These increased responses (Fig. 2) cause invalid assays because of the lack of parallelism of the response lines. Since the two papers do not meet the requirement of parallel dose responses for a valid assay, potency assays of the two drugs on B-225 and B-676 paper are inaccurate.

For the same reasons, *i.e.*, increased dose responses and, therefore, not parallel response lines, assays of neomycin on B-676 paper and tobramycin on B-126 paper are of no value. Grade B-676 caused a response of 6 mm to neomycin while the other papers resulted in slopes closer to 5 mm. Slopes of essentially 4.5 mm were observed for tobramycin except with B-126, which caused a response of 6 mm. The diminished response to tetracycline on B-126 paper, discussed earlier, similarly renders assay of this product worthless, because a significantly greater quantity of this antibiotic is released from the paper at the low dose, compared with A-740-E, than is released at the higher levels. This results in a nonparallel response line and a nonlinear line from the B-126 paper.

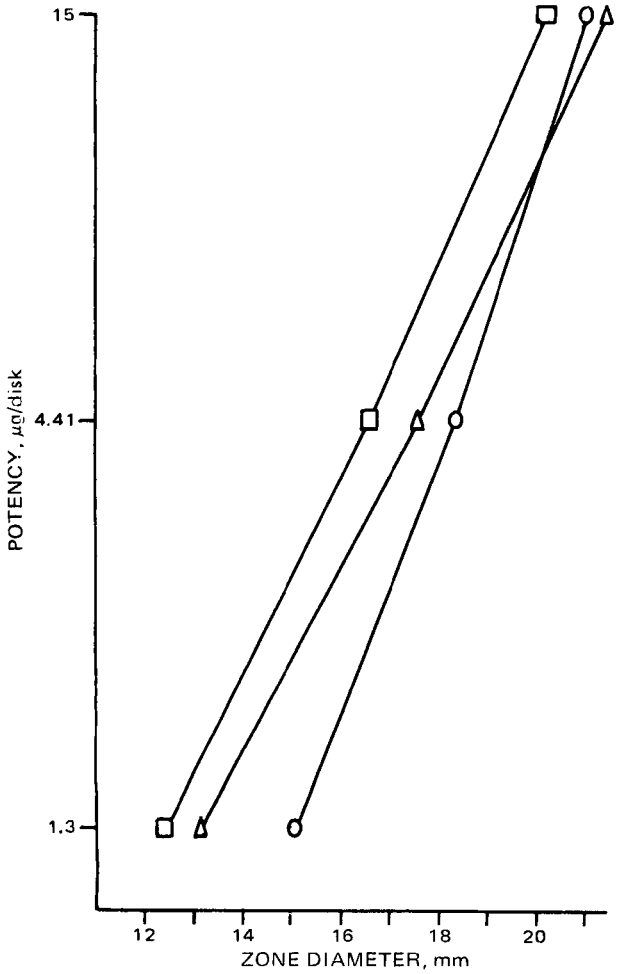

Figure 2—*Graph showing intersecting response lines due to enhanced dose response of* Bordetella bronchiseptica *to colistin on B-225 (△) and B-676 (□) papers compared with the response evoked by A-740-E (○). The same effect was noticed for aminoglycoside antibiotics with one or another of all commercial production papers. None of these assays produces meaningful results because of the lack of parallelism of the respective response lines produced by the production papers compared to the responses from the standard A-740-E paper.*

