Identification of Antibiotics in Sensitivity Disks

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Identity tests for antibiotic sensitivity disks have been developed. Suitable tests previously reported in the literature have been modified and adapted to the microquantities involved in these disks. Once a system of testing had been established, it was screened for interference from all other disk antibiotics. Penicillins are identified generally by an iodometric method and specifically by thin-layer chromatography (TLC). The polypeptide antibiotics are differentiated by TLC in combination with nitration, reduction, and diazotization to further distinguish colistin from polymyxin. Lincomycin, oleandomycin, and kanamycin are identified by TLC systems, the tetracyclines by paper chromatography. Under varying conditions of solvents and wavelengths, streptomycin, erythromycin, nystatin, vancomycin, novobiocin, cephalothin, and viomycin are identified spectrophotometrically. Color tests have been devised for chloramphenicol, dihydrostreptomycin, and neomycin.

THE FIRST antibiotic disks, made by Bondi *et al.* in 1947, were widely recognized as "diagnostic aids for the rapid determination of the susceptibility of microorganisms to antibiotics" (1). Since then, they have proved as valuable as anticipated, and have widespread use in hospitals and clinical laboratories throughout the world. Because of the reliance placed upon information elicited from their use, confirmatory evidence of the labeled identity of the antibiotics in the disks is of great importance. This paper describes laboratory procedures developed for this purpose.

Regulations describing the tests and methods of assay and requirements for certification of antibiotic disks are published in the Code of Federal Regulations, title 21, chapter 1, part 147. Table I lists the antibiotic disks now included in these regulations with references to the applicable identity tests described in this paper.

Although antibiotic disks are tested by a "performance" method for inhibitory response against specific microorganisms and are compared with control disks of known potencies, the zone of inhibition obtained does not always specifically identify the antibiotic. For example, from microbiological assay alone, positive differentiation cannot be made between colistin and polymyxin, and between chloramphenicol and the tetracyclines; among the various tetracyclines, and among the various penicillins; between kanamycin and neomycin, and between dihydrostreptomycin and streptomycin, particularly if the potencies deviate greatly from the label claims. While it may not be of the utmost medical importance to differentiate one tetracycline from another, it is important to distinguish a tetracycline from chloramphenicol, or a semisynthetic penicillin from cephalothin or penicillin G. Some antimicrobial agent other than an antibiotic may conceivably be present and under certain conditions may be undetected by microbial assay. Therefore, a system of chemical identity tests for antibiotics in disks was established to supplement the quantitative information obtained by microbiological assay techniques.

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IDENTIFICATION PROCEDURES

I. Penicillins—(*a*) *Iodometric Test*—This general screening procedure for penicillin and cephalothin involves a modification of the iodometric assay method (2). Place enough disks in each of two tests tubes to provide 30 mcg. of sodium cephalothin, 20 units of penicillin G, or 4 mcg. of other penicillins. To both tubes add 1 ml. of 1% phosphate buffer (pH 6) and shake well. To one tube, add 1 ml. of 1 *N* sodium hydroxide, shake, and let stand 15 min. Then add 1 ml. of 1.2 *N* hydrochloric acid and 0.2 ml. of 0.001 *N* iodine solution; after 15 min. add 1 drop of starch indicator solution. To the second tube add 2 ml. of distilled water, 0.2 ml. of iodine solution, and 1 drop of starch indicator solution. If penicillin or cephalothin is present, the first tube remains colorless, while the second develops the characteristic blue of the starch–iodine complex.

(*b*) *Chromatography*—Blend 30 Gm. of cellulose MN 300 without binder (Macherey, Nagel, Westbury, N.Y.) with 200 ml. of distilled water at high speed for 15 min. Apply this slurry to 20 × 20 cm. glass plates with a Brinkmann-Desaga applicator set for a film depth of 0.250 mm. Air dry the plates overnight and use without preactivation. Place enough disks to provide not less than 10 mcg. of penicillin in a sintered-glass microfi ter. Pipet 1.0 ml. of methanol onto the disks and allow to steep, with occasional stirring, for 5 min. Then apply suction and collect the filtrate in a small, flat-bottomed vial (15 × 75 mm.). Evaporate the extract to dryness under a stream of air, and redissolve in 50 μl. of methanol. Spot this solution on a TLC plate in 1-μl. increments until 1 mcg. has been applied.

