CHROM. 3682

CLASSIFICATION OF CRUDE ANTIBIOTICS BY INSTANT THIN-LAYER CHROMATOGRAPHY (ITLC)

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SUMMARY

We have presented a thin-layer chromatographic method for identifying antibiotics contained in a crude mixture during the early stages of isolation of these compounds. The method attempts to assess rapidly the probability that the antibiotic in question is an already known one. A total of 84 antibiotics was included in this study.

Used alone, this method will not identify an individual antibiotic in a crude mixture, but it will narrow the choice of identities to a small number. Additional chemical, physical, and microbiological testing are required to distinguish individual antibiotics.

The method is applicable to samples of crude antibiotics, which are themselves easily prepared. It does not require the parallel evaluation of standard samples of antibiotics.

INTRODUCTION

Much time and effort are wasted in identifying supposedly "new" antibiotics that then turn out to be already known. A simple, rapid means of identifying antibiotics is of great value.

Chromatographic analysis, which had earlier been used to identify such natural products as steroids and alkaloids, has been applied to the systematic identification of antibiotics.

The summarized paper chromatogram, obtained when the R_F values of antibiotics tested in several solvent systems are represented graphically, was employed by ISHIDA *et al.*[1]. When AMMANN AND GOTTLIEB[2] used this technique to characterize antifungal antibiotics, they found that it sometimes failed to differentiate between closely related antibiotics. SNELL *et al.*[3], who used the same technique to differentiate 19 polypeptide antibiotics that inhibit Gram-positive organisms, pointed out the danger of attaching any significance to the specific R_F values in summarized paper chromatograms obtained from samples of crude antibiotics. Their observation that other constituents of the culture medium were able to influence the rates of migration

of the antibiotics led them to suggest the use of solvent systems that would differentiate antibiotics by giving widely different rates of movement for each substance.

Both BRODASKY[4] and FANG *et al.*[5] employed paper chromatography to identify the individual components in mixtures of antibiotics. BRODASKY, working with three pure antibiotics, found that R_F values determined by this technique were more reproducible than those determined by silica gel thin-layer chromatography. FANG *et al.* used six solvent systems simultaneously to separate approximately 50 mixed antibiotics.

MIYAZAKI *et al.*[6] refined paper chromatographic analysis by adding a salting-out process to their technique for classifying antibiotics. BETINA[7] and BETINA AND NEMEC[8] characterized antibiotics by means of their ionic character and of their R_F values, revealed by use of paper chromatography with buffered solutions.

BETINA[9] also classified 62 antibiotics by employing four solvent systems for a primary analysis, which yielded five classes, and additional solvent systems for a secondary analysis, which yielded 14 subclasses.

IKEKAWA *et al.*[10] used color-forming reagents to locate macrolide antibiotics isolated on thin-layer chromatograms. BETINA AND BARATH[11] and BICKEL *et al.*[12] subsequently combined thin-layer chromatography with bioautography for the detection of antibiotics.

SCHUURMANS *et al.*[13] employed strains of mammalian cells to screen for antitumor activity several antibiotics previously isolated by paper chromatography and paper strip electrophoresis.

The essential characteristics sought in a method for identifying unknown antibiotics were rapidity and applicability to easily prepared samples of crude antibiotics. The elimination of parallel evaluation of standard samples of antibiotics, after the chromatographic system had been standardized in a particular laboratory, was considered a worthwhile goal.

The time-consuming preparation of thin-layer chromatographic plates was avoided by use of Eastman Chromagram sheets, which also require only a short time for development. The method to be described below proved applicable to samples prepared by three different techniques, all based on simple solvent extraction.

It was taken for granted that the method would not be useful in the case of antitumor substances that lack antibacterial activity and that additional chemical, physical, and microbiological testing would be required for final identification of the antibiotics.

MATERIALS AND METHODS

Solvent systems

Antibiotics were first analyzed in three solvent systems: α = methanol; β = 10 % methanol in chloroform; γ = chloroform.

The four groups yielded by this initial analysis were divided into 15 subgroups by the use of 11 additional solvent systems (see Table I).