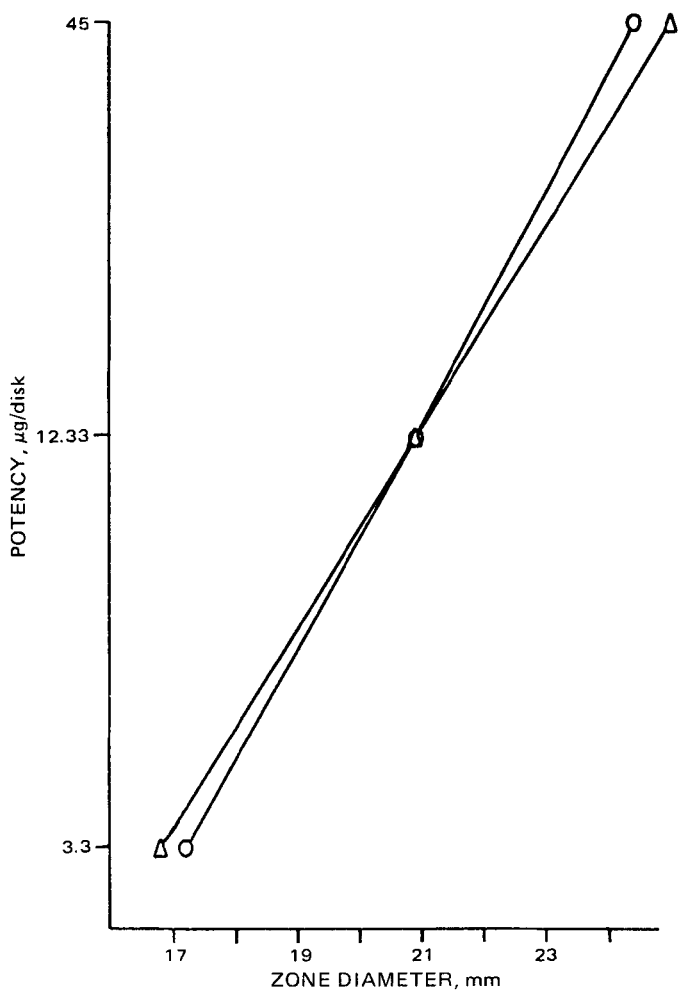

Figure 3—*Graph of valid assay of tetracycline on B-225 (△) paper against* Sarcina lutea. *Penicillin on B-126 paper showed the same relationship to A-740-E (○) standard disks. The respective response lines are not sufficiently divergent to invalidate the assay, but an assay of any batch of disks with antibiotic concentration close to either end of the standard curve will result in a potency value quite dissimilar to the actual content of antibiotic.*

The significant changes in slope of these respective dose responses reflect effects of the papers on bacterial growth. Thus, H-126 masks the effectiveness of tetracycline against the test organism. The opposite effect is caused by one or more of all three commercial papers in combination with colistin, gentamicin, neomycin, and tobramycin.

When analyzed by the statistical methods presented in USP XIX (8), penicillin assays on B-126 paper and tetracyline assays on B-225 paper are both valid. In these instances (Fig. 3), the slope from the B-126 paper for penicillin was approximately 6.5 mm while the slope of the standard A-740-E paper line was about 5.5 mm. The slope generated by B-225 paper with tetracycline was close to 7.5 mm, while the standard paper created a response of about 6.5 mm. The two response lines intersected nearly at the center. Thus, a lot of disks made with this paper and formulated at a potency near the center of the curve will assay in close agreement with theory. However, if the lot is formulated closer to either end of the response line to achieve the labeled content, assay potency will diverge proportionately from theory.

Tetracycline susceptibility disks are marketed in the United States with labeled concentrations of 5, 10, and 30 μg. The Code of Federal Regulations, §460.6(f) (7), requires the potency of a batch to be between 67 and 150% of the labeled strength to be certified for use. With the differences in dose response of the test organism promoted by B-225 and A-740-E papers impregnated with this antibiotic, a 5-μg disk produced on B-225 paper that actually contains 3.8 μg of tetracycline will act as though it only holds 3.3 μg when tested beside A-740-E paper. Conversely, a 30-μg disk of grade B-225 paper formulated to contain 37 μg of drug can be expected to produce the same response by the test organism as an A-740-E disk containing 45 μg.

Thus, a commercial disk of the B-225 paper that actually contains 3.8 μg of tetracycline is apt to fail the performance test as being subpotent; a disk containing 37 μg, well within legal requirements for a 30-μg disk, is likely to fail the performance test as superpotent. Such results tend to confound both production and analytical departments.

The differences in the diffusion characteristics of the various grades of paper clearly outline the need for one specified paper for the preparation of standard control disks. Data presented here indicate that the two lots of A-740-E paper act similarly with regard to diffusion of all currently used concentrations of each antibiotic studied, as Kramer and Kirshbaum (1) previously reported. The performance of the single lot of A-470 paper was also comparable to that of the A-740-E papers. Since A-740-E paper is readily available as punched 6.35-mm disks, the use of such disks could help fulfill the need for uniform standard disks by control laboratories.

Because paper is, in essence, a natural product and subject to certain variation in both the source fiber and the manufacturing process, it might be advisable for all control laboratories, insofar as possible, to use the same lot of paper for the preparation of standard disks. In this way, analytical discrepancies due to possible variations in lots of the same grade of paper would be reduced to the greatest extent, at least for the majority of assays that are valid in design.

