PAPER CHROMATOGRAPHY OF ANTIBIOTICS OF THE MACROLIDE GROUP

M. IGLÓY, A. MIZSEI AND I. HORVÁTH
Research Institute for Pharmaceutical Chemistry, Budapest (Hungary)
(Received April 12th, 1965)

INTRODUCTION

In the search for antibiotics progress very much depends upon developments in paper chromatographic techniques used in the screening of productive strains. Various procedures have been described[1–7], which enable a definite discrimination between groups of antibiotics to be made but are unsuitable for the differentiation of antibiotics which are closely related chemically. Nowadays, when looking for new antibiotics it would be expected that such substances would show only slight structural differences, when compared with substances already known, but that these minute structural variations would be found to involve pharmacological advantages. Thus, it is more than ever necessary to achieve with precision the screening, by paper chromatography, for example, of single antibiotics of a group when several members of it are present simultaneously. This communication deals with the paper chromatography of the macrolide antibiotics.

Data from the literature[8–11] also showed the need to use special techniques for the separations, but the present authors found that the solvent systems proposed were not completely satisfactory. The underlying principle in these techniques consists in the use of a non-polar solvent on paper impregnated with a polar solvent. For special tasks special solvents with a strong resolving effect have been described[12–13]. In the use of these the principle of pH-independent separation has been abandoned, and the basicity of the molecules has been utilized[14–17].

In experiments carried out here we endeavoured to formulate solvent systems which allow maximum separation, of macrolide group antibiotics from other antibiotics, together with maximum separation of individual members within this group, and furthermore, allow the reproducible estimation of samples of low biological activity.

The principle of the separation method is based on the structural specificity of macrolide antibiotics. The members of this group contain, without exception, a multi-carbon, so-called macrocyclic, lactone ring and an amino sugar residue. Thus the dissimilarity of the several members of this group resides partly in the structure of the non-basic moiety, and partly in the dissociation constants of the basic group. These dissimilarities can be demonstrated by the differences between R_F values, provided a suitable chromatographic method can be found. Accordingly, various mixtures, of polar and non-polar solvents were tried in different proportions and the differences in basic strength of the substances were exploited by varying the pH of the chromatographic system. This latter method, called "pH-chromatography",

has been proposed by several authors[18–24], generally for the separation of acidic, basic and amphoteric substances, including antibiotics. According to this principle, by suitable adjustment of polarity and acidity of the developing system, satisfactory separation could be achieved. Variation of acidity was carried out in some of our experiments by saturation of the paper with a buffer, while in other experiments organic acids or bases were added to the solvent mixture.

EXPERIMENTAL

Development of chromatograms was carried out in tubes of 1.5 cm diameter, by the ascending method, on Schleicher-Schüll 2043/b paper strips of 1 × 40 cm. Chromatograms were bio-autographed with *B. subtilis* ATCC 6633. Solvent systems contained in every case a polar and non-polar solvent; the water content of the mixtures was standardized by shaking with one tenth volume of a 30 % aqueous sodium chloride solution.

The following polar solvents were used in these experiments: methanol, ethanol, *n*-propanol, isopropanol, *n*-butanol, *tert.*-butanol; the non-polar solvents were: benzene, toluene, *p*-xylene, cyclohexane, ether, chloroform and dichloroethane.

The first of the non-polar solvents to be investigated was benzene, which was mixed with each of the alcohols enumerated in three concentration ratios. In formulating further solvent mixtures, only methanol and *n*-butanol were used as the polar solvents since these had proved the most suitable as far as separation was concerned.

The adjustment of the pH of the paper was carried out by impregnating it with $^1/_{15}$ *M* phosphate buffers of pH 4.7, 6.0, 7.0 and 8.0, respectively. When the organic solvent was buffered the following five mixtures, in ascending order of their basicity, were used (see Table I).

TABLE I

BUFFER MIXTURES FOR USE WITH SOLVENT SYSTEMS

No of mixture	*Moles of acid per 100 ml solvent*	*Moles of base per 100 ml solvent*
1	0.018	nil
2	0.018	0.009
3	0.018	0.018
4	0.009	0.018
5	nil	0.018

In the case of polybasic acids the quantity of the acid taken was divided by the number of carboxylic groups. Dimethylamine, di-isopropylamine, butylamine, and pyridine were investigated in combination with acetic acid; then pyridine, having shown most promise, was combined in succession with tartaric acid, citric acid, oxalic acid, and decanoic acid.