Development of chromatograms and detection of antibiotics

Eastman Chromagram sheets, silica gel type 6060, henceforth referred to as ITLC (instant thin-layer chromatography) sheets, were partially deactivated by

TABLE I

Group	*Solvent system*	
I	pyridine–water (1:1)	Ia
	pyridine–water–absolute ethanol (1:1:1)	Ib
	pyridine–water–absolute ethanol (1:1:3)	Ic
II	butanol–methanol (1:1)	IIa
	chloroform–methanol (1:1)	IIb
	absolute ethanol	IIc
III	methanol–benzene (12:88)	IIIa
	methanol–benzene (6:94)	IIIb
	methanol–benzene (4:96)	IIIc
IV	methanol–benzene (1:99)	IVa
	methanol–benzene–chloroform (1:49:50)	IVb

exposure to air at room temperature (50–65 % relative humidity) for 24 h prior to use. Single glass chambers, lined with filter paper, were used for development of the chromatograms. For each antibiotic, the solvent systems used for development are given in Tables III and IV.

After development, the chromatograms were scanned under ultraviolet light and then bioautographed. The usual incubation time was 18 h at 37°, after the dried chromatogram had been placed directly on a filter paper resting on the seeded agar.

Methods of preparing samples of crude antibiotics

Three different methods were employed for the preparation of samples of crude antibiotics obtained from the 19 model fermentations.

Preparation A. To 6 ml whole broth, 0.4 ml 6 *N* HCl was added in one test tube, 0.5 ml 2 *N* NH_4OH was added in a second, and no addition was made to a third. After the addition of 3 ml butanol to each tube, they were shaken for 15 min and centrifuged. From each tube, 0.02 ml of the supernatant was applied by micropipette to the ITLC sheet.

Preparation B. After the addition of 10 ml isopropanol to 6 ml whole broth, the test tube was shaken for 15 min and centrifuged. A total of 0.04 to 0.06 ml of the supernatant was applied by micropipette to the ITLC sheet.

Preparation C. Ten milliliters of the isopropanol solution described in Preparation B were dried *in vacuo*. The residue was triturated twice with 1 ml ethanol and the ethanolic solutions were combined.

To the ethanol-insoluble triturate, 2 ml water were added, followed by 2 ml acetone, leading to the precipitation of proteins. The resulting suspension was centrifuged and a total of 0.02 ml, drawn from the supernatant as well as from the earlier ethanolic solution, was applied by micropipette to the ITLC sheet.

Antibiotics

Standard samples of 84 antibiotics were dissolved, each in its proper solvent, to give 1 % concentrations. The volume of solution applied to the ITLC sheets ranged from 1 to 60 λ, depending on the level of activity of each antibiotic against the micro-

TABLE II

ANTIBIOTICS INCLUDED IN THE ITLC CLASSIFICATION STUDY

Antibiotic	*Volume of antibiotic solution [1% conc.] (λ)*	*Microörganism used for bioautography*
Actidione	4	*Saccharomyces cerevisiae*
Actinobolin	5	*Staphylococcus aureus*
Actinomycin C_2	2	*Staph. aureus*
Actinomycin C_3	2	*Staph. aureus*
Amicetin	10	*Staph. aureus*
Aminosidin	10	*Staph. aureus*
Amphomycin	5	*Staph. aureus*
Anisomycin	20	*S. cerevisiae*
Azacolutin	30	*S. pastorianus*
Bacitracin	10	*Staph. aureus*
Bostrycoidin	2	*Bacillus subtilis*
Candicidin	10	*Candida albicans*
Candidin	2	*C. albicans*
Celesticetin	10	*Staph. aureus*
Cephalothecin	5	*Staph. aureus*
Chartreusin	3	*Staph. aureus*
Chloramphenicol	1	*Staph. aureus, Escherichia coli*
Citrinin	10	*Staph. aureus*
Clavacin	3	*E. coli*
C-73	20	*C. albicans*
Dihydrostreptomycin	20	*E. coli*
DON	40	*Staph. aureus*
Duramycin	20	*B. subtilis*
Echinomycin	5	*Staph. aureus*
Erythromycin	3	*Staph. aureus*
Esperin	10	*Staph. aureus*
Etamycin	10	*Staph. aureus*
Filipin	30	*S. cerevisiae*
Fungimycin	2	*S. cerevisiae*
Fusanin B	10	*B. subtilis*
Fusarubin	4	*Staph. aureus*
Gentamycin D	3	*Staph. aureus*
Gentamycin C_1	3	*Staph. aureus*
Gentamycin C_2	3	*Staph. aureus*
Gramicidin S	5	*Staph. aureus*
Griseofulvin	60	*C. albicans*
Hamycin	10	*C. albicans*
Humatin	10	*E. coli*
Hygromycin B	10	*Staph. aureus*
Javanicin	10	*B. subtilis*
Kanamycin	5	*Staph. aureus*
Lincomycin	3	*Staph. aureus*
Lucensomycin	5	*S. cerevisiae*
Mitomycin	5	*Staph. aureus*
Mycostatin	10	*C. albicans*
Neomycin C	10	*B. subtilis*
Nocardorubin	1	*Staph. aureus*
Nogalamycin	1	*Staph. aureus*
Novobiocin	10	*Staph. aureus*
Oleandomycin	10	*Staph. aureus*
Oligomycin	1	*S. cerevisiae*
Oxytetracycline	1	*Staph. aureus*
Polymyxin B	30	*E. coli*
Prasinomycin	3	*Staph. aureus*