SUMMARY

Three grades of papers used in the production of antibiotic susceptibility disks and two grades of papers used by control laboratories to assay

susceptibility disks were compared for performance. Grades A-740-E, A-470, and B-126 papers perform in the same manner. Grade B-676 paper appears to bind a high percentage of most antibiotic solutions applied to it. This binding is particularly pronounced with polypeptide and aminoglycoside drugs. Grade B-225 paper, on the other hand, frequently releases antibiotics at greater rates than the A-470 and A-740-E papers.

Analysis of slopes of dose–response lines did not show the same effects on classes of antibiotics from one paper to another. Again, the A-470 and A-740-E papers were generally equivalent for each drug. In a few instances, one or another of the production papers appeared to enhance the dose response compared to the slopes produced by A-740-E paper. Grade B-126 decreased the dose response to tetracycline. These types of differences in dose responses between papers nullify the concept of the design of the performance test used to certify production lots of the respective disks, rendering assay of these products quite inaccurate. Thus, it has been shown that, contrary to the intent of the Code of Federal Regulations, §460.1(a) (7), each grade of paper used in the manufacture of antibiotic susceptibility disks affects bacterial growth and/or the antibiotic in one or more instances.

Most potency assays are statistically valid. However, since sample disks of production papers frequently do not evoke the same response as a like quantity of antibiotic on a standard disk, universal agreement among control laboratories as to the type of paper to be used to prepare standard control disks is highly desirable. Such agreement could extend to the use of a common lot of paper by those laboratories and would greatly enhance the likelihood of interlaboratory concurrence in potency values of commercial lots of antibiotic disks.

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Microdetermination of Procainamide in Human Serum

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Abstract □ An electron-capture GLC method to measure procainamide (0.1–1 μg/sample) in human serum was developed. An internal standard, *p*-amino-*N*-[2-(dipropylamino)ethyl]benzamide, is added to the serum before the sample is alkalinized with pH 10.5 phosphate buffer and extracted with ethyl acetate. The ethyl acetate phase is evaporated to dryness, and the residue is reacted with pentafluoropropionic anhydride. *N*-Pentafluoropropionyl derivatives of the drug and the internal standard had retention times of 5 and 8 min, respectively, when chromatographed at 235° on a 1-m (4-mm i.d.) glass column packed with 5% OV-17 (carrier gas flow of 40 ml/min). The coefficient of variation was less than 5% for spiked standards. Furthermore, *N*-acetylprocainamide added to samples did not interfere. One hundred and eighty-six samples from 16 patients receiving procainamide intravenously were assayed by this GLC procedure and by a standard colorimetric method. Linear regression analysis yielded a correlation coefficient of 0.985 (slope, 1.040; intercept, 0.015).

Keyphrases □ Procainamide—electron-capture GLC analysis in human serum □ GLC, electron capture—analysis, procainamide in human serum □ Cardiac depressants—procainamide, electron-capture GLC analysis in human serum

Procainamide has been used clinically over the last 25 years for the prevention and treatment of ventricular arrhythmias. Several studies (1–4) demonstrating the utility of plasma procainamide concentrations in dosage regimen design have stimulated interest in procainamide assays.

The colorimetric technique of Mark *et al.* (5) is commonly used to determine procainamide in biological fluids. However, Gibson *et al.* (6) reported that *N*-acetylprocainamide, a major metabolite of procainamide in humans (7), may be hydrolyzed to procainamide under certain conditions. Recently, it was found (8) that the colorimetric method (5) as modified by Sitar *et al.* (9) did not result in the hydrolysis of *N*-acetylprocainamide. A specific flame-ionization detection GLC assay for procainamide was developed (10) and gave results similar to those of the colorimetric analysis.

Several GLC techniques (10–13) and a high-pressure liquid chromatographic (HPLC) procedure (14) were described for procainamide analysis, but these methods require at least 0.5–1 ml of serum. This report describes an electron-capture GLC determination of procainamide that requires as little as 0.1 ml of plasma. In addition, the results of this new procedure are compared to those obtained using the modified colorimetric procedure (9).

EXPERIMENTAL

Reagents—Stock solutions of procainamide hydrochloride[1], *N*-acetylprocainamide hydrochloride[2], and the internal standard, *p*-

[1] ICN Pharmaceuticals, Plainview, N.Y.

[2] Astra Pharmaceutical Products, Worcester, Mass.