A SYSTEMATIC ANALYSIS OF ANTIBIOTICS USING PAPER CHROMATOGRAPHY

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(Received November 19th, 1963)

INTRODUCTION

Paper chromatographic studies of compounds in several solvent systems for the purpose of their classification and identification have proved useful in systematic analysis[1]. At a recent symposium[2] papers were presented on the systematic analysis of alkaloids[3], antibiotics[4], synthetic dyes[5], inorganic cations and anions[6,7], aromatic compounds[8] and steroids[9].

Several methods for the systematic analysis of antibiotics are known. The oldest of them is the so-called "summarized paper chromatogram"[10], where the antibiotics are analysed in eight solvent systems and the R_F values of an antibiotic are presented graphically as a "summarized chromatogram". This principle has been used with various modifications by many authors[11–17].

MIYAZAKI *et al.*[18] classified antibiotics using a salting-out paper chromatography technique, in which nine solvent systems with different concentrations of ammonium chloride in water were used. According to their R_F values in these systems antibiotics can be divided into six groups[18,19]. NEMEC *et al.*[20] have also applied it to antibiotics from fungi.

BETINA[21] and BETINA AND NEMEC[22] determined the ionic character of unknown antibiotics and also the possibility of their isolation and purification from the "pH chromatograms".

From the chemical point of view antibiotics are a very heterogeneous group of biologically active compounds, which causes some difficulty in working out a systematic chromatographic analysis. Bearing this fact in mind a systematic analysis was developed along the following lines. The antibiotics are first analysed in four solvent systems, and then are divided into five classes with fourteen subclasses which are further analysed in additional solvent systems for each class.

MATERIALS AND METHODS

Principal solvent systems

The antibiotics were first analysed in the following four solvent systems:

1. Distilled water,
2. *n*-Butanol saturated with water,
3. Ethyl acetate saturated with water,
4. Benzene saturated with water.

The antibiotics were divided into five classes with fourteen subclasses, according to their R_F values in the above solvent systems.

*Additional solvent systems**

The following additional solvent systems were used for individual classes of antibiotics:

Class I:

A. Methanol–water (40:60).
B. *n*-Propanol–water (40:60).
C. Methanol–3 % ammonium chloride in water (70:30).
D. Methyl ethyl ketone–*n*-butanol–water (30:5:65).

Classes II and III**:

E. 3 % Ammonium chloride in water.
F. Isoamyl acetate–methanol–99 % formic acid–water (65:20:5:10).
G. *n*-Butyl acetate–methyl ethyl ketone–0.15 *M* phosphate buffer, pH 7.4 (50:25:5).
H. Ethyl acetate–*n*-hexane–0.15 *M* phosphate buffer, pH 6.0 (65:15:20).

Classes IV and V***:

I. Isoamyl acetate–methanol–99 % formic acid–water (40:20:10:30).
J. *n*-Butanol–methanol–water (40:10:50).
K. Methanol–*n*-hexane (60:40).
L. Benzene–cyclohexanone–0.15 *M* phosphate buffer, pH 7.4 (5:35:60).

Development and detection of chromatograms

Strips of the Whatman No. 1 paper 1 × 35 cm were used, the origin being 3 cm from the lower end of the strips.

For the development of the chromatograms 500 ml glass cylinders were used with 25 ml of the solvents. After applying the antibiotics to the origin and drying in air, the chromatographic strips were immersed to a depth of 1 cm in the solvent. Ascending development at 20 ± 1°, without preliminary saturation of the chromatograms with the vapours of the solvents, was used. The development was stopped when the solvent front reached a distance of 15 cm from the origin.

After drying the chromatograms in air, detection was carried out either bioautographically, using sensitive test microorganisms[23], or, in some special cases, chemically (see Table I).

Antibiotics

Sixty-two antibiotics from actinomycetes, fungi and lichens were studied. Data about the amount of each antibiotic used, the solvent for its solution before application on to paper strips, and the detection technique are given in Table I.

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* All proportions are given by volume.
** Upper layers of systems F, G, and H were used.
*** Bottom layers of systems I, J, K, and L were used.

TABLE I

SOLVENTS AND DETECTION METHODS FOR THE ANTIBIOTICS ANALYZED

Solvents used for the application of antibiotics on to chromatograms: A = acetone; AW = 50% acetone in water; C = chloroform; E = ethyl acetate; M = methanol; W = water.
Detection methods: a = bioautographically with *Bacillus subtilis*; b = bioautographically with *Escherichia coli*; c = bioautographically with *Candida pseudotropicalis*; d = 3% $FeCl_3$ in methanol; e = 3% NH_4Cl in water; f = acidic $KMnO_4$ (according to ref. 25, p. 737); g = conc. H_2SO_4; h = 3% NH_4OH in water; i = original colour.