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TABLE II (*continued*)

Antibiotic	*Volume of antibiotic solution [1% conc.] (λ)*	*Microörganism used for bioautography*
Psicofuranine	20	*Staph. aureus*
Puromycin	20	*Staph. aureus*
Rhodomycetin	2	*Staph. aureus*
Rhodomycin	3	*Staph. aureus*
Rimocidin	10	*S. cerevisiae*
Ristocetin	5	*Staph. aureus*
Rubiflavin	1	*Staph. aureus*
Saramycetin	10	*Paecilomyces varioti*
Septacidin	20	*Trichophyton mentagrophytes*
Spiramycin	40	*Staph. aureus*
Streptomycin	5	*Staph. aureus*
Streptothricin BII	10	*Staph. aureus*
Streptovaricin	10	*Staph. aureus*
Streptozotocin	5	*Staph. aureus*
Streptovitacin	40	*S. pastorianus*
Subtilin	10	*Staph. aureus*
Sulfocidin	5	*Staph. aureus*
Tetracycline	1	*Staph. aureus*
Thiolutin	3	*Staph. aureus*
Thiostrepton	5	*Staph. aureus*
Tyrothricin	10	*Staph. aureus*
Toyocamycin	10	*C. albicans*
Trichomycin	10	*Staph. aureus*
Tubercidin	5	*C. albicans*
Tylosin	5	*C. albicans*
Unamycin A	25	*S. cerevisiae*
Usnic acid	10	*B. subtilis*
Vancomycin	10	*Staph. aureus*
Vernamycin A	5	*Staph. aureus*
Viomycin	10	*Proteus vulgaris*

organism used for bioautography. Data on volumes and microorganisms used are given in Table II.

Buffered ITLC

The ionic character of various antibiotics was determined by chromatography on buffered ITLC sheets. Prior to application of the antibiotic samples, each sheet was immersed in 0.5 *M* phosphate buffer solution of pH 2 or pH 11, then dried in air. The chromatograms were developed with the least polar solvent in which the antibiotic still showed movement (see Table V). Development of the chromatogram was followed by standard bioautography.

RESULTS

The prime criterion employed in our method of classification was the occurrence of movement of an antibiotic in a specific solvent system. Analysis of 84 pure antibiotics with three primary solvent systems produced a scheme containing four primary groups: group I = antibiotics showing no motion in solvents α, β, or γ; group II = antibiotics moving only in α; group III = antibiotics moving in α and β,