The following standard substances were used:

Magnamycin base (Pfizer) in alcoholic solution.

Erythromycin stearate (Abbot) in alcoholic solution.
Oleandomycin phosphate (Hoffmann-LaRoche) in alcoholic solution.
Picromycin base, prepared here in crystalline form, in chloroform.
Methymycin base, prepared here in crystalline form, in alcoholic solution.

RESULTS AND DISCUSSION

In a first series of experiments the behaviour on buffered paper of the five macrolide type antibiotics was investigated with the solvent pairs mentioned. Results indicate that the R_F values of the substances change not only according to the solvent pair, but that also the pH value of the paper has a considerable effect. However in the course of these experiments elongated spots were formed in some instances and this caused some uncertainty in the evaluation of the results. This occurred chiefly when substances of low molecular weight were chromatographed.

Therefore, in a second series of experiments buffered solvents were used and this completely prevented the tailing of the spots. A detailed investigation of the behaviour of the substances was made for all the solvent pairs, with all the combinations of the acids and bases previously mentioned. The observations can be summarized as follows: starting at the solvent front, the sequence of the substances is generally magnamycin, methymycin, picromycin, oleandomycin, erythromycin. Within a series of one solvent pair, *i.e.* a non-polar + polar combination, the R_F value is greater the higher the % concentration of the alcohol component. From the point of view of a choice of solvent, it was found that with an increase of the number of carbon atoms in the alcoholic component the R_F value increases, whereas it decreases with an increase of the number of carbon atoms in the non-polar component. A further tendency, *viz.* that with a decrease of acidity the R_F value increases, is only consis-

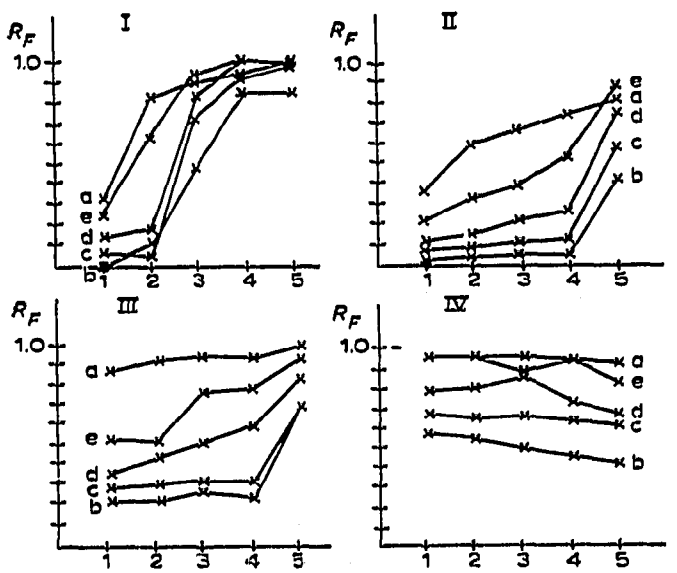

Fig. 1. R_F values obtained with buffered organic solvents. I. Methanol–benzene (20:70), buffered with di-isopropylamine–acetic acid. II. Methanol–benzene (20:70), buffered with pyridine–acetic acid. III. Methanol–dichloroethane (10:80) buffered with pyridine–acetic acid. IV. Methanol–benzene (20:70), buffered with pyridine–oxalic acid. a = magnamycin; b = erythromycin; c = oleandomycin; d = picromycin; e = methymycin. 1, 2, 3, 4, 5 = solvents buffered differently (see Table I).

tently observable when mono-basic acids are used for the buffering of the organic phase; when di- or tribasic acids are used the R_F value remains unchanged or even decreases somewhat with an increase of base content. In the case of aliphatic amines neither the length of the carbon chain nor the order, primary or secondary, of the amine have much effect. With pyridine the increase of the R_F value, in conjunction with higher concentrations of the base, is not as pronounced as with the aliphatic amines, but separation is better.