Antibiotic	*Application*		*Detection*	*Colour reaction*
	Amount (μg)	*Solvent*		
Aburamycin	20	M	a	—
Actinomycin C·HCl	20	A	a	—
Alternariol	60	A	d	dark green
Amphomycin	40	W	a	—
Amphotericin B	250	W	g	blue
Aspergillic acid	60	A	d	red
Azalomycin B	80	M	a	—
Azalomycin F	60	M	c	—
Bryamycin	40	C	a	—
Chloramphenicol	10	M	a	—
Chlorotetracycline·HCl	30	M	d	light brown
Citrinin	80	A	a	—
Congocidin·HCl	40	W	a	—
Cyanein	80	A	c	—
Cyclopaldic acid	40	A	d	dark green
Cycloserine	40	W	a	—
Dihydrostreptomycin (sulfate)	10	W	b	—
Eniantin B	90	A	g	light red
Erythromycin (lactobionate)	20	W	a	—
Etamycin	5	A	a	—
Flavipin	50	M	d	dark green
Flavofungin	80	M	f	decolorized
Fomecin A	30	A	d	blue-green
Frequentin	50	C	d	brown-red
Fuscin	40	A	h	violet
Geodin	30	A	a	—
Gladiolic acid	50	A	e	dark green
Gliotoxin	30	A	a	—
Griseofulvin	100	C	g	yellow
Illudin M	40	A	a	—
Illudin S	20	A	a	—
Javanicin	20	A	i	red-violet
Kanamycin (sulfate)	80	W	b	—
Kojic acid	40	A	d	brown-red
5-Methoxy-*p*-toluquinone	40	A	a	—
Mycophenolic acid	80	A	d	dark green
Neomycin B (base)	250	W	b	—
Novobiocin (Na salt)	10	E	a	—

(Table continued on p. 382)

TABLE I (*continued*)

Antibiotic	*Application*		*Detection*	*Colour reaction*
	Amount (μg)	*Solvent*		
Oleandomycin (base)	30	M	a	—
Oxytetracycline·HCl	60	W	d	light brown
Patulin	100	A	a	—
Penicillic acid	160	M	a	—
Penicillin G (K salt)	0.5*	W	a	—
Penicillin V (K salt)	0.5*	W	a	—
Pleuromutilin	120	A	a	—
Puromycin	120	W	a	—
Quadrilineatin	40	A	e	dark green
Ristocetin	40	W	a	—
Rugulosin	20	A	d	yellow-brown
Sclerotiorin	20	A	g	orange-red
Spinulosin	50	A	d	brown
Spiramycin	80	M	a	—
Streptomycin (sulfate)	10	W	b	—
Synnematin B (cephalosporin N)	10	W	a	—
Telomycin	40	AW	a	—
Terreic acid	80	M	d	brown-red
Tetracycline·HCl	60	W	d	light brown
Trichothecin	20	A	c	—
Usnic acid	80	A	g	brown
Vancomycin	20	W	a	—
Viomycin (sulphate)	160	W	b	—
Viridin	80	A	a	—

* Units

RESULTS

The principles whereby the antibiotics were divided into five classes with fourteen subclasses, according to their R_F values in four solvent systems, are given schematically in Table II. Each subclass is represented by a typical antibiotic in Fig. 1.

The distribution of the antibiotics studied into the classes and subclasses is given in Tables III to VII. R_F values in the four principal and in four of the additional solvent systems are presented graphically as "summarized chromatograms" (or "R_F spectra"[7]) in Figs. 2 to 6.

Class I of antibiotics is characterized as follows: R_F values are equal to zero in the principal solvent systems 3 and 4, and 0.00–0.30 in system 2. There are greater differences in system 1, these were used for the division of this class into three subclasses: 1a, 1b, and 1c (see Table II and Fig. 1).

Class I includes antibiotics of the streptomycin–neomycin group, tetracyclines, cycloserine, synnematin B (cephalosporin N), vancomycin, ristocetin and other antibiotics (Table III). The "summarized chromatograms" of tetracyclines are very similar. There are also similarities between the "summarized chromatograms" of

TABLE II

CLASSIFICATION OF ANTIBIOTICS INTO CLASSES (PART A) AND INTO SUBCLASSES (PARTS B, C, D, E AND F)

Part A	*Classes*				
R_F values in principal systems	*I*	*II*	*III*	*IV*	*V*
R_{F1}	$>$ 0.00	$>$ 0.60	0.31–0.60	0.00–0.30	0.00–1.00
R_{F2}	0.00–0.30	$>$ 0.30	$> R_{F1}$	$> R_{F1}$	$>$ 0.60
R_{F3}	0.00	$>$ 0.00	$> R_{F4}$	$\geqq R_{F4}$	$>$ 0.60
R_{F4}	0.00	0.00	0.00–0.60	0.00–0.60	$>$ 0.60

Part B	*Class I*		
R_F values	*Subclasses*		
	Ia	*Ib*	*Ic*
R_{F1}	$>$ 0.60	0.31–0.60	$\leqslant$ 0.30

Part C	*Class II*		
	Subclasses		
	IIa	*IIb*	*IIc*
	$R_{F1} > R_{F2} > R_{F3}$	$R_{F1} > R_{F2} < R_{F3}$	$R_{F1} < R_{F2} < R_{F3}$

Part D	*Class III*	
R_F values	*Subclasses*	
	IIIa	*IIIb*
R_{F4}	0.00	0.05–0.60

Part E	*Class IV*		
R_F values	*Subclasses*		
	IVa	*IVb*	*IVc*
R_{F2}	$> R_{F1}$	$> R_{F1}$	$> R_{F1}$
R_{F3}	$> R_{F1}$	0.00	$> R_{F4}$
R_{F4}	0.00	0.00	0.05–0.60

