PAPER CHROMATOGRAPHIC SEPARATION OF MULTI-COMPONENT ANTIBIOTIC MIXTURES

THE ACETYLATED OLEANDOMYCINS*

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The use of paper chromatography in separating, classifying and identifying biological products has in the last ten years become a well established technique. When used in conjunction with bioautographic methods it has proven particularly fruitful in the field of antibiotics. Recently, two exceptionally challenging antibiotic mixtures, the quinocycline complex of six components[1] and the group of seven acetylated oleandomycins[2,3,4] have been examined by CELMER *et al.* We wish to report the details of the paper chromatographic methods that have proven successful in our laboratories for resolving these mixtures.

The use of formamide-impregnated paper along with a non-polar mobile phase has been described by ZAFFARONI[5] for the separation of steroids and by SCHINDLER[6] for the separation of alkaloids. The utility of this technique for the separation of the same materials has been confirmed in our laboratories and also it has proven very useful in separating many solvent-soluble antibiotics, especially those of the macrolide group[9]. These methods described more fully here have been particularly successful in resolving naturally occurring mixtures such as the quinocyclines, and closely related derivatives such as the acetylated oleandomycins. The toxicity of formamide for some of the bacteria used in preparing bioautographic plates may have discouraged other workers from using these systems; however, there are several micro-organisms that can be employed.

The use of paper chromatography in following the preparation and recovery of the six members of the quinocycline complex, along with the solvent system** and a table of R_F's has been mentioned by CELMER *et al.*[1]. In the following procedure we shall elaborate on the specific techniques of paper chromatography employed using as an example separation of the acetylated oleandomycins achieved by these systems.

METHODS

Whatman No. 4 paper is cut into 6½ in. × 19 in. strips with the long axis in the machine direction of the paper. A 1-in. hem is sewn on one end of the paper to

* Triacetyloleandomycin is commercially available from the J. B. Roerig Division of Chas. Pfizer and Co., Inc. The registered trademark for this material is TAO.

** Chloroform–ethyl acetate (3:1) saturated with formamide.

serve as a holder for a solid glass anchor rod, which also serves as a convenient grip in handling the sheets in later steps. The strips are impregnated by passing them through a 50% solution of formamide in methanol and then blotted on clean white blotters. Spots of the material* to be examined are then placed on the sheets. Normally we place on a sheet five or six evenly spaced 10-λ spots containing from 1 to 10 γ of antibiotic material per spot; the concentration depending upon the potency and number of components. Usually one control spot containing oleandomycin base and its seven acetylated derivatives is used on each sheet.

The sheets are then developed by a descending solvent in a 12 in. × 24 in. cylindrical jar using an 8 in. × 3 in. × 1½ in. porcelain tray to contain the developing solvent. No anti-siphon rods are needed with these trays. Solvents used were of analytical grade not further purified. The solvent is allowed to run to about 1 or 2 in. from the end of the paper, usually requiring between 1 and 2 hours. Longer runs, wherein the solvent drips off the sheets, will achieve greater separation of the components.

The bioautographic plate is prepared from a sheet of Pyrex plate glass 19 in. × 15 in. × $^3/_{16}$ in. to which are cemented** two strips 13½ in. × ¾ in. × ¼ in. and two strips 19 in. × ¾ in. × ¼ in. to make a shallow glass tray. A second sheet of glass can be used as a lid for the plate. Each plate is filled with 300 ml of Bacto Streptomycin assay agar*** inoculated with *B. subtilis* (ATCC No. 6633). After the agar has set, the chromatogram sheet, previously air dried for about 30 min to remove the volatile solvents but not the formamide, is gently pressed onto its surface by means of a lipless test tube, and left in contact from 15 to 30 min. Similar bioautographic techniques have been reported by other workers[7,8]. The reverse side of the plate can be marked with the point of origin, and suitable sample identification marks. The sheets are removed, the plates incubated overnight, and on the following day the outlines of the inhibition zones are delineated with a glass marking crayon. By using heavier inoculum and allowing the plates to preincubate while the chromatogram sheets are developing, a sample can be run and visualized within 6 to 8 hours. Photographs of the plates can be made either with a camera or by direct contact on Kodagraph paper, but for routine work, copies made on tracing paper are more rapid and less expensive.