Prepare a standard for each antibiotic from standard disks and spot each on the plate in the same manner. Standard and sample extracts may be spotted both individually and as a mixture to

insure homogeneity. Use only freshly extracted solutions. When the plate has been spotted, place it in a rectangular tank lined with heavy filter paper saturated with the solvent system, a 0.1 *M* sodium chloride solution (3). Place a glass trough in the bottom of the tank with solvent and allow the plate to rest directly in it. Allow the solvent front to travel 10 cm. from the starting line, then remove the plate from the development tank and allow to air dry. Lightly spray the plate with a freshly prepared solution of 2 parts 10% aqueous ferric chloride, 1 part 5% aqueous potassium ferricyanide, and 7 parts 20% sulfuric acid. After 5 min. for color development, the penicillins appear as blue spots on a green background. Directly compare the positions of the sample spots with the standards. The average R_f values of 10 runs are: sodium nafcillin, 0.45; cloxacillin, 0.62; sodium oxacillin, 0.68; potassium phenethicillin, 0.76; penicillin G, 0.85; and methicillin 0.89.

The tetracyclines have R_f values from 0.50 to 0.65 but are yellow before the color reagent is applied and in addition, yield negative iodometric reactions. Vancomycin hydrochloride appears at an R_f of about 0.90, but also yields a negative iodometric test.

(*c*) *Ninhydrin Test*—Ampicillin is the only penicillin that reacts with ninhydrin. After identifying it as a penicillin by the iodometric test, staple one disk to a sheet of Whatman No. 1 chromatographic paper, and apply one drop of ninhydrin reagent (1% ninhydrin in 100 ml. of water-washed *n*-butanol containing about 1 ml. of pyridine). Dry and heat the disk with a hot-air gun. Ampicillin yields a characteristic purple color.

II. Bacitracin, Colistin Sulfate, Kanamycin Sulfate, Neomycin Sulfate, and Polymyxin B Sulfate—(*a*) *Chromatography*—Apply Silica Gel G on glass plates (20 × 20 cm.) manually with a Brinkmann-Desaga applicator to a depth of 0.250 mm. Air dry overnight. Commercial TLC sheets may also be used.

Extract the antibiotics from the disks in the manner described for the penicillins, using water instead of methanol. Extract a minimum of 20 mcg. from the disks so that, when the residue is dissolved in 50 µl. of water, a total of 4 mcg. will be spotted on a TLC plate. For each antibiotic, extract standard disks and spot the extracts on the plate. Prepare the tank in the manner described for the penicillins, and use the same type of tank. The two-phase resolving system consists of chloroform–methanol–17% ammonia (40:25:20) (4), and the portion in the glass trough must be freshly prepared for each run. Both phases are used together. After the plate has been placed in the tank to develop, seal the lid with masking tape. When the solvent front has moved 10 cm. (in about 1 hr.), remove the plate from the tank and allow it to air dry. Spray the plate with ninhydrin reagent (see Ic) or use a commercial aerosol containing 0.3% ninhydrin in ethanol. Heat the plate with a hot-air gun to make the spots visible. Bacitracin yields a very characteristic spot at the front. The R_f values are: colistin sulfate, 0.80; polymyxin B sulfate, 0.70; kanamycin sulfate, 0.50; and neomycin sulfate, 0.30.