Part F	*Class V*		
R_F values	*Subclasses*		
	Va	*Vb*	*Vc*
R_{F1}	$>$ 0.60	0.31–0.60	0.00–0.30

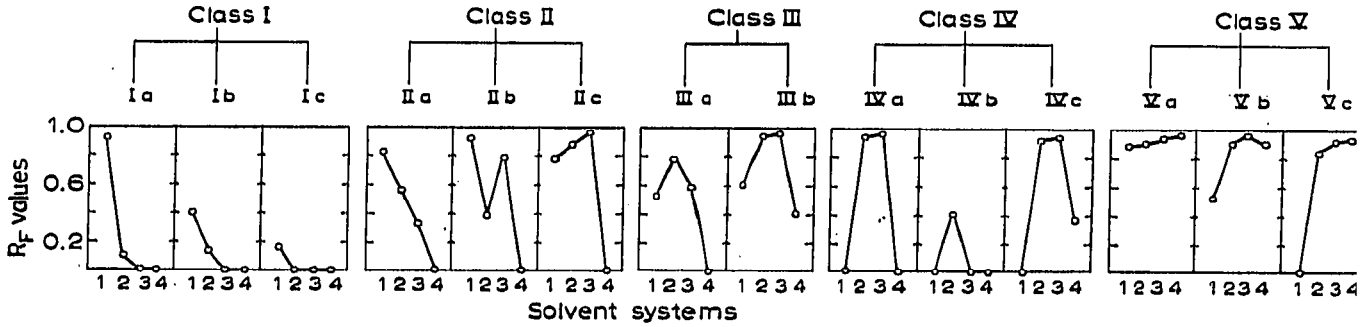

Fig. 1. Typical antibiotics belonging to five classes according to their R_F values in principa solvent systems 1, 2, 3 and 4. From left to right: cycloserine (subclass Ia), chlorotetracycline (Ib) streptomycin (Ic), kojic acid (IIa), penicillin G (IIb), chloramphenicol (IIc), oleandomycin (IIIa) etamycin (IIIb), cyanein (IVa), azalomycin F (IVb), azalomycin B (IVc), illudin M (Va), pleuro mutilin (Vb) and viridin (Vc).

