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Paper Chromatography of the Streptomycins and Some Related Compounds

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A partition chromatography system capable of resolving all the components of the streptomycin group of antibiotics has been developed. Amylalcohol containing di-2-ethyl-hexyl phosphate as carrier was used as the mobile phase. The stationary phase was a borate buffer.

A simple chromatographic method to separate and detect the components of the streptomycin group of antibiotics is of great importance, as the different components often occur simultaneously. In the streptomycin fermentation some mannosidostreptomycin is usually formed. This compound is toxic and has little antimicrobial effect, and is therefore highly undesirable in the final preparations. The procedures for isolation and purification of streptomycin from a fermentation broth may cause some contamination of the product with streptidine. The sulphate of this compound easily forms oversaturated solutions, especially in the presence of streptomycin sulphate. The streptidine sulphate may crystallize spontaneously after a storage of several months as sharp big crystals, making the preparation unfit for use. Finally, the dihydrostreptomycin obtained by hydrogenation of streptomycin is not always free from some streptomycin.

The classic solvent system described by Winsten and Eigen [1] is water-saturated butanol containing 2 % *p*-toluene sulfonic acid as a carrier. This system does not separate streptomycin and dihydrostreptomycin. Furthermore the time needed for the development is 72 h. Hunter *et al.*[2] have modified this system. However, no data describing the resolutions obtained are available. We have tested the system and have found it to be time-consuming and the resolution is poor.

The circular paper chromatography system developed by Horowitz and Schaffner [3] for the resolution of the streptotricin antibiotics has also been examined. The R_F values were very small and badly defined.

The extensive systematic study of the chromatographic classification of sixty-two antibiotics from fungi actinomycetes and lichens described by

Bettina [4] does not offer any method for the separation of streptomycin and dihydrostreptomycin. The investigation does not include mannosidostreptomycin nor hydroxystreptomycin and streptidine.

Kondo *et al.*[5] classify a number of basic water-soluble antibiotics produced by actinomycetes by means of eight different solvent systems. This leads to four groups called the streptomycin, the streptotricin, the fradiomycin, and the kanamycin groups. The identification of the single components is effected by either descending chromatography using *p*-toluene sulfonic acid in water-saturated butanol or by ascending chromatography in 80 % aqueous methanol containing 1.5 % sodium chloride. The authors do not give details of the resolution of the single components of the streptomycin group.

Ito *et al.*[6] obtain very good separations of a number of antibiotics by means of thin-layer chromatography on cellulose powder. Hydroxystreptomycin, with an R_F value of 0.32, is clearly separated from dihydrostreptomycin and streptomycin which both have an R_F value of 0.44. The latter compounds are thus not separated.

Nussbaumer and Schorderet [7] have described a separation of streptomycin and dihydrostreptomycin by thin-layer chromatography after prior treatment of the mixture with phenylhydrazine. The one spot is phenylhydrazine which has the same R_F value as dihydrostreptomycin whereas the phenylhydrazone of streptomycin has a considerably smaller R_F value. The method has, however, not been tried with compounds other than the two mentioned, and furthermore the smallest amount of streptomycin detectable in the presence of dihydrostreptomycin is about 10 %.

RESULTS AND DISCUSSION

A complete resolution of a mixture of streptomycin, dihydrostreptomycin, streptidine, hydroxystreptomycin and mannosidostreptomycin was obtained by the descending method using Whatman paper No. 20. The development time was 8 h. Fig. 1 shows a chromatogram sprayed with the colour reagent, Fig. 2 shows the zones of inhibition caused by an identical chromatogram on an agar plate seeded with bacteria.

In Table 1 the R_F values relative to streptomycin are given.

Table 1. R_F values relative to streptomycin.

Streptomycin	1.00
Dihydrostreptomycin	0.83
Hydroxystreptomycin	0.73
Streptidine	0.66
Mannosidostreptomycin	0.43

The real R_F value for streptomycin varies considerably from run to run, in most cases being 0.7.

The absolute R_F values vary considerably from run to run, a very important factor being the moisture content of the paper. The paper which was found most satisfactory was Whatman No. 20 if resolution of all the antibiotics mentioned was desired.

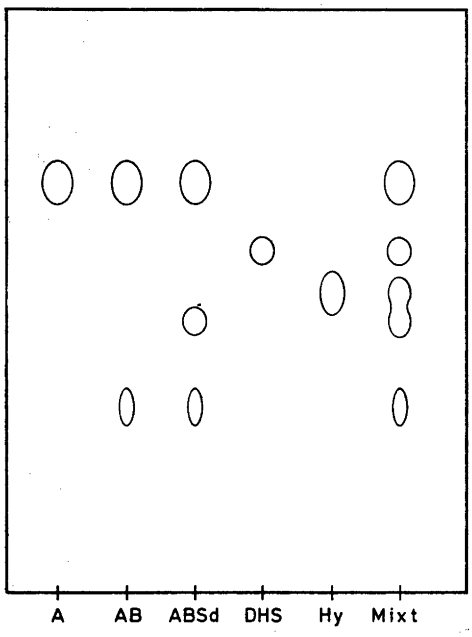

Fig. 1. Descending chromatogram of streptomycin (A), streptidine (Sd), hydroxystreptomycin (Hy), dihydrostreptomycin (DHS), and mannosidostreptomycin (B) sprayed with the diacetyl, α-naphtol reagent.