Based upon the foregoing the following solvents have been found the most suitable for effecting separations:

I. Methanol–benzene (20:70) buffered with di-isopropylamine–acetic acid.
II. Methanol–benzene (20:70) buffered with pyridine–acetic acid.
III. Methanol–dichloroethane (10:80) buffered with pyridine–acetic acid.
IV. Methanol–benzene (20:70) buffered with pyridine–oxalic acid.

Prior to their buffering, all the organic solvents were saturated with water by adding 10 parts of a 30% aqueous sodium chloride solution (see Experimental). Results with these solvent systems are presented in Fig. 1.

SUMMARY

A method has been developed for the paper chromatographic separation of macrolide antibiotics, which is suitable for the identification of the active substances in the fermentation broth of the productive microorganism. The most effective solvent mixtures are composed of an alcohol and a non-polar solvent, saturated with a concentrated solution of sodium chloride, and buffered to various pH values with a mixture of organic acids and bases.

REFERENCES

1 N. ISHIDA, T. SHIRATORI, S. OKAMOTO AND J. MIYAZAKI, *J. Antibiotics*, 4 (1951) 505.
2 N. ISHIDA AND J. MIYAZAKI, *J. Antibiotics*, 5 (1952) 481.
3 J. MIYAZAKI, K. OMACHI AND T. KAMATA, *J. Antibiotics*, 6 (1953) 6.
4 J. URI, *Nature*, 183 (1959) 1188.
5 V. ŠEVČÍK, M. PODOJIL AND A. VRTIŠKOVÁ, *Folia Biol. (Prague)*, 3 (1957) 218.
6 V. ŠEVČÍK, *Microbiology (USSR)*, 28 (1959) 105.
7 V. BETINA, *J. Chromatog.*, 15 (1964) 379.
8 K. MURAI, B. A. SOBIN, W. D. CELMER AND F. W. TANNER, *Antibiot. Chemotheraphy*, 9 (1959) 485.
9 T. M. LEES, P. J. DEMURIA AND W. H. BOEGEMANN, *J. Chromatog.*, 5 (1961) 126.
10 D. DOSKOCILOVÁ AND M. VONDRACEK, *Antibiotiki*, 6 (1961) 649.
11 H. BICKEL, E. GÄUMANN, R. HUETTER, W. SACKMANN, E. VISCHER, W. VOSER, A. WETTSTEIN AND H. ZAEHNER, *Helv. Chim. Acta*, 45 (1962) 1396.
12 J. F. PAGANO, M. J. WEINSTEIN AND C. M. MCKEE, *Antibiot. Chemotherapy*, 3 (1953) 899.
13 W. T. SOKOLSKI, S. ULLMANN, H. KOFFLER AND P. A. TETRAULT, *Antibiot. Chemotherapy*, 4 (1954) 1057.
14 K. MAEDA, T. TAKEUCHI, K. NITTA, K. YAGISHITA, R. UTAHARA, T. OSATO, M. UEDA, S. KONDO, Y. OKAMI AND H. UMEZAWA, *J. Antibiotics (Tokyo)*, 9 (1956) 75.
15 T. OSATO, K. YAGISHITA AND H. UMEZAWA, *J. Antibiotics (Tokyo)*, 8 (1955) 161.
16 T. WATANABE, *Bull. Chem. Soc. Japan*, 33 (1960) 1100.
17 C. W. PETTINGA, W. M. STARK, F. R. VAN ABEELE, *J. Am. Chem. Soc.*, 76 (1954) 569.
18 G. BÜCHI AND M. SOLIVA, *Pharm. Acta Helv.*, 30 (1955) 154; 195; 265 and 297.
19 V. BETINA, *Nature*, 182 (1958) 796.
20 V. BETINA AND P. NEMEC, *Nature*, 187 (1960) 1111.
21 V. BETINA, *Chem. Zvesti*, 13 (1959) 51.
22 V. BETINA, *Chem. Zvesti*, 15 (1961) 750.
23 V. BETINA, *Chem. Zvesti*, 15 (1961) 661.
24 M. IGLÓY, *Magy. Kém. Lapja*, 18 (1963) 622.