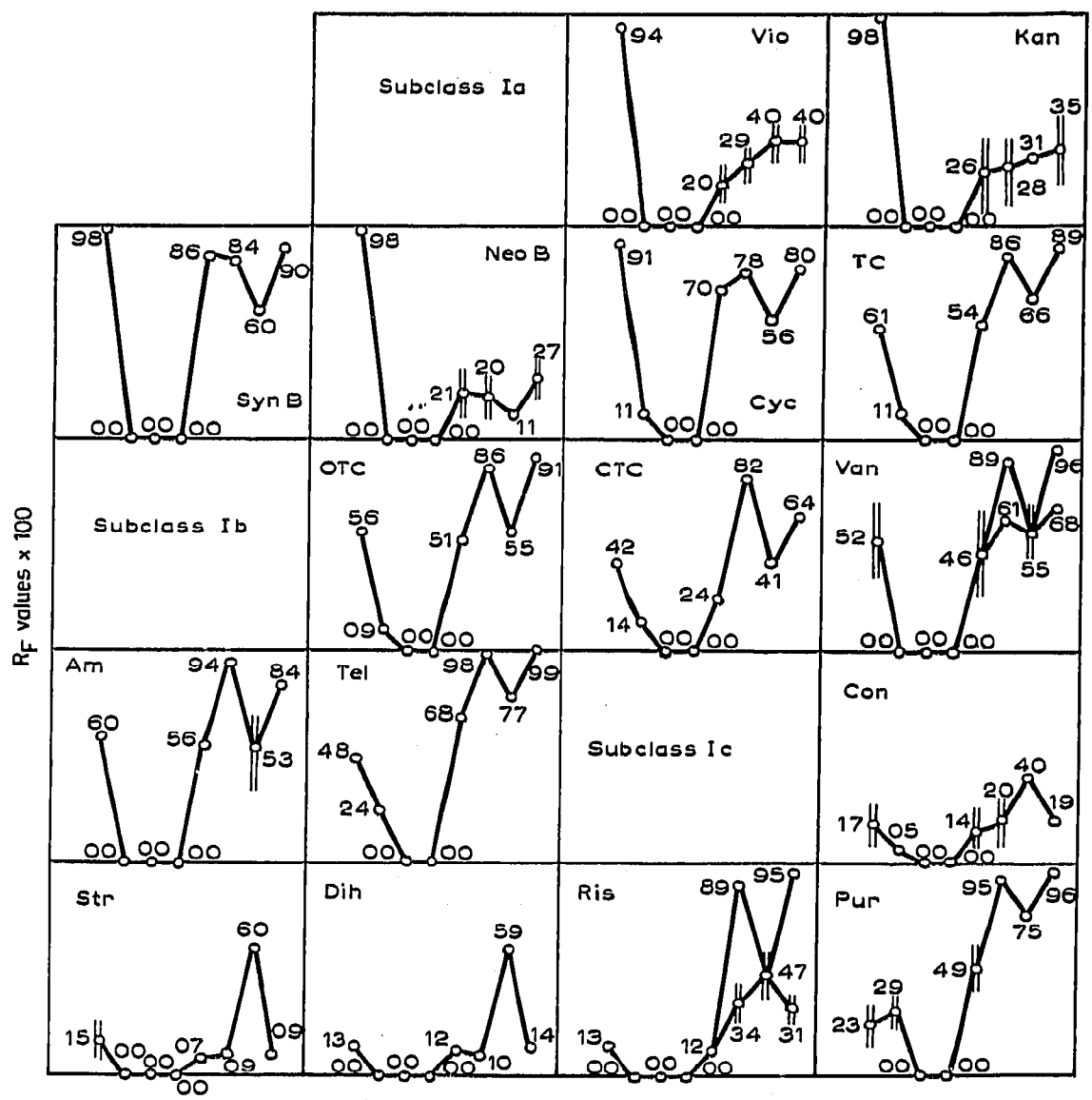

Fig. 2. "Summarized chromatograms" of antibiotics of class I. Subclass Ia: viomycin (Vio), kanamycin (Kan), synnematin B (Syn B), neomycin B (Neo B), cycloserine (Cyc) and tetracycline (TC). Subclass Ib: oxytetracycline (OTC), chlorotetracycline (CTC), vancomycin (Van), amphomycin (Am) and telomycin (Tel). Subclass Ic: congocidin (Con), streptomycin (Str), dihydrostreptomycin (Dih), ristocetin (Ris) and puromycin (Pur). Solvent systems: 1, 2, 3, 4, A, B, C, and D.

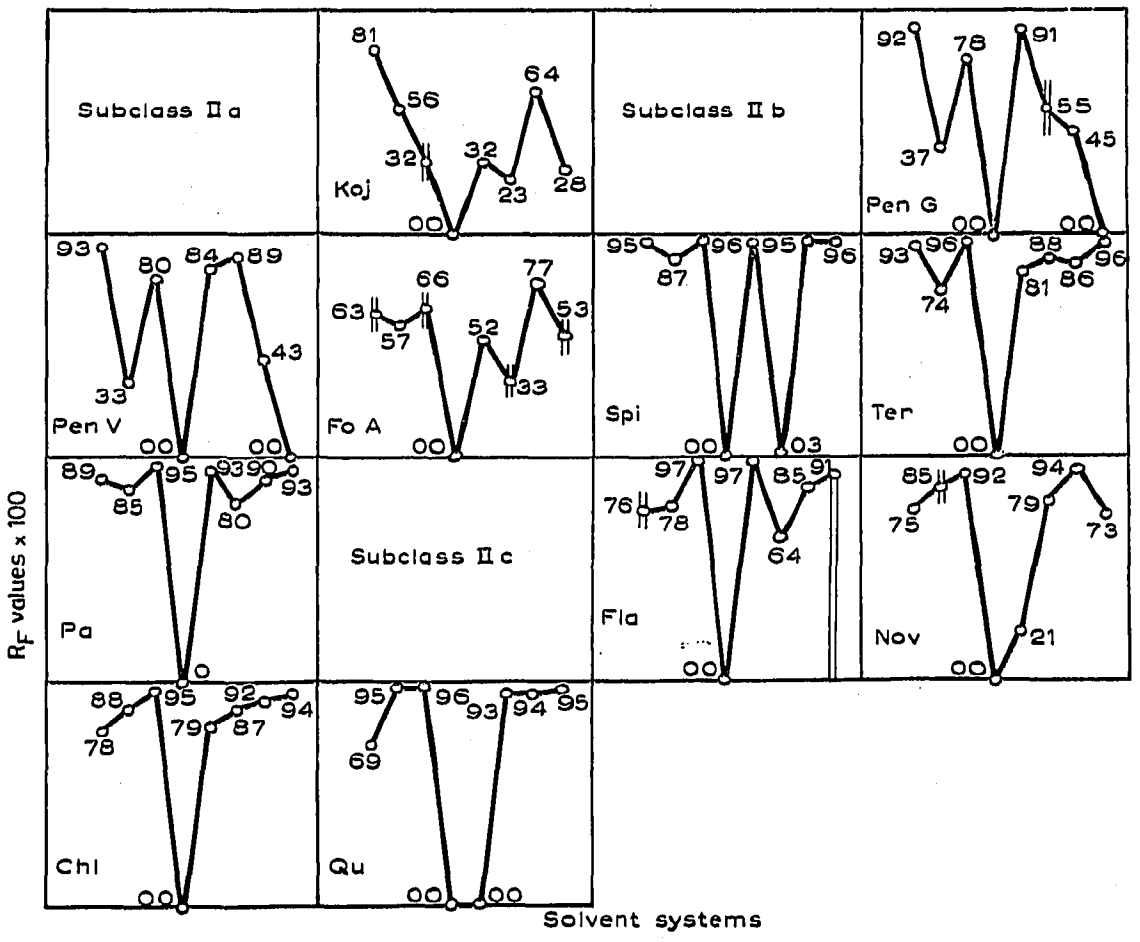

Fig. 3. "Summarized chromatograms" of antibiotics of class II. Subclass IIa: kojic acid (Koj). Subclass IIb: penicillin G (Pen G), penicillin V (Pen V), fomecin A (Fo A), spiramycin (Spi), terreic acid (Ter) and penicillic acid (Pa). Subclass IIc: flavipin (Fla), novobiocin (Nov), chloramphenicol (Chl) and quadrilineatin (Qu). Solvent systems: 1, 2, 3, 4, E, F, G, and H.