TABLE III

R_F VALUES OF ANTIBIOTICS INCLUDED IN THE ITLC CLASSIFICATION STUDY

Subgroup	*Antibiotic*	*R_F values in specific solvent systems*[a]					
		α	*β*	*γ*	*Ia*	*Ib*	*Ic*
I-1							
	Gentamycin D	0	0	0	0	0	0
	Gentamycin C_1	0	0	0	0	0	0
	Gentamycin C_2	0	0	0	0	0	0
	Humatin	0	0	0	0	0	0
	Neomycin	0	0	0	0	0	0
I-2	(Ia)[b]						
	Aminosidin	0	0	0	0–0.8	0	0
	Dihydrostreptomycin	0	0	0	0.25	0	0
	Hygromycin B	0	0	0	0.1	0	0
	Streptomycin	0	0	0	0.15	0	0
	Streptothricin	0	0	0	0.3	0	0
	Viomycin	0	0	0	0.3–0.5	0	0
I-3	(Ia, Ib)						
	Kanamycin	0	0	0	0–0.3	0–0.2	0
	Rubiflavin	0	0	0	0–0.2	0–0.15	0
I-4	(Ia, Ib, Ic)						
	Polymyxin B	0	0	0	0.7	0.56	0.4
	Ristocetin	0	0	0	0.9	0.7	0.7
	Vancomycin	0	0	0	0.3–0.8	0.7	0–0.6
		α	*β*	*γ*	*IIa*	*IIb*	*IIc*
II-1							
	Candidin	0–0.32	0	0	0	0	0
	Gramicidin S	0–0.2	0	0	0	0	0
	Prasinomycin	0.28	0	0	0	0	0
	Duramycin	0.6	0	0	0	0	0
II-2	(α, IIa, or IIb)						
	Amphomycin	0.35	0	0	0.08	0	0
	Candicidin	0.4–0.6	0	0	0	0.3–0.4	0
	Mycostatin	0.5	0	0	0.22	0	0
II-3	(α, IIa, IIb)						
	Bacitracin	0.23	0	0	0.2	0.15	0
	Subtilin	0.7	0	0	0.5	0.2	0
	Trichomycin	0.5	0	0	0–0.15	0.1	0
	Unamycin A	0.5	0	0	0.3	0.2	0
II-4	(α, IIa, IIb, IIc)						
	Azacolutin	0.7	0	0	0.1	0.1	0.05
	Cephalothecin	0.8	0	0	0.6	0.6	0.6
	Hamycin	0–0.6	0	0	0–0.1	0–0.1	0.05
	Lucensomycin	0.4	0	0	0.22	0.12	0.06
	Oxytetracycline	0–0.4	0	0	0–0.4	0–0.25	0–0.2
	Rimocidin	0.5	0	0	0–0.3	0–0.3	0–0.15
	Septacidin	0.6	0	0	0.55	0.5	0.45
	Tetracycline	0–0.3	0	0	0–0.5	0–0.2	0–0.2

[a] See MATERIALS AND METHODS section for details.
[b] Designation in parentheses identifies the solvent systems in which the antibiotic moved.

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TABLE III (*continued*)

Subgroup	*Antibiotic*	*R_F values in specific solvent systems*[a]					
		α	*β*	*γ*	*IIIa*	*IIIb*	*IIIc*
III-1	(α, β)						
	Fungimycin	0–0.75	0–0.2	0	0	0	0
	Novobiocin	0.71	0.7	0	0	0	0
	Oleandomycin	0.48	0.3	0	0	0	0
	Rhodomycetin	0.05	0.05	0	0	0	0
	Saramycetin	0.62	0.62	0	0	0	0
III-2	(α, β, IIIa)						
	Amicetin	0.6	0.28	0	0.1	0	0
	Citrinin	0.72	0.8	0	0.16	0	0
	Erythromycin	0.42	0.26	0	0.1	0	0
	Filipin	0.6	0.15	0	0.18	0	0
	Nogalamycin	0.46	0.4	0	0.3	0	0
	Rhodomycin	0.73	0.5	0	0.3	0	0
	Streptozotocin	0.6	0–0.4	0	0–0.2	0	0
	Toyocamycin	0.65	0.28	0	0	0.1	0
	Tubercidin	0.62	0.22	0	0.05	0	0
III-3	(α, β, IIIa, IIIb)						
	Anisomycin	0.5	0.35	0	0.2	0.1	0
	Fusanin B	0.65	0.58	0	0.2	0.1	0
	Lincomycin	0.41	0.25	0	0–0.2	0–0.1	0
	Puromycin	0.7	0.55	0	0.25	0–0.15	0
	Streptovitacin	0.41	0.25	0	0.23	0.1	0
	Sulfocidin	0.65	0.5	0	0.85	0–0.5	0
	Thiostrepton	0.62	0.56	0	0.25	0.15	0
	Tylosin	0.7	0.6	0	0.35	0.1	0
III-4	(α, β, IIIa, IIIb, IIIc)						
	Actidione	0.64	0.67	0	0.5	0.22	0.2
	Actinomycin C_2	0.82	0.8	0	0.5	0.26	0.05
	Actinomycin C_3	0.82	0.75	0	0.5	0.26	0.05
	Celesticetin	0.76	0.74	0	0.4	0.25	0.05
	Chloramphenicol	0.72	0.57	0	0.3	0.15	0.09
	Echinomycin	0.7	0.7	0	0.5	0.22	0.05
	Esperin	0.75	0–0.5	0	0.2	0.09	0.05
	Javanicin	0.7	0.68	0	0.72	0.71	0.71
	Mitomycin	0.72	0.48	0	0.25	0.1	0.05
	Nocardorubin	0.55	0.5	0	0.45	0.3	0.25
	Streptovaricin	0.79	0.78	0	0–0.5	0–0.4	0–0.4
		α	*β*	*γ*	*IVa*	*IVb*	
IV-1	(α, β, γ)						
	Actinobolin	0.42	0.2	0.08	0	0	
	DON	0.78	0.76	0.65	0	0	
	Psicofuranine	0.7	0.15	0–0.1	0	0	
	Spiramycin	0.6	0.4	0–0.3	0	0	
IV-2	(α, β, γ, IVa)						
	Etamycin	0.8	0.8	0–0.2	0.1	0	
	Oligomycin	0.43	0.43	0.32	0.2	0	
	Thiolutin	0–0.6	0.65	0.35	0.3	0	
	Vernamycin A	0.6	0.63	0.2	0.12	0	