(*b*) *Diazotization*—Since phenylalanine is one of the decomposition products of polymyxin B sulfate but not of colistin sulfate, one may be differentiated from another by nitration, reduction, and diazotization of this aromatic moiety (5). Extract 10–15 polymyxin B sulfate 50-unit or colistin sulfate 10-mcg. disks with 1 ml. of water into a 50-ml. conical flask and evaporate the extract to dryness under a stream of air. Simultaneously evaporate 1.0 ml. of solution containing 10 mcg. of phenylalanine. Add 1.0 ml. of fuming nitric acid to each flask, cover with a watch glass, and heat on a vigorously boiling steam bath for 30 min. Remove from heat and add 10 ml. of water, 2 ml. of concentrated ammonium hydroxide, and about 50 mg. of sodium hydrosulfite. Mix and allow to stand 20 min. Then cool the flasks in a large evaporating dish containing crushed ice. Add 5.0 ml. of a 5% sodium nitrite solution and 1.5 ml. concentrated hydrochloric acid. Let stand 30 min., maintaining the temperature below 5°. Add 10 ml. of 5% sulfamic acid, shake, and dispel the nitrous oxide fumes with a current of air. Add 4.0 ml. of freshly prepared 0.8% solution of *N*-(1-naphthyl) ethylenediamine dihydrochloride. If polymyxin B sulfate is present, a red-purple color similar to that of the phenylalanine will develop. Colistin sulfate will remain colorless or only faintly pink tinged.

Bacitracin gives a positive result in this test, but may be identified by the TLC system described for group II*a*. Ampicillin yields a positive reaction with ninhydrin and diazotization; it also moves with the solvent front in the TLC system for group II*a*, but may be distinguished from them by the iodometric test for penicillin.

III. Tetracyclines—*Chromatography*—Extract the disks in the same manner described for the penicillins, using methanol as solvent. Extract enough disks to yield 50 mcg. in 50 µl. Subject the solutions, along with appropriate standards, to ascending paper chromatography, using the method of Selzer and Wright (6). Use a stationary phase of MacElvaine's buffer (pH 3.5) and a solvent system of chloroform–nitromethane–pyridine (10:20:3). The resulting spots are visible under ultraviolet light in the presence of ammonia fumes. No interference arises from any of the other 23 antibiotics listed in Table I.

IV. Lincomycin Hydrochloride and Oleandomycin Phosphate—*Chromatography*—Extract ten 2-mcg. disks in the manner described for penicillins, but with 0.05% phosphate buffer (pH 6) instead of methanol. After evaporation, dissolve the residue in 50 µl. of the buffer and apply ten 1-µl. spots to a TLC plate prepared as for procedure II*a*. Treat standard disks in the same manner. Use the resolving system, methylethyl ketone–acetone–water (9.3:2.6:1), developed by Brodasky and Lummis (7). Develop the chromatogram and air dry the plate. Place the plate in an iodine atmosphere (a closed rectangular glass tank containing a small beaker with several iodine crystals warmed on a steam bath) until yellow spots appear. Remove the plate from the tank, allow it to stand several minutes, and then spray it lightly with starch indicator solution until the lincomycin hydrochloride or oleandomycin phosphate shows up as a dark purple spot. Make direct comparisons of the positions of the spots with the standards.

All the penicillins give two spots in this system. Cloxacillin, methicillin, penicillin G, potassium phenethicillin, sodium nafcillin, and sodium oxacillin

TABLE I—IDENTIFICATION PROCEDURES FOR ANTIBIOTIC SENSITIVITY DISKS

Antibiotic	Usual Potency Level[a] Low	High	Identification Procedure
Ampicillin	2	10	I*a,c*
Bacitracin	2 units	10 units	I*a*,II*a*
Sodium cephalothin	30	...	XI
Chloramphenicol	5	30	VI
Chlortetracycline hydrochloride	5	30	III
Sodium cloxacillin	1	...	I*a,b*
Colistin sulfate	2	10	II*a,b*
Demethylchlortetracycline hydrochloride	5	30	III
Dihydrostreptomycin sulfate	2	10	VIII*a,b*
Erythromycin	2	15	XIII
Kanamycin sulfate	5	30	II*a*
Lincomycin hydrochloride	2	...	IV
Methacycline hydrochloride	5	30	III
Methicillin	5	...	I*a,b*
Sodium nafcillin	1	...	I*a,b*
Neomycin sulfate	5	30	II*a,V*
Sodium novobiocin	5	30	VII
Nystatin	100 units	...	IX
Oleandomycin phosphate	2	15	IV
Sodium oxacillin	1	...	I*a,b*
Oxytetracycline	5	30	III
Penicillin G	2 units	10 units	I*a,b*
Potassium phenethicillin	3 units	...	I*a,b*
Polymyxin B sulfate	50 units	300 units	II*a,b*
Streptomycin sulfate	2	10	VIII*a,b*
Tetracycline hydrochloride	5	30	III
Vancomycin hydrochloride	5	30	XII
Viomycin sulfate	2	10	X