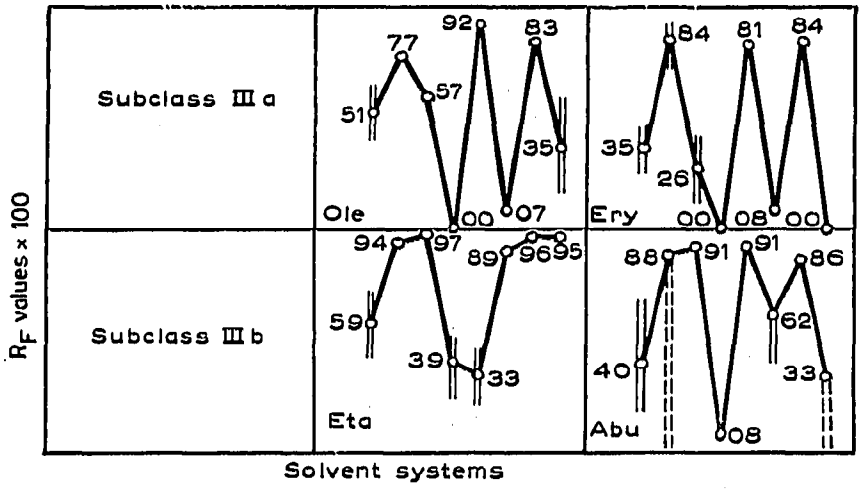

Fig. 4. "Summarized chromatograms" of antibiotics of class III. Subclass IIIa: oleandomycin (Ole) and erythromycin (Ery). Subclass IIIb: etamycin (Eta) and aburamycin (Abu). Solvent systems: 1, 2, 3, 4, E, F, G, and H.

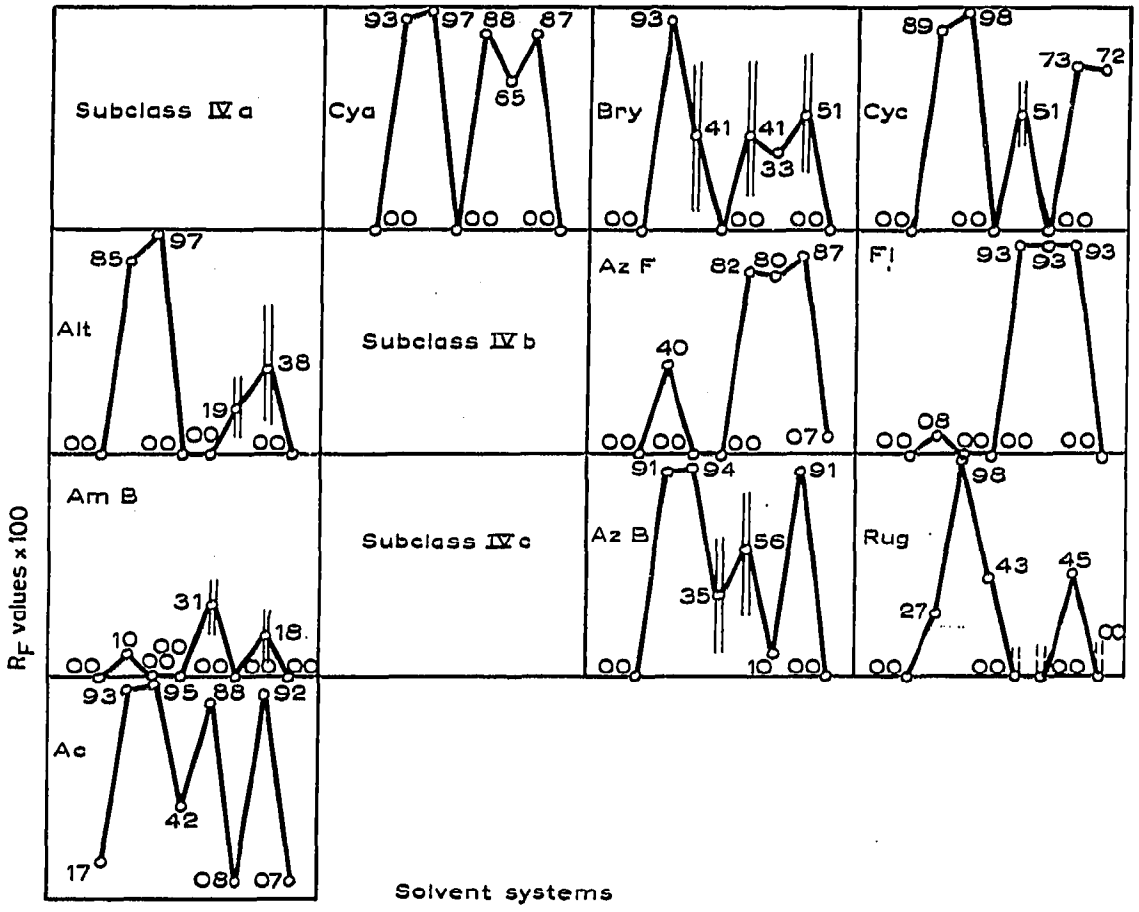

Fig. 5. "Summarized chromatograms" of antibiotics of class IV. Subclass IVa: cyanein (Cya), bryamycin (Bry), cyclopaldic acid (Cyc) and alternariol (Alt). Subclass IVb: azalomycin F (Az F), flavofungin (Fl) and amphotericin B (Am B). Subclass IVc: azalomycin B (Az B), rugulosin (Rug) and actinomycin C (Ac). Solvent systems: 1, 2, 3, 4, I, J, K, and L.