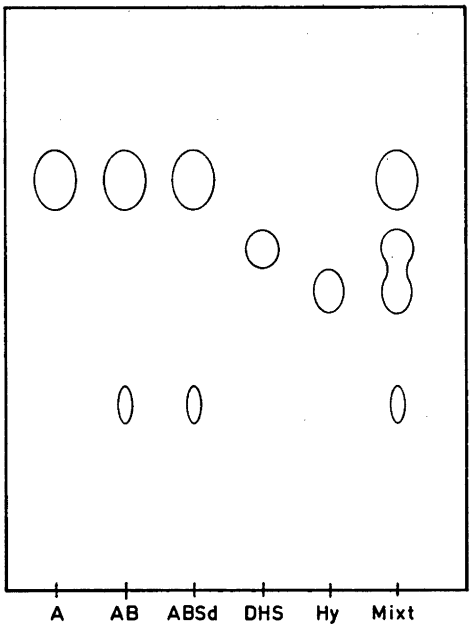

Fig. 2. Zones of inhibition on an agar plate seeded with bacteria caused by an identical chromatogram as the one shown in Fig. 1.

When separation of only two or three compounds is desired a paper with a faster flow rate may be used. Good separation of streptomycin and mannosidostreptomycin was obtained on Whatman No. 1 paper, the development taking only 6 h. Streptomycin and streptidine could also be separated on this paper but not streptomycin and dihydrostreptomycin.

A good separation of streptomycin, streptidine and mannosidostreptomycin can be obtained in a few hours by ascending chromatography using the same solvent system. The front of the solvent moves 10—12 cm during development. Streptomycin, hydroxystreptomycin, and dihydrostreptomycin are, however, not separated using this technique.

An important feature of the chromatographic system described is its insensitivity to high salt and protein concentrations. This makes it possible to chromatograph fermentation broths and other impure samples without previous desalting and deproteinizing.

The smallest amount of streptomycins detectable by the colour reaction is about 1 μg and 0.5 μg by the biological method. The detection of even a very small cross contamination between the streptomycins is, however, possible because the method allows the application of considerable amounts of

material without interfering with the separation. In particular the presence of small amounts of streptomycin in dihydrostreptomycin is readily detected, the lower limit being about 1 %.

Occasionally it may be difficult to distinguish between hydroxystreptomycin and streptidine by the colour reaction due to the small difference between the R_F values. The microbiological test, however, makes this possible since hydroxystreptomycin is highly active and streptidine inactive.

The simplicity of the method described makes it a useful tool in testing the purity of streptomycin. Contamination of streptomycin with streptidine and dihydrostreptomycin is easily detected.

MATERIALS AND METHODS

Materials. The chromatography papers used were Whatman Nos. 1, 4, 20 and Schleicher and Schüll No. 2043 b Mg. 1. All the chemicals were of analytical grade except the di-2-ethyl-hexyl phosphate which was of technical grade (Virginia-Carolina, Chm. Corp., Carolina, U.S.A.). The streptomycin standard was a commercial technical preparation. The streptidine was prepared from streptomycin by acid hydrolysis and recrystallized twice as the sulphate from water, as decribed by Pech *et al.*[8] The mannosidostreptomycin containing some streptomycin was obtained from the mother liquid of the streptomycin calcium chloride crystallization. Streptidine was removed from the preparation by precipitation as its insoluble complex with 2,6-dibromo-4-nitro-phenol.[9] Hydroxystreptomycin was obtained from Abbott Laboratories, North Chicago, Ill. U.S.A.

Methods. Equal volumes of amylalcohol containing 1 %, v/v, di-2-ethylhexyl phosphate and 0.5 %, w/v, of sodium chloride in borate buffer (0.62 g boric acid, 0.21 g borax/l) were mixed. The pH was adjusted to 8.0 with sodium hydroxide while stirring. Stirring was continued for 30 min. After separation of the phases the chromatography paper was dipped in the lower phase. The paper was blotted between two sheets of filter paper, and the samples were applied to the paper as aqueous solutions. The paper was allowed to dry for a few minutes and still wet developed with the organic phase in either the ascending or descending mode. After development the paper was dried in a current of air and either sprayed or dipped in the alkaline α-naphtol-diacetyl colour reagent described by Halliday.[10] Equal volumes of 40 %, w/v, potassium-hydroxide in 50 % methanol, 2.5 %, w/v, α-naphtol in methanol and 0.1 % w/v, diacetyl in methanol was mixed immediately before use in the order given. Testing for biological activity was made by applying the carefully dried chromatogram for one minute on the surface of a nutrient agar seeded with spores of *B. subtilis*. After removal of the chromatogram the plate was incubated for 8 h at 37°C.

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