Great care must be taken to avoid contaminating the chromatogram sheet with antibiotic particles such as might be floating in the air and the same precautions must be taken with the bioautographic plates. Airborne bacterial contamination is seldom a serious problem providing the surface of the agar is allowed to dry slightly before applying the chromatogram sheet. It is a characteristic of the formamide systems that at different temperatures and also in different chambers the R_F and degree of separation of the components may vary considerably. The separation of 1,2-diacetyl-

* Antibiotic samples were supplied by Dr. W. D. CELMER and his co-workers.

** At present we are using Flintkote Rubber Adhesive No. 979 obtained from the Flintkote Co., 30 Rockefeller Plaza, New York 20, N.Y.

*** Difco Laboratories, Inc., Detroit, Mich.

oleandomycin (1,2-DAO) and triacetyloleandomycin (TAO) is especially sensitive to temperature. By altering the proportions of the solvents in the system or by using a more or less polar solvent combination the desired resolution can be obtained. Two general precautions should be observed: the first is not to overload the chromatogram spots; the other is to use control spots and not depend entirely on R_F values.

RESULTS

In Table I the R_F's of the various acetylated oleandomycins are shown for the four most useful solvent combinations arranged in order of increasing polarity. These values were obtained from a series of runs made at the same time; chromatograms run at other times may show different R_F's but the components will appear in the same order. Figs. 1 and 2 illustrate bioautographs of the separations obtainable by this method. Fermentation broths, broth isolation samples, reaction mixtures, urines, and solvent extracts of blood have all been examined by using these paper chromatographic systems.

TABLE I

R_F VALUES OF ACETYLATED OLEANDOMYCINS*

Compound	R_F in solvent system**			
	A	B	C	D
Oleandomycin base	0.02	0.05	0.05	0.30
3-MAO	0.05	0.10	0.15	0.50
2-MAO	0.10	0.25	0.40	0.70
2,3-DAO	0.20	0.35	0.70	0.95
1-MAO	0.30	0.50	0.95	0.95
1,3-DAO	0.40	0.65	0.95	0.95
1,2-DAO	0.80	0.90	0.95	0.95
TAO	0.95	0.95	0.95	0.95

* The nomenclature of the acetylated oleandomycins is that of CELMER[3,4]: 3-MAO = 3-monoacetyloleandomycin; 2,3-DAO = 2,3-diacetyloleandomycin; TAO = triacetyloleandomycin, etc.
** Solvent system: A = benzene–cyclohexane (1:1); B = benzene–cyclohexane (2:1); C = benzene–chloroform (3:1); D = benzene–chloroform (1:1); all systems saturated with formamide.

Several trials were made to see if quantitative results could be determined from the size of the inhibition zones. Reasonably accurate values could be obtained for most of the derivatives if the following conditions were observed: (A) uniform spot volumes were used; (B) reference standards of the same derivative to be measured were used; and (C) the component to be measured must be in the region of R_F 0.2 to 0.5. Plots of area *versus* logarithm of concentration usually gave smooth curves. However, a sample containing several components required a multiplicity of standards and more than one solvent system, resulting in a cumbersome and error-prone procedure. It was usually satisfactory, and also simpler, to make an estimate of the relative amounts of the components by visual inspection of the bioautographic plates.

Samples of blood and urine taken at various intervals after the ingestion of

TAO have been examined and the appearance of the several de-acetylated products can be readily detected. The metabolic fate of other derivatives was similarly investigated[4]. In some blood samples an unidentified component is often noted. This material is slightly more polar than TAO and is characterized by a less distinct zone edge on the bioautographic plates. It is thought to be a conjugate or additive compound composed of one of the oleandomycins and some blood constituent.