TABLE III

ANTIBIOTICS BELONGING TO CLASS I

Subclass I a	*Subclass I b*	*Subclass I c*
Viomycin	Oxytetracycline	Streptomycin
Kanamycin	Chlorotetracycline	Dihydrostreptomycin
Synnematin B	Vancomycin	Congocidin
Neomycin B	Amphomycin	Ristocetin
Cycloserine	Telomycin	Puromycin
Tetracycline		

TABLE IV

ANTIBIOTICS BELONGING TO CLASS II

Subclass II a	*Subclass II b*	*Subclass II c*
Kojic acid	Penicillin G	Flavipin
	Penicillin V	Novobiocin
	Fomecin A	Chloramphenicol
	Spiramycin	Quadrilineatin
	Terreic acid	
	Penicillic acid	

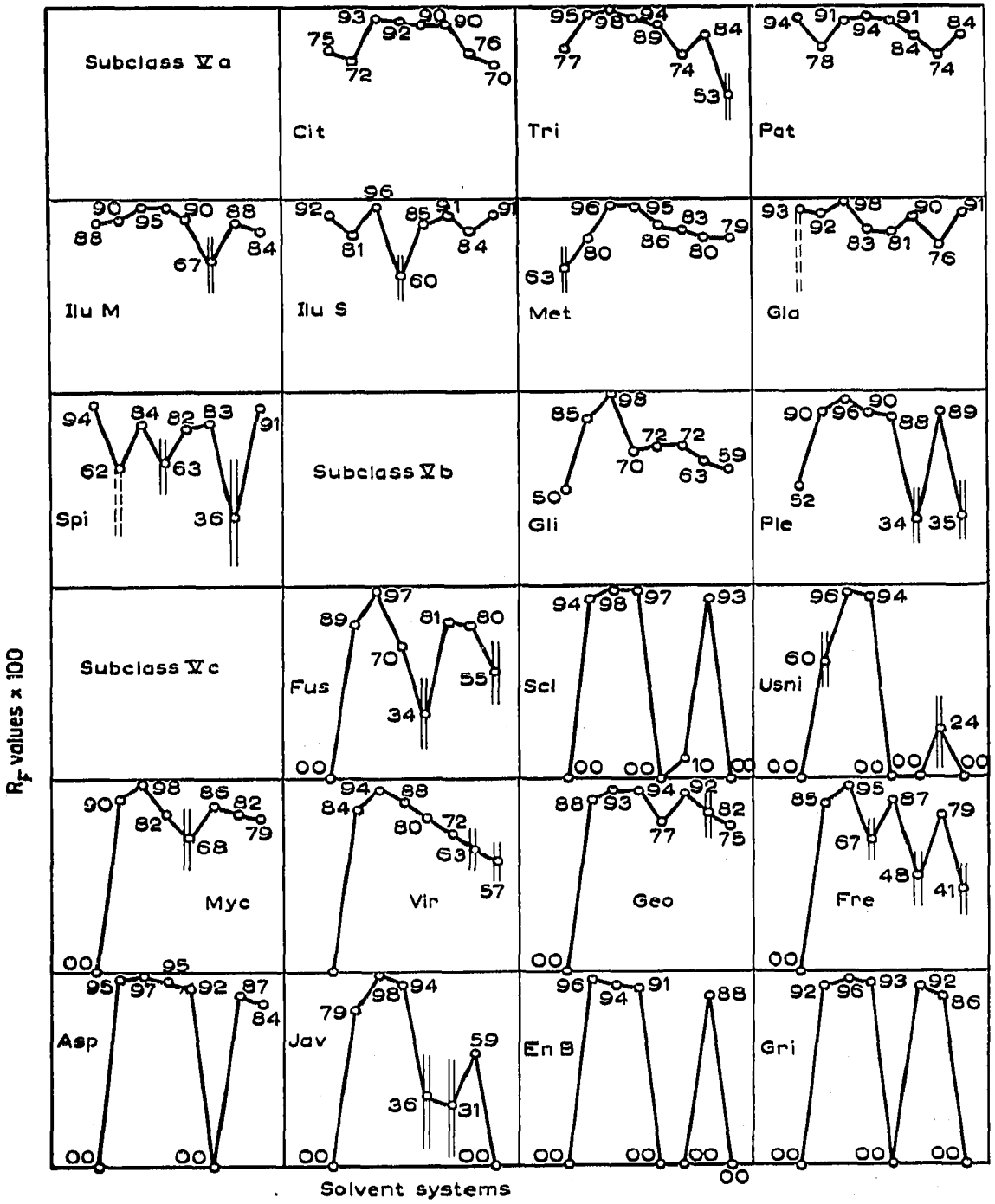

Fig. 6. "Summarized chromatograms" of antibiotics of class V. Subclass Va: citrinin (Cit), trichothecin (Tri), patulin (Pat), illudin M (Ilu M), illudin S (Ilu S), 5-methoxy-*p*-toluquinone (Met), gladiolic acid (Gla) and spinulosin (Spi). Subclass Vb: gliotoxin (Gli) and pleuromutilin (Ple). Subclass Vc: fuscin (Fus), sclerotiorin (Scl), usnic acid (Usni), mycophenolic acid (Myc), viridin (Vir), geodin (Geo), frequentin (Fre), aspergillic acid (Asp), javanicin (Jav), eniantin B (En B) and griseofulvin (Gri). Solvent systems: 1, 2, 3, 4, I, J, K, and L.

