RIFOMYCIN

XII.* CHROMATOGRAPHIC STUDIES OF SOME PRODUCTS OF TRANSFORMATION OF RIFOMYCIN B**

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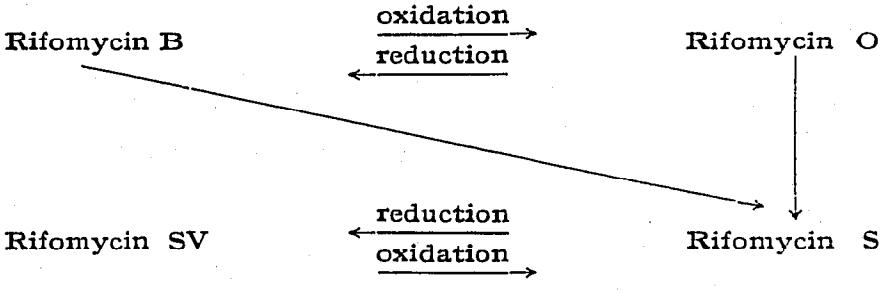
Rifomycin B is a new antibiotic of clinical interest, which has been isolated by SENSI *et al.*[1,2]. When treated with oxidizing agents, rifomycin B is transformed into rifomycin O, which can be again reduced to rifomycin B[3]. Both rifomycin B and rifomycin O in aqueous solutions show the interesting property of undergoing a transformation into another substance with a higher antibiotic activity, rifomycin S[4]. Rifomycin S can be transformed by mild reduction with ascorbic acid into rifomycin SV[4].

In short, we have already proved that the relationships amongst the above-mentioned antibiotics can be represented by the following scheme:



In the course of chromatographic studies aimed at developing test methods for the antibiotics of this family, we encountered some difficulties due to the ease with which each substance is transformed into the others.

The activation process of rifomycin B and rifomycin O was followed by paper chromatography in order to detect the newly formed compound. *n*-Amyl alcohol–*n*-butyl alcohol (9:1), saturated with an aqueous solution containing ascorbic acid, was currently used as solvent system. The addition of ascorbic acid proved to be necessary in order to make the system reducing. With this method, the only antibiotics separated by paper chromatography are rifomycin B and rifomycin SV.

Figs. 1 and 2 represent the paper chromatography of rifomycin B, rifomycin O, rifomycin S and rifomycin SV, the solvent system being *n*-amyl alcohol–*n*-butyl alcohol (9:1) saturated with phosphate buffer pH 8.6, without and with 0.1 % sodium

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ascorbate. In presence of sodium ascorbate (Fig. 2), rifomycin B and rifomycin O give spots with the same R_F value (0.40); these spots are due to rifomycin B. Rifomycin S and rifomycin SV behave in the same fashion, each presenting a spot with the same R_F value (0.87); these spots are due to rifomycin SV.

Using the same solvent system without sodium ascorbate (Fig. 1), rifomycin B develops two spots and this is also the case with rifomycin O. It is assumed that the first spot (R_F 0.25) is rifomycin B, also when rifomycin O is the starting material.

Fig. 1. (1) Rifomycin B; (2) rifomycin O; (3) rifomycin S; (4) rifomycin SV. Solvent system: *n*-amyl alcohol–*n*-butyl alcohol (9:1) saturated with phosphate buffer pH 8.6.

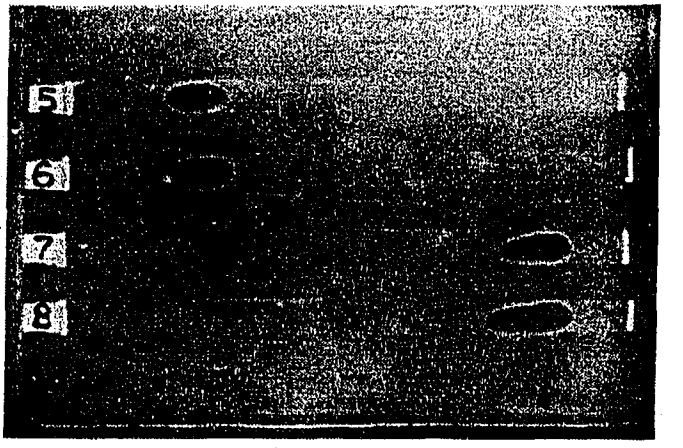

Fig. 2. (5) Rifomycin B; (6) rifomycin O; (7) rifomycin S; (8) rifomycin SV. Solvent system: *n*-amyl alcohol–*n*-butyl alcohol (9:1) saturated with phosphate buffer pH 8.6 containing 0.1 % sodium ascorbate.