[a] Potency is given as mcg./disk unless otherwise stated.

have R_f values ranging from about 0.35–0.44 for the major spot, while ampicillin has its major spot at 0.16. Sodium cephalothin has one spot at R_f 0.35; the tetracyclines and nystatin all have one or two spots which are yellow *before* being exposed to iodine; and oleandomycin phosphate has one spot at R_f 0.24. Lincomycin hydrochloride is readily distinguished from all these, since it yields a single spot at an R_f of 0.56.

V. Neomycin Sulfate—Extract enough disks with 1 ml. of water to give at least 75 mcg. of antibiotic. Collect the filtrate in a 50-ml. round-bottom distillation flask. Add 5 ml. of 40% sulfuric acid; reflux for 100 min. and cool. Transfer the solution to a glass-stoppered test tube and extract with 2.5 ml. of xylene. Transfer the upper xylene layer with a syringe with a cannula tip to a second tube. Add 2.5 ml. of *p*-bromoaniline reagent (8), and shake vigorously. If the disks contain neomycin sulfate, a pink color develops on standing.

No other disk antibiotic has been found to interfere in this test but the pink color will not develop in the presence of water.

VI. Chloramphenicol—Steep at least four 30-mcg. disks in 1 ml. of methanol with occasional stirring for 2 min. Filter with suction into a test tube and wash into a 50-ml. glass-stoppered centrifuge tube with 10 ml. of water. Add 10 ml. of 12 *N* sodium hydroxide and 10 ml. of α-naphthol reagent (a 2 mg./ml. solution in 6 *N* sodium hydroxide, freshly prepared) (9). Heat the tube for 30 min. in a boiling water bath, cool in tap water for about 5 min., and dilute to mark with methanol. Immediately upon the addition of the methanol a blue color appears which deepens slightly upon standing.

None of the other 27 antibiotics in Table I gives a blue color.

VII. Sodium Novobiocin—Extract enough disks with 2 ml. of methanol to give 40 mcg. of novobiocin. Evaporate to dryness under a stream of air and dissolve the residue in 2 ml. of 0.1 *N* sodium hydroxide. Record the UV spectrum from 200 to 400 mμ in 1-ml. cells. A characteristic absorption curve is obtained with a maximum at 305 mμ, shoulders at 288 and 248 mμ, and a minimum at 260 mμ. Standard disks are similarly treated for comparison.

VIII. Streptomycin Sulfate and Dihydrostreptomycin—(*a*) Place single disks of sample and standard side by side on a spot plate. Add one drop of oxidized nitroprusside reagent to each disk (10). With either streptomycin sulfate or dihydrostreptomycin a red-orange color develops rapidly. Viomycin sulfate gives a faint response. None of the other disk antibiotics interferes. If a ninhydrin spot test on a single disk (method I*c*) is negative, viomycin sulfate is eliminated.

(*b*) Differentiate streptomycin sulfate from dihydrostreptomycin by extracting 60 mcg. from disks into a test tube with 2 ml. of water. To the extract add 0.4 ml. of 1 *N* sodium hydroxide. Place the same solution in both 1-ml. cells and, with a double-beam spectrophotometer, record the spectrum from 300 to 350 mμ. Return the contents of the cell to the tube and heat it in a boiling water bath for 5 min. Cool the tube to room temperature and record the spectrum from 300 to 350 mμ with the unheated solution in the reference cell. Streptomycin sulfate produces a broad maltol peak at 320 mμ (11). The ferric chloride color of this method (11) is too faint to be visible at this concentration.

No other antibiotic in Table I gives this maltol reaction; however, the lower potency streptomycin sulfate disks give inconclusive results, possibly

because of methylcellulose interference from the higher proportion of paper.