streptomycin and dihydrostreptomycin. Commercial samples of vancomycin (Vancocin) and ristocetin (Spontin) were used in our studies. These preparations contain mixtures of vancomycins and of ristocetins, respectively. Double spots in systems

TABLE V

ANTIBIOTICS BELONGING TO CLASS III

Subclass III a	*Subclass III b*
Oleandomycin	Etamycin
Erythromycin	Aburamycin

TABLE VI

ANTIBIOTICS BELONGING TO CLASS IV

Subclass IV a	*Subclass IV b*	*Subclass IV c*
Cyanein	Azalomycin F	Azalomycin B
Bryamycin	Flavofungin	Rugulosin
Cyclopaldic acid	Amphotericin B	Actinomycin C
Alternariol		

TABLE VII

ANTIBIOTICS BELONGING TO CLASS V

Subclass V a	*Subclass V b*	*Subclass V c*
Citrinin	Gliotoxin	Fuscin
Trichothecin	Pleuromutilin	Sclerotiorin
Patulin		Usnic acid
Illudin M		Mycophenolic acid
Illudin S		Viridin
5-Methoxy-*p*-toluquinone		Geodin
		Frequentin
Gladiolic acid		Aspergillic acid
Spinulosin		Javanicin
		Eniantin B
		Griseofulvin

B and D were observed in both cases; these solvent systems could be used for the paper chromatographic separation of the antibiotics mentioned.

Class II of antibiotics differ from class I with respect to their R_F values in the solvent systems 2, 3 and partially in system 1 (see Table II and Fig. 1). Differences in the relationships of the R_F values in systems 1, 2, and 3 served as limits for the subclasses IIa, IIb and IIc.

Class III of antibiotics differ from class II by the R_F values in solvent system 1 and partially (subclass IIIb) in solvent system 4. Differences of R_F values in system 4 were used for the characterization of subclasses IIIa and IIIb.

Both class II and class III have the same additional solvent systems because of some similarities in antibiotics belonging to these two classes. Such antibiotics as penicillins G and V, chloramphenicol, novobiocin and macrolide antibiotics can be shown in these classes. Against this, synnematin B (cephalosporin N), a penicillin with a more hydrophilic side chain in its molecule, does not belong to class II like

penicillins G and V, but to class I which includes antibiotics of a more hydrophilic character.

Antibiotics of class IV have some similarities with classes I, II, and III, when their R_F values in solvent systems 2, 3 and 4 are considered. However, they are akin to the Vc subclass by virtue of their R_F values in solvent system 1. They form a transitional class in our systematic analysis.

Class V is characterized by the R_F values in systems 2, 3 and 4, which are always higher than 0.60, showing a maximum in system 3 in most cases. The characterizations of subclasses Va, Vb, and Vc were established according to differences of the R_F values in solvent system 1.

DISCUSSION

DROZEN[24] discussed the classification and identification procedures of systematic chromatographic analysis in the light of information theory. He mentioned two main different methods of analysis, *viz.* the so-called sequential (step-wise) method and the simultaneous method.

In the chromatographic analysis of antibiotics hitherto known, both principles have been used. SNELL *et al.*[14] classified and identified several peptide antibiotics according to the principles of sequential analysis. ISHIDA *et al.*[10] and most other authors used the principles of simultaneous analysis in several solvent systems. Both methods have their advantages and disadvantages[24].

The advantage of our method of systematic analysis of antibiotics is the use of a combination of both principles. In the first step, antibiotics are analysed simultaneously in the principal solvent systems and are classified into classes and subclasses. The classification is then completed by the second step using the additional solvent systems. Using our combination of sequential and simultaneous analysis, it is not necessary to analyse a compound in many solvent systems as is done in simultaneous analysis. On the other hand, it is possible to compare the "summarized chromatogram" of an antibiotic with others belonging to the same class or subclass, respectively. The chromatographic identification of an unknown antibiotic, of course, must be confirmed by other data (I. R. spectrum, U. V. spectrum and other physical, chemical and biological properties).

MACEK AND PROCHÁZKA[25] mention two possibilities for the evaluation of solvent systems for paper chromatography. Solvent systems are classified according to their decreasing polarity in the eluotropic scale. Another classification of solvent systems is based on their possibility (or impossibility) to be hydrogen donors or acceptors, forming hydrogen bridges.

Both criteria were taken into consideration in our attempt to establish a systematic analysis of antibiotics. The polarity of the principal solvent systems decreases from system 1 to system 4. Solvent system 1 consists of water, which can accept or donate hydrogen, forming hydrogen bridges with other molecules of water. *n*-Butanol, the main component of system 2, also possesses the property of accepting or donating hydrogen and forms hydrogen bridges with molecules of other compounds. Ethyl acetate in solvent system 3 can only accept hydrogen, and benzene in system 4 has the least possibility (compared to the three solvents mentioned) of forming hydrogen bridges.