(*continued on p.* 494)

TABLE III (*continued*)

Subgroup	*Antibiotic*	*R_F values in specific solvent systems*[a]				
		α	*β*	*γ*	*IVa*	*IVb*
IV-3	(α, β, γ, IVa, IVb)					
	Bostrycoidin	0.76	0.73	0.26	0.22	0.15
	Chartreusin	0.74	0.65	0.15	0.16	0.1
	Clavacin	0.7	0.65	0.3	0.25	0.16
	C-73	0.8	0.7	0.55	0.4	0–0.22
	Fusarubin	0.3	0.73	0.45	0.23	0.15
	Griseofulvin	0.75	0.75	0.52	0.5	0.25
	Tyrothricin	0.7	0.7	0.25	0.7	0.1
	Usnic acid	0.75	0.75	0.75	0.5	0.45

but not in γ; group IV = antibiotics moving in α, β, and γ. Of the 84 antibiotics tested, 16 were in group I, 19 in group II, 33 in group III, and 16 in group IV.

Application of 11 additional solvent systems to the members of the four primary groups yielded 15 subgroups. R_F values of all antibiotics in both the primary and secondary solvent systems are shown in Table III.

Nineteen of the 84 antibiotics tested were chosen as models and produced by fermentation. They included four from group I, five from group II, seven from group III, and three from group IV. Preparation methods A, B, and C (see MATERIALS AND METHODS) were applied to each of the 19 fermentation broths and all 57 resulting samples were tested in the ITLC system.

Table IV shows which methods of preparation yielded samples of these 19 antibiotics that could be satisfactorily applied to the ITLC sheets. It also gives the R_F values of the bioactive spots found in chromatograms of these compounds.

Thirty-six of the original 84 pure antibiotics were tested in the buffered ITLC system, as were 18 of the 19 crude antibiotic preparations named in Table IV. R_F values for compounds tested in the buffered ITLC system are given in Table V.

DISCUSSION

The purpose of this study has been to develop a screening technique that can reveal, within a few days, whether a microbiologically active principle found in a fermentation broth is an already known antibiotic. We worked with 84 known antibiotics. Because the method is partially dependent on bioautography, its application to the screening of antitumor antibiotics is limited to those in which antibacterial activity parallels *in vivo* antitumor activity. Such parallelism exists for about 85 % of antitumor antibiotics.

The method of SCHUURMANS *et al.*[13] for detecting antitumor antibiotics is based on the inhibition of cellular dehydrogenase activity and requires cultivation of mammalian cell strains under conditions that are sometimes difficult to achieve in a chemical laboratory. Like the method presented in this paper, it is not applicable to some antitumor antibiotics. It is, perhaps, of greatest value in the later stages of isolation of these compounds.