This assumption is supported by experiments which demonstrate the possibility of rifomycin O being transformed into rifomycin B by the mild reducing power of the chromatographic paper. The second spot (R_F 0.73) is caused by the conversion of rifomycin B or rifomycin O into rifomycin S. Furthermore, we have demonstrated that rifomycin S can be reduced by chromatographic paper to rifomycin SV; no doubt the second spot must be due to rifomycin SV.

The R_F values obtained with the solvent systems with and without sodium ascorbate do not correspond exactly. We suppose that the differences are caused by the presence of sodium ascorbate in the first solvent system. These differences are even more marked if the percentage of sodium ascorbate is increased.

The reduction of rifomycin O to rifomycin B and of rifomycin S to rifomycin SV by the very mild reducing properties of chromatographic paper was demonstrated by the following experiments.

A methanolic solution of rifomycin O was micropipetted onto four Whatman No. 1 paper strips (50 γ on each strip) and chromatographed using as solvent system *n*-amyl alcohol–*n*-butyl alcohol (9:1), saturated with phosphate buffer pH 8.6. The spots corresponding to R_F 0.25 were cut out, collected and eluted with 25 ml methanol. The methanolic solution was concentrated under vacuum to 5 ml. The visible spectrum of this solution clearly shows the characteristics of rifomycin B (Fig. 3).

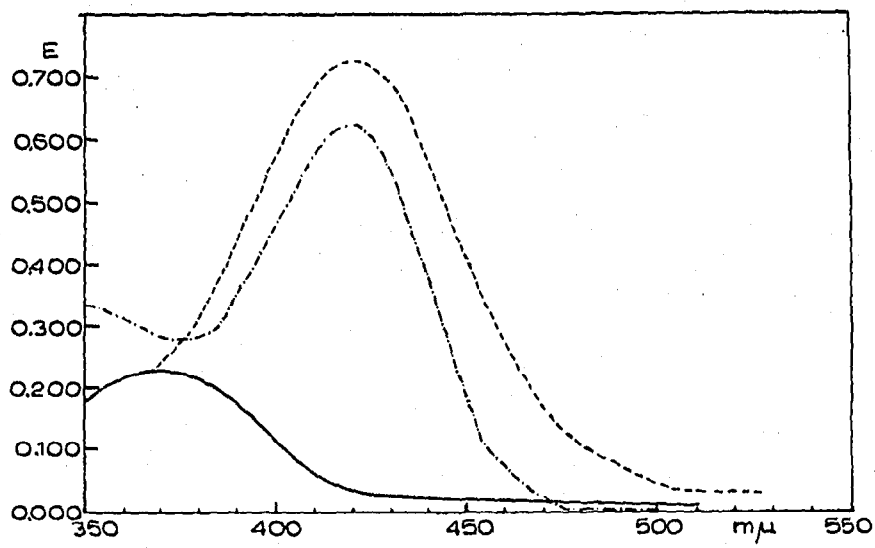

Fig. 3. Reduction of rifomycin O to rifomycin B by chromatographic paper. Visible spectra in methanolic solution at 40 γ/ml concentration of: ——— rifomycin O; – – – – – rifomycin B; —·—·— rifomycin O after paper chromatography.

The same experiment was performed with rifomycin S. The spectrum of the methanolic eluate from four chromatographic strips is identical with that of rifomycin SV (Fig. 4). Elution must be performed very carefully in absence of oxygen, to avoid the possibility of the eluted rifomycin SV being oxidized to rifomycin S. In this case the resulting spectrum of the eluate is a combination of the two forms — one oxidized and one reduced — of the antibiotic.

Various attemps to separate rifomycin O from rifomycin B and rifomycin S from rifomycin SV failed because of the reducing power of the chromatographic paper.