According to our classification, the hydrophilic character of the antibiotics analysed generally diminishes from subclass Ia to subclass Vc. Structurally related antibiotics (such as tetracyclines, streptomycins, penicillins G and V) have very similar "summarized chromatograms". In some cases the additional solvent systems may help to separate closely related antibiotics (see vancomycin and ristocetin in systems B and D, and penicillins G and V in system F).

In searches for new antibiotics from fungi our systematic chromatographic analysis can be applied when only crude concentrates of the antibiotics are available. An antibiotically active strain is cultivated in liquid medium up to the maximum of the antibiotic activity. Then the mycelium of the fungus is separated from the medium by filtration. Both the filtrate of the cultivation medium and the mycelium of the producing strain are used separately for the preparation of crude concentrates of antibiotics. The crude concentrates are prepared as follows[26]:

(1) 50 ml of acetone are added to 50 ml of the filtrate to precipitate proteins and other compounds that might interfere in the chromatographic analysis of the antibiotics in the filtrate. The mixture is heated to 50° for 10 min and then cooled to laboratory temperature. After precipitation and filtration, the filtrate is evaporated *in vacuo* to dryness. The dry residue is dissolved in 5 ml of 80 % aqueous acetone, filtered and used for the chromatographic studies.

(2) The mycelium of the producing strain is extracted twice with ethyl acetate, filtered and the filtrate is evaporated *in vacuo* to dryness. The dry residue is dissolved in 5 ml of 80 % aqueous acetone, filtered and used for the chromatographic studies of antibiotics from the mycelium.

Results of such chromatographic studies[26] of antibiotics from filtrates of media and from mycelia of 62 strains of antibiotically active fungi are given in Table VIII.

DOSKOČILOVÁ AND VONDRÁČEK[17] in their studies of antibiotics from actinomycetes prepared only concentrates from media and used them for chromatographic analysis of antibiotics. Other authors[27] prepared mixed concentrates of antibiotics

TABLE VIII

CLASSIFICATION OF ANTIBIOTICS FROM 62 STRAINS OF FUNGI

Subclasses	*Antibiotics found in*		
	Media	*Mycelia*	*Both media and mycelia*
Ia	10	—	—
Ib	1	—	—
Ic	—	—	—
IIa	5	—	—
IIb	1	—	—
IIc	5	—	—
IIIa	—	—	—
IIIb	—	1	—
IVa	5	5	5
IVb	7	—	—
IVc	—	1	—
Va	1	6	3
Vb	—	—	—
Vc	—	28	1
	35	41	9

from both the cultivation media and the mycelia of fungi and used them for chromatographic studies.

We found that it is necessary to prepare concentrates from both the cultivation media and the mycelia of strains studied, and that a separate chromatographic analysis of both concentrates of the same strain provides more precise results. We found in many cases that antibiotics in the concentrate from the medium and in the concentrate from the mycelium of the same strain belonged to different classes or subclasses of our systematic analysis. In some cases it was also possible to identify chromatographically antibiotics in concentrates from the medium and from the mycelium of the same strain. Such findings are also very useful for isolation procedures of unknown antibiotics.

In our laboratory data of these chromatographic studies are used not only for the classification but also for the tentative identification of unknown antibiotics. In our screening programme of new antibiotics from fungi it was possible to identify three antibiotics which had been isolated as strictly identical with citrinin[28], gliotoxin[29] and kojic acid[29]. Chromatographic identifications (the "summarized chromatograms", the "pH chromatograms" and the salting-out paper chromatograms) were confirmed by the I. R. spectra and by other physical, chemical and biological properties of these antibiotics.

Data about antibiotics in crude concentrates from filtrates of cultivation media and from mycelia of fungi that are obtained from the "summarized chromatograms" and from the "pH chromatograms"[21,22] help us to determine their ionic character and general possibilities of the isolation procedures[21,22,29]. This principle was applied during isolation and purification procedures of the three antibiotics mentioned and of the new antibiotic cyanein[30].

Of the known antibiotics studied, antibacterial compounds belong mainly to classes I, II and III, whereas antifungal antibiotics or antibiotics with simultaneous antibacterial and antifungal activity belong mostly to classes IV and V, respectively. It is well known that solubility, distribution coefficients and other physico-chemical properties of antibiotics have some relationship to their spectra of antimicrobial activity. This aspect of our systematic chromatographic analysis of antibiotics, of course, needs more detailed studies.

ACKNOWLEDGEMENT

The author wishes to thank Prof. Dr. PAVEL NEMEC, Corresponding member of the Slovak Academy of Sciences and Head of the Department of Technical Microbiology and Biochemistry, for his warm interest in this work.

SUMMARY

An attempt to establish a systematic chromatographic analysis of antibiotics has been made. Sixty-two known antibiotics were distributed into five classes and into fourteen subclasses, according to their R_F values in four principal solvent systems. Principles of the classification of antibiotics are given. For more detailed comparison and characterization of antibiotics further additional solvent systems were used. Additional solvent systems A, B, C, and D were used for class I, systems E, F, G, and H for classes II and III, systems I, J, K, and L were used for classes IV and V.

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