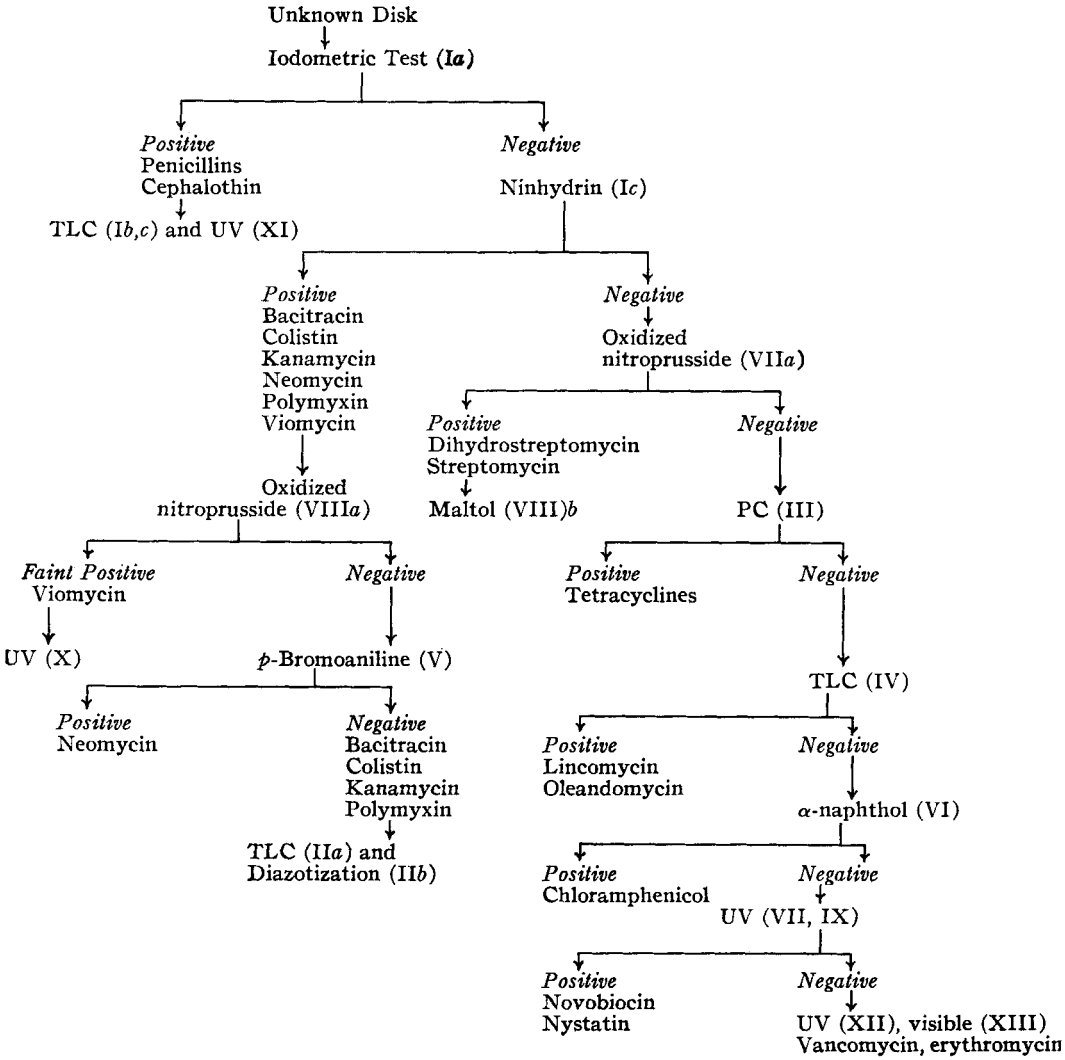
IX. Nystatin—Extract one 100-unit disk with three 1-ml. portions of methanol; collect and dilute to volume with methanol in a 5-ml. volumetric flask. Record the UV spectrum from 210 to 400 mμ. Characteristic peaks occur at 318, 304, and 291 mμ. Treat standard disks in a similar manner and compare spectra. No other antibiotic has a UV spectrum closely resembling that of nystatin.

X. Viomycin Sulfate—Extract five 10-mcg. or ten 2-mcg. disks with 1 ml. of 0.05% phosphate buffer, pH 6.0 Record the UV spectrum from 250–350 mμ against a buffer blank, using 1-ml. cells. Viomycin sulfate has a strong absorbance at 268 mμ. Although potassium phenethicillin absorbs at this wavelength, it is 10 times weaker in magnitude, and the shape of the potassium phenethicillin curve is more complex.