Rifomycin B can be separated from rifomycin SV by means of several solvent

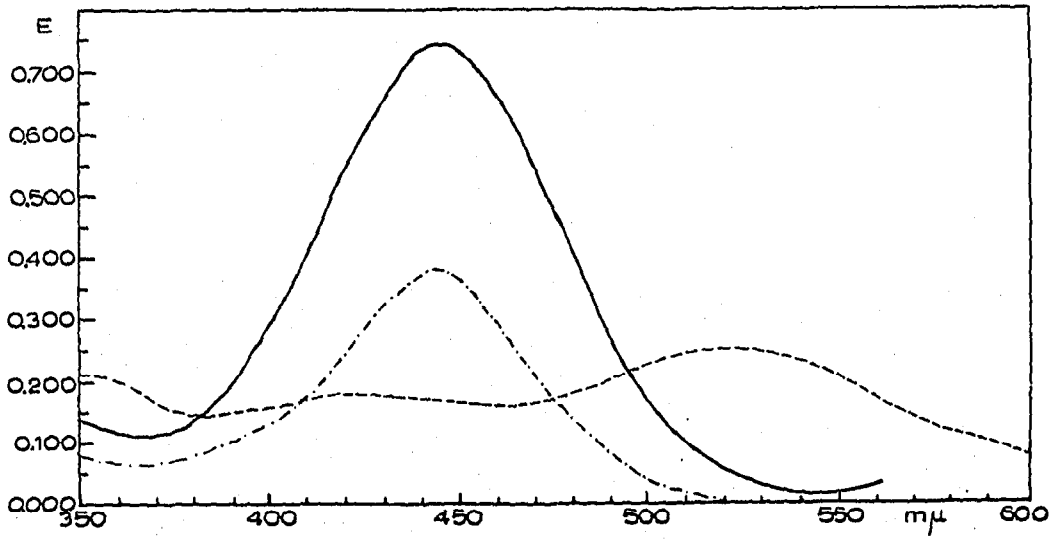

Fig. 4. Reduction of rifomycin S to rifomycin SV by chromatographic paper. Visible spectra in buffer solution pH 7.3 at 40 γ/ml concentration of: ——— rifomycin SV; – – – – – rifomycin S; —·—·— rifomycin S after paper chromatography.

systems. The most suitable systems that we employed for routine analyses are the following:

(1) *n*-Amyl alcohol–*n*-butyl alcohol (9:1) saturated with phosphate buffer pH 8.6 containing 0.1 % sodium ascorbate. The ratio amyl alcohol–butyl alcohol is not critical; the small percentage of butyl alcohol markedly increases the mobility of the two antibiotics. The pH value of the aqueous phase must be neutral or slightly alkaline. In fact, both rifomycin B and rifomycin SV are acidic substances of different strength. Rifomycin B is a dibasic acid whilst rifomycin SV is a monobasic acid. Therefore, it is obvious that at a neutral or slightly alkaline pH, rifomycin SV has a higher mobility in organic solvents than rifomycin B (rifomycin B, R_F = 0.40; rifomycin SV, R_F = 0.87) (Fig. 2).

(2) Phosphate buffer pH 7.3 containing 0.1 % sodium ascorbate, saturated with *n*-amyl alcohol–*n*-butyl alcohol (9:1). In this aqueous solvent rifomycin B shows a higher mobility than rifomycin SV (rifomycin B, R_F = 0.67; rifomycin SV, R_F = 0.51) (Fig.5).

(3) *n*-Butyl alcohol saturated with phosphate buffer pH 7.3 containing 0.1 % sodium ascorbate (rifomycin B, R_F = 0.75; rifomycin SV, R_F = 0.95) (Fig. 6).

(4) Phosphate buffer pH 8.6 containing 0.1 % sodium ascorbate saturated with *n*-butanol (rifomycin B, R_F = 0.86; rifomycin SV, R_F = 0.67) (Fig. 7).

All the chromatographic experiments were performed in glass jars, saturated for 6 hours at 22° with the solvent system. Whatman No. 1 paper was dipped in the aqueous buffered phase and dried. About 18 hours were necessary for a chromatographic run of 25 cm if the organic solvents were employed and, only 6 hours if the aqueous solvents were used. The strips were dried at room temperature and developed on agar plates buffered at pH 5.9 using *Sarcina lutea* as the micro-organism test. The optimal quantities of the two antibiotics were about 1 γ of rifomycin SV and 5–10 γ of rifomycin B for each strip.

CENTRIFUGAL CIRCULAR CHROMATOGRAPHY

In order to avoid the activation of rifomycin B in the course of the chromatographic run, centrifugal circular chromatography[5] was employed. With this technique, the time of a chromatographic run is greatly reduced (about 4 min). Rifomycin B was micropipetted at 3 cm from the center of the paper disk (Whatman No. 1). The paper disk was placed on a circular, stainless steel frame in a saturated air-tight chamber and rotated at about 1500 r.p.m. while a thin flow of solvent was aimed at a point about 2 cm from the center. The solvent reached the border of the disk in 4 min. The paper disk was dried and then placed for 15 min on the agar assay plates buffered at pH 5.9 using *Sarcina lutea* as the micro-organism test.