Our ITLC system, which combines the sequential analysis technique of SNELL *et al.*[3] with the simultaneous analysis technique of ISHIDA *et al.*[1], reduces the number

TABLE IV

R_F VALUES OF CRUDE PREPARATIONS OF ANTIBIOTICS IN THE ITLC SYSTEMS

Antibiotic	*R_F values[a] of bioactive spot[b] in specific solvent systems[c]*						*Types of crude preparation[c] successfully applied*
Group I	*α*	*β*	*γ*	*Ia*	*Ib*	*Ic*	
Dihydrostreptomycin	0 (0.5)	0	0	0.3 (0.4)	0	0	BC
Polymyxin B	0 (0.62)	0(0.53)	0 (0.62)	0.75	0.6	0.65	ABC
Rubiflavin	0	0	0	0–0.3	0–0.2	0	ABC
Streptomycin	0 (0.5)	0	0	0–0.15	0	0	BC
Group II	*α*	*β*	*γ*	*IIa*	*IIb*	*IIc*	
Azacolutin	0.75 (0)	0	0	0.14 (0)	0.12 (0)	0.05	ABC
Duramycin	0.7	0	0	0	0	0	ABC
Oxytetracycline	0.5	0	0	0–0.5	0–0.3	0–0.3	ABC
Prasinomycin	0.3	0	0	0	0	0	ABC
Tetracycline	0–0.4	0	0	0–0.5	0–0.5	0–0.3	ABC
Group III	*α*	*β*	*γ*	*IIIa*	*IIIb*	*IIIc*	
Actidione	0.64	0.7	0	0.5	0.23	0.2	ABC
Chloramphenicol	0.74	0.54 (0.57)	0 (0.3)	0.32 (0.45)	0.14 (0.35)	0.09 (0.28)	ABC
Erythromycin	0.43 (0.65)	0.28 (0.65)	0 (0.15)	0.1 (0.3)	0 (0.35)	0 (0.5)	ABC
Novobiocin	0.71	0.71 (0)	0	0	0	0	ABC
Saramycetin	0.62	0.6	0	0	0	0	ABC
Thiostrepton	0.62	0.6 (0)	0	0.28 (0)	0.13 (0)	0	ABC
Toyocamycin	0.65	0.3	0	0.1	0	0	ABC
Group IV	*α*	*β*	*γ*	*IVa*	*IVb*		
Bostrycoidin	0.76	0.73	0.28 (0.5)	0.25 (0.4)	0.15		ABC
Chartreusin	0.74	0.65	0.18	0.18	0.1		ABC
Vernamyacin A	0.6	0.63	0.1–0.25	0–0.12	0		ABC

[a] The R_F value in parentheses designates a minor second bioactive spot.
[b] Microörganisms used for bioautography are listed in Table II.
[c] See MATERIALS AND METHODS section for details.

TABLE V

R_F VALUES[a] OF ANTIBIOTICS IN BIOACTIVE SPOTS[b] IN A BUFFERED ITLC SYSTEM[c]

Antibiotic[d]	*Solvent system used to develop chromatogram*	*R_F values*	
		pH 2 buffer	*pH 11 buffer*
Actidione	β	0.61 (0.63)	0.61 (0.65)
Actinomycin C_2	β	0.71	0.75
Actinomycin C_3	β	0.70	0.75
Amicetin	β	0	0.3
Azacolutin	α	0.5	—
Bacitracin	α	0–0.65	0–0.1
Bostrycoidin	γ	0.4 (0.42)	0.55 (0.55)
Chartreusin	γ	0.5–0.7 (0.65)	0.1 (0.25)
Chloramphenicol	β	0.4 (0.45)	0.4 (0.43)
Citrinin	β	0.2	0
Dihydrostreptomycin	α	0	0.2 (0.25)
Erythromycin	β	0.4 (0.3–0.5)	0.58 (0.6)
Etamycin	β	0.8	0.8
Filipin	β	0.55	0.5
Fusarubin	γ	0.48	0.28
Griseofulvin	γ	0.4	0.4
Javanicin	γ	0.48	0.59
Mycostatin	α	0.5	0.45
Nogalamycin	β	0.36	0.44
Novobiocin	β	0.4 (0.42)	0 (0)
Oligomycin	α	0.26	0.26
Oxytetracycline	α	0–0.1 (0–0.2)	0.5 (0–0.6)
Polymyxin B	β	0–0.8 (0.65)	0.6 (0.5)
Prasinomycin	α	0.6 (0–0.6)	0.25 (0.2)
Rhodomycin	β	0–0.7	0.65
Rubiflavin	β	0 (0)	0–0.2 (0–0.2)
Saramycetin	α	0.57 (0.4–0.5)	0.3 (0.25)
Spiramycin	β	0.25	0.6
Streptomycin	α	0 (0–0.05)	0.3 (0.2–0.3)
Streptovitacin	β	0.45	0.45
Tetracycline	α	0–0.1 (0–0.18)	0–0.5 (0–0.54)
Thiostrepton	β	0.65 (0.68)	0.75 (0.75)
Toyocamycin	β	0–0.03 (0.05)	0.28 (0.3)
Tylosin	β	0.58	0.65
Unamycin A	α	0.45	0.35
Vernamycin A	γ	0–0.2 (0–0.22)	0–0.2 (0–0.2)