XI. Sodium Cephalothin—Extract four 30-mcg. disks with 1 ml. of water. Wash the disks with two additional 1-ml. portions of water and collect all filtrates in a 5-ml. volumetric flask. Dilute to 5 ml. with water and record the UV spectrum from 220 to 310 mμ. Treat standard disks similarly. Sodium cephalothin yields a peak at 237 mμ and a shoulder at 265 mμ. No other antibiotic in Table I gives this spectrum.

XII. Vancomycin Hydrochloride—Extract enough disks to yield 50 mcg. in 1 ml. of water. Evaporate the extract to dryness under a stream of air and redissolve in 1 ml. of 0.02 *N* sulfuric acid. Record the UV spectrum from 250–350 mμ, using water in the 1-ml. reference cell. With vancomycin hydrochloride a peak occurs at about 278 mμ.

Extract the same number of disks, evaporate the extract to dryness, and redissolve in 1 ml. of 0.02 *N* sodium hydroxide. Record the UV spectrum from 250–350 mμ, using 1-ml. cells. The vancomycin hydrochloride absorption peak shifts to about 302 mμ. In each case standard disks are similarly treated. This acid-base shift in absorbance wavelength is characteristic only of vancomycin hydrochloride (12) among the antibiotics listed in Table I.



XIII. Erythromycin—Extract two to four 15-mcg. disks with 1 ml. of chloroform, evaporate the extract to dryness under a stream of air, and redissolve in 1 ml. of 40% sulfuric acid. Heat in a water bath at 50° for 30 min. and cool to room temperature. Treat standard disks in the same manner. Record the visible spectrum from 450–500 mμ, using water in the 1-ml. reference cell. Erythromycin has a strong absorbance at about 482 mμ (13). Under these conditions none of the other 27 antibiotics listed in Table I absorbs strongly at 482 mμ.

DISCUSSION

The system of chemical testing outlined in the flow chart (Scheme I) and described here in detail can completely identify an unknown disk sample, if enough disks (80–100) are available. Four unknown disk samples were carried through this identification system and successfully identified as colistin, kanamycin, nafcillin, and oxytetracycline.

In practice, testing begins from the labeled identity and proceeds to confirm or deny the label. In the identification system described, as in many others, positive reactions to any single test may not be sufficient to characterize definitively the compound in question. The composite picture from supplemental chemical tests, in combination with microbiological response and potency data, results in satisfactory identification. For example, ampicillin and bacitracin react with ninhydrin, move with the solvent front in the TLC method for group II*a* antibiotics (although bacitracin has a very distinctive spot), and undergo the nitration, reduction, and diazotization procedure for the differentiation of colistin sulfate and polymyxin B sulfate. The iodometric test would identify a properly labeled ampicillin disk as a penicillin, but if an ampicillin disk were labeled as bacitracin and tested as such, the error might not be discovered. Because of this possibility, the iodometric test is carried out on bacitracin disks routinely. However, the discrepancy would be detected by microbial assay, because in that test the responses of ampicillin and bacitracin are very different.

The identity tests described have been applied satisfactorily to 250 lots of disks received as part of the antibiotic certification program. Each antibiotic was represented at least twice. Often these disks are color coded with dyes. Thus far, none of the dyes has been found to interfere in the tests.

This system of identity tests applies to those antibiotics presently in use in sensitivity disks but the use of new antibiotics may bring new problems. If such new antibiotics are closely related to the ones now in use, some of these identity tests may not be as specific as at present. The special problems of antibiotic sensitivity disk identity testing, dealing with microgram quantities of highly complex, related materials on a paper matrix, require frequent reevaluation for the possible improvement of the existing tests. Nevertheless, the system of methods just described should serve as a useful starting point for developing new test methods as the need arises.

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Keyphrases

Antibiotics in sensitivity disks—identity
Iodometric assay
TLC—identity
Ninhydrin test—identity
Diazotization-reaction—identity
UV spectrophotometry—identity