Rifomycin B could be separated from rifomycin SV by using *n*-amyl alcohol–*n*-butyl alcohol (9:1) saturated with phosphate buffer pH 8.6.

The quantities of rifomycin B and SV were 1 γ and 0.15 γ, respectively. There was no evidence of "activation" of rifomycin B although the solvent system did not contain sodium ascorbate as stabilizer (Fig. 8).

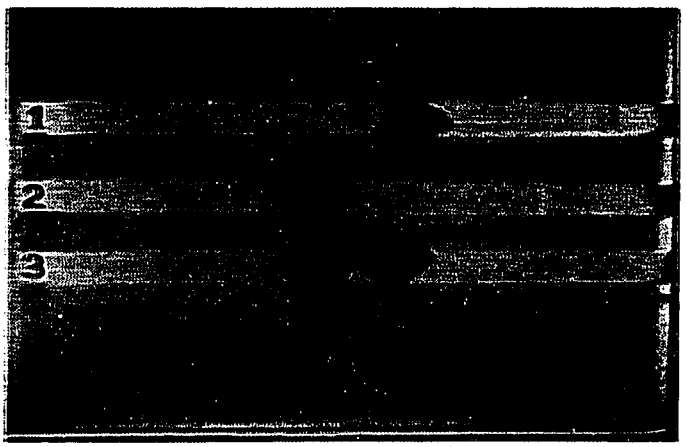

Fig. 5. (1) Rifomycin B; (2) rifomycin SV; (3) rifomycin B and rifomycin SV. Solvent system: phosphate buffer pH 7.3 containing 0.1 % sodium ascorbate saturated with *n*-amyl alcohol–*n*-butyl alcohol (9:1).

Fig. 6. (1) Rifomycin B; (2) rifomycin SV; (3) rifomycin B and rifomycin SV. Solvent system: *n*-butyl alcohol saturated with phosphate buffer pH 7.3 containing 0.1 % sodium ascorbate.

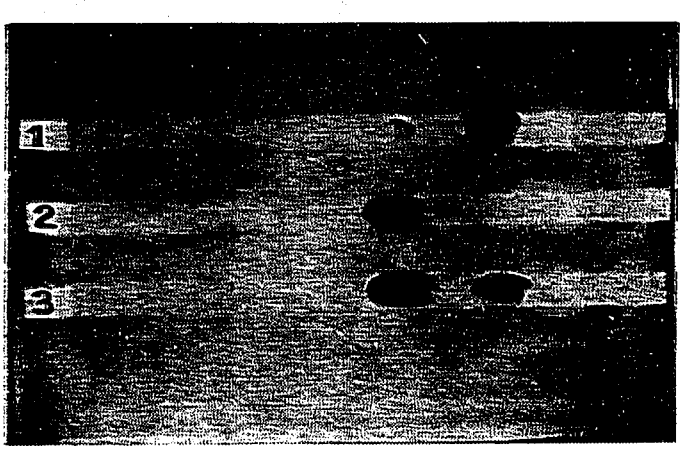

Fig. 7. (1) Rifomycin B; (2) rifomycin SV; (3) rifomycin B and rifomycin SV. Solvent system: phosphate buffer pH 8.6 containing 0.1 % sodium ascorbate saturated with *n*-butanol.

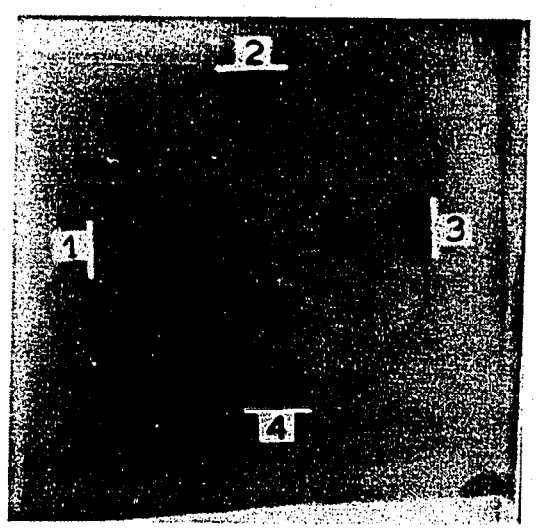

Fig. 8. Centrifugal circular chromatography. (1) Rifomycin B; (2) rifomycin SV; (3, 4) rifomycin B and rifomycin SV. Solvent system: *n*-amyl alcohol–*n*-butyl alcohol (9:1) saturated with phosphate buffer pH 8.6.