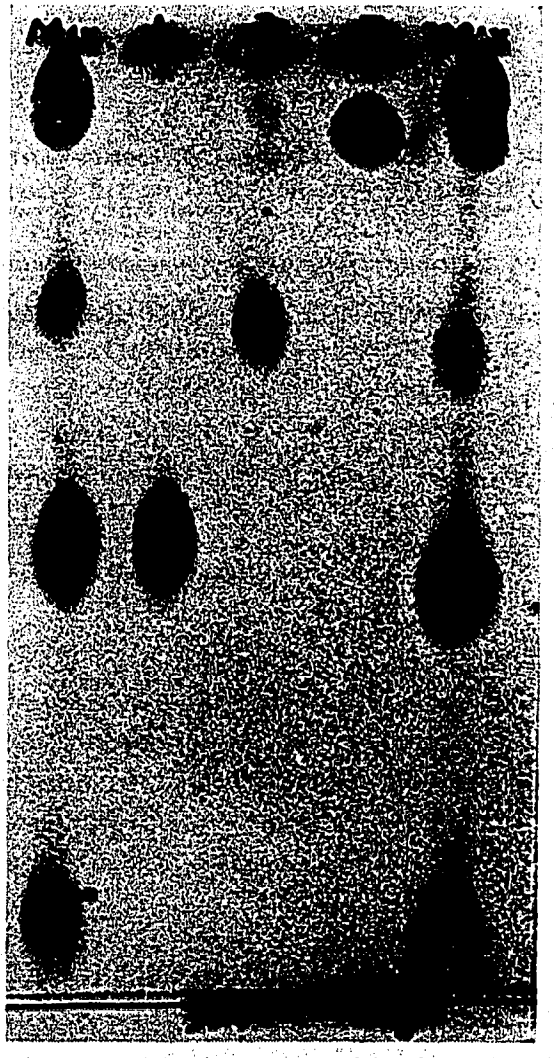

Fig. 1. *B. subtilis* bioautographic plate showing separation of the MAO's by solvent system B. The mix also contains oleandomycin base and TAO.

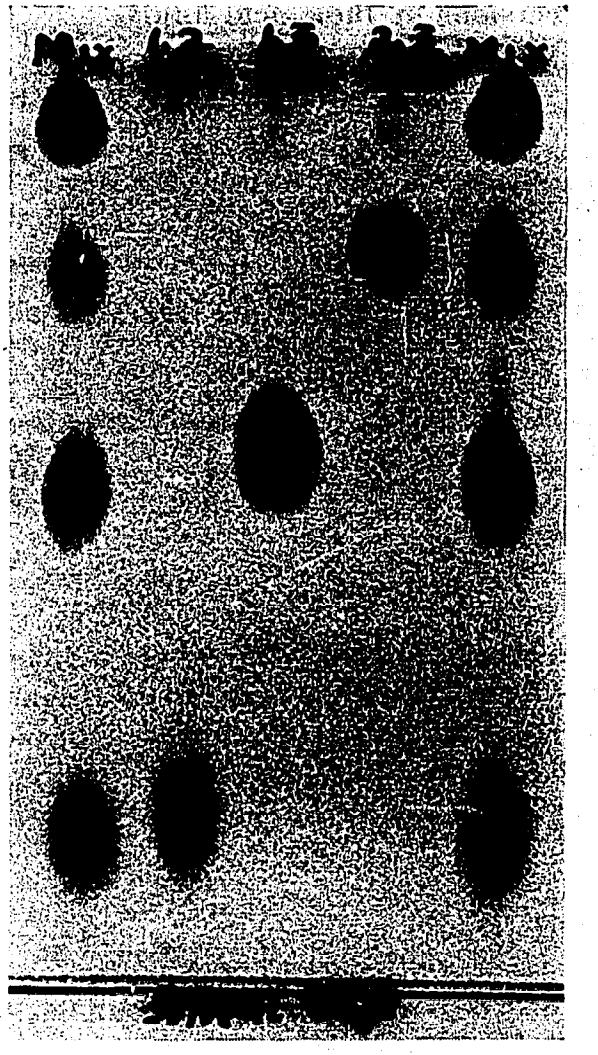

Fig. 2. *B. subtilis* bioautographic plate showing separation of the DAO's by solvent system A. The mix also contains oleandomycin base.

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SUMMARY

A paper chromatographic procedure has been described which has proved to be a powerful tool for separating the solvent-soluble antibiotics such as the macrolides and the quinocyclines. The method is rapid, versatile and capable of handling a wide variety of sample types. It has proven extremely valuable in following the synthesis of the several acetylated derivatives of oleandomycin and their appearance and interconversions in biological fluids.

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