[a] R_F value in parentheses indicates a bioactive spot from the crude antibiotic preparation, in contrast to main value obtained from pure antibiotic.

[b] Microörganisms used for bioautography are listed in Table II.

[c] Amphoteric antibiotics may appear acidic, basic, or neutral in this two-buffer ITLC system.

[d] Same volume as shown in Table II.

of solvent systems required. The unknown antibiotic is classified as a member of a subgroup that contains only a few antibiotics. Use of a buffered ITLC system, as well as of additional microbiological and chemical tests, permits these few antibiotics to be distinguished from each other. Differentiation of antibiotics based on the characteristics revealed by such tests, as advocated by SNELL *et al.*[3], is more reliable than is differentiation based on the potentially misleading differences in R_F values. The only use made of R_F values in our system of classification is to demonstrate the movement of an antibiotic.

Examination of the data in Table III makes it apparent that the solvent systems employed have not placed into one subgroup all antibiotics with a close chemical relationship. This fact is both advantageous and disadvantageous. Antibiotics in any one subgroup that are not closely related chemically can be differentiated by chemical and microbiological means more easily than can those that are. On the other hand, mere assignment of an antibiotic to a subgroup does not reveal much about what type of antibiotic it is.

All three methods of preparing samples of crude antibiotics were, in general, equally useful. Method A failed with streptomycin and dihydrostreptomycin, both of which are insoluble in butanol. The inability of butanol to dissolve some antibiotics may be turned to advantage in screening a large number of fermentation broths. Method A may also give some information about the ionic character of the unidentified active principle. Method B, which was successful with all 19 model fermentations, required the application of only a single sample to each ITLC sheet. Method C, the most time-consuming one, yielded the purest samples and R_F values of these samples showed good reproducibility, even with variations in the fermentation procedure.

A crude preparation of an antibiotic does not give the same R_F values as does the pure form of the same substance (see Tables III and IV).

Bioautography of thin-layer chromatograms was described by BETINA AND BARATH[11], who employed a "fingerprinting" technique. BICKEL *et al.*[12] were able to place the thin-layer chromatogram, on a paper base, directly on to the seeded agar plate. We were able to place developed ITLC sheets on to a filter paper already resting on the seeded agar. Well-defined zones of inhibition were visible after the usual incubation period.

The present technique is inherently simple. An ITLC sheet is easy to handle. Chromatograms can be developed in a single glass chamber within 20 to 30 min, except for solvent systems containing pyridine, which require 3 to 4 h.

We have chromatographed antibiotics on columns, employing either silicic acid or silica gel deactivated in a manner similar to that used for the ITLC sheets. Our results have been in very good agreement with those obtained by means of the ITLC system.

On the ITLC sheets, some antibiotics streaked rather than moving as circular spots (Tables III, IV and V). Such streaking occurred in all solvent systems with aminosidin, fungimycin, gramicidin S, hamycin, kanamycin, oxytetracycline, and tetracycline. In other cases, like azacolutin, bacitracin, echinomycin, lincomycin, polymyxin B, rubiflavin, streptovaricin, sulfocidin, and vernamycin A, the streaking took place only in some solvents. The streaking behavior of an antibiotic may sometimes be used as an identifying characteristic.

The ITLC system does not require the use of standard samples of antibiotics for parallel evaluation after the system has been standardized in a particular laboratory. Results obtained by its use have shown good reproducibility. The initial step in identifying an unknown antibiotic is the determination of movement in specific solvent systems and the nature of the microbiological spectrum. Final identification depends on further chemical and microbiological tests.

Although the present study included only 84 antibiotics, application of the ITLC classification system to additional antibiotics is now in progress.

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