THIN-LAYER CHROMATOGRAPHY

The difficulties encountered in the detection of rifomycin O and rifomycin S by paper chromatography mentioned above, can be overcome by using thin-layer chromatography according to STAHL[6–11].

The plates were prepared as described earlier by one of us[12], using 25 g of Silicagel G (a mixture of Silicagel and calcium sulfate) and 50 g of water. A thin layer of Silicagel was obtained on glass plates by a roller machine. The plates were dried at

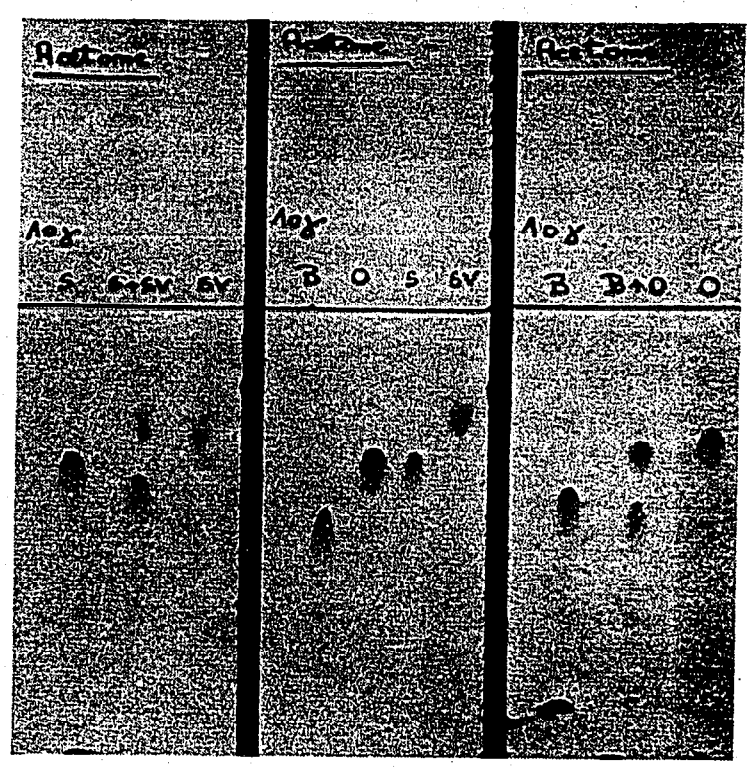

Fig. 9. Thin-layer chromatography of rifomycin B, O, S and SV.

105–110° for 30 min and cooled in a desiccator over Sikkon. The thin layer is fixed on the glass plates by the calcium sulfate. The activity of the Silicagel layer is equivalent to about II–III degrees.

The separation of rifomycin B from rifomycin O and of rifomycin SV from rifomycin S can be achieved by using acetone as solvent (Fig. 9).

10 γ of each of the antibiotics rifomycin B (in methanol solution), O, S and SV (in acetone solution) were applied at 2 cm from one end of the plate at distances of 1 cm. After drying for a short time the end was placed into an air-tight jar (saturated for 6 h with the solvent) containing a 1 cm layer of acetone at the bottom. After a run of 10 cm (15 min) the plate was removed and air-dried. Rifomycin B, O and SV are visualized as clear yellow spots, rifomycin S as a red-violet one.

Fig. 9 shows a good separation of rifomycin B from rifomycin O and of rifomycin S from rifomycin SV. The R_F values of rifomycin O and rifomycin S are about the same and no clear separation occurs with acetone. A mixture of these antibiotics can nevertheless be detected since rifomycin S is red and rifomycin O yellow. In this case a yellow spot appears with a red halo.

SUMMARY

Several chromatographic techniques were used in order to separate rifomycin B, rifomycin O, rifomycin S and rifomycin SV.

Rifomycin B and rifomycin SV can be easily separated by paper chromatography using organic or aqueous systems containing ascorbic acid as stabilizer.

Rifomycin O and rifomycin S are reduced by the paper during the chromatographic run, to rifomycin B and rifomycin SV respectively.

The four antibiotics can be separated by means of thin-layer chromatography on Silicagel.

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