

**NOTE**

**CHROMATOGRAPHIC ASSAY  
OF MIXED PENICILLINS,  
AMPICILLIN AND CLOXACILLIN  
IN BODY FLUIDS**

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A thin-layer chromatographic assay method for the separation and determination of ampicillin and cloxacillin when present as a mixture was developed using a bioautographic technique. The measurement of the diameters of the inhibition zone was used as the means of quantitation. This method was useful for the separate determination of ampicillin and cloxacillin in the serum or urine from the animal or human subject administered with both penicillins.

**Methods and Results**

Samples of Body Fluids

Prior to the application to thin-layer chromatography (TLC), the samples were pre-treated as follows:

(a) Serum: One ml of ethanol was added to 0.5 ml of each serum sample, and was stirred well. The mixture was centrifuged at  $1,000 \times g$  for 10 minutes to obtain supernatants for the assay.

(b) Urine: Urine samples were diluted with 1/15 M phosphate buffer (pH 7.0) to contain 3~20 mcg/ml of each penicillin.

Standard Solutions

Standard solutions for the assay were prepared as follows:

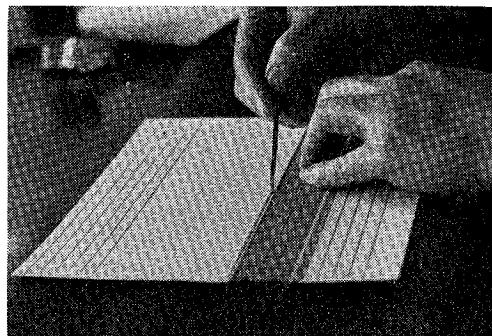
(a) Serum: Ampicillin and cloxacillin, each the same in quantity, were added to human serum at concentrations of 2.5, 5, 10 and 20 mcg/ml.

(b) Urine and other body fluids: A similar method to (a) was used except for the use of a 1/15 M phosphate buffer (pH 7.0) instead of the human serum, and for omission of ethanol treatment.

Thin-layer Chromatography

Eastman Chromagram Sheet No. 6061 (20  $\times$  20 cm) was used. The silica-gel layer of the sheet was grooved with a small spatula and a scale so as to make parallel zones each about 7 mm in width (Fig. 1).

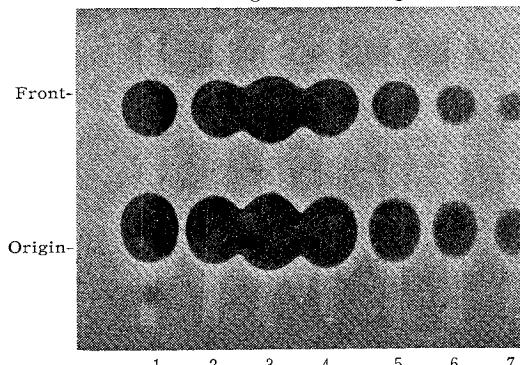
Fig. 1. Thin-layer plate grooved



For the assay of urine and other body fluid, 10  $\mu$ l of each standard solution (or sample) was applied on the grooved zone by means of micropipette or microsyringe.

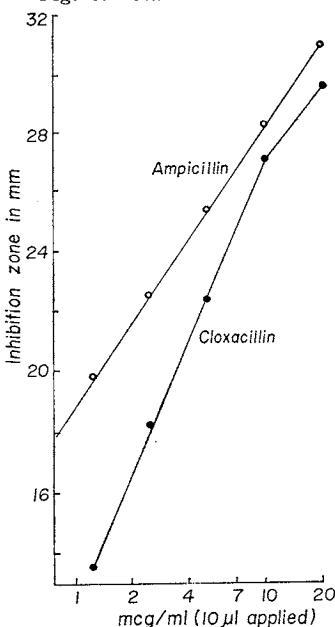
For the assay of serum, 30  $\mu$ l of each standard solution (or sample) was used because the standard solutions or samples were diluted with ethanol to three-fold in the pretreatment described above. The spot was dried at room temperature with the aid of a fan, so as to make the diameter of each spot 7 mm, which is identical with the width of the zone. For samples of low antibiotic concentration, multiple application of 10 or 30  $\mu$ l were made so that the total volume contained at least 0.01  $\mu$ g of each antibiotic. The solvent system used was ethyl acetate-acetic acid-water (8:1:1). The distance developed between the origin and the front was about 6 cm.

Fig. 2. Bioautograms



No. 1.	Urine sample
2.	Serum sample
3.	Standard solution each 20 mcg/ml (10 µl applied)
4.	" " " 10 " ( " " )
5.	" " " 5 " ( " " )
6.	" " " 2.5 " ( " " )
7.	" " " 1.25 " ( " " )

Fig. 3. Standard curves



### Bioautography

After development the sheets were dried at room temperature using a fan and the strips were cut separately and plated individually on agar plates (thin: 2 mm) seeded with *B. subtilis* ATCC 6633. After 30 minutes the strips were removed. A 0.2% inoculum of spore suspension ( $3 \times 10^8$ /ml) was used in the agar medium containing 1% of sodium citrate, 0.5% of Polypeptone, 0.25% of meat extract and 1% of agar. After an overnight incubation at 37°C, the diameter of inhibition zone was measured. The inhibition zone near the origin is due to ampicillin and the other zone near the front to cloxacillin.

An example of the bioautograms is shown in Fig. 2. The diameters of the inhibition zones of the standard solutions were plotted against the amount of each penicillin on semi-logarithmic paper (Fig. 3). The accuracy of this method was checked by the use of an authentic sample in the buffer or serum. When each sample was run in triplicate, the error was estimated to be less than 10%.

### Discussion

One method of estimating each penicillin individually, is to use two organisms, one sensitive to ampicillin but resistant to cloxacillin, and the other sensitive to cloxacillin but resistant to ampicillin. This method, however, has the disadvantage of variable sensitivity of the test organisms to the penicillins and the results may also be affected if the concentration of one penicillin greatly exceeds that of the other.

Our present method, though somewhat complicated in its procedure, does not suffer from the above disadvantage, since each penicillin is separated and assayed individually. The method can be widely applied to the individual determination of penicillins not only in body fluids but also in tissue extracts and enzymatic reaction mixtures.

Furthermore the method may be used in the individual determination of other mixtures of antibiotics by selecting suitable adsorbents, solvent system and assay organisms.