Paper Chromatographic Techniques for the Determination of Cephalothin and Desacetylcephalothin in Body Fluids

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Paper chromatographic procedures may be used to detect cephalothin and its metabolite desacetylcephalothin in urine, plasma, synovial fluid, and cerebrospinal fluid. Protein-bound antibiotics are released from plasma, cerebrospinal fluid, and synovial fluid by dilution with an equal volume of dimethylformamide. Data are presented on the sample preparation, paper chromatographic system, and other specific techniques.

Metabolic studies of certain of the cephalosporin antibiotics (1,6) revealed the necessity to determine the titers of microbiologically active metabolites in the presence of the parent compounds. R. P. Miller (5) developed a paper chromatographic assay for certain cephalosporins. Lee et al. (4) used this method for pharmacological and toxicological studies of cephalothin. Although the method is satisfactory for the analysis of urine samples, a more sensitive method for the detection of low titers of antibiotics in other body fluids is needed. A method was devised which afforded quantitative disassociation of these antibiotics from plasma proteins, and an improved paper chromatographic technique was developed to measure cephalothin and low concentrations of its metabolite desacetylcephalothin.

MATERIALS AND METHODS

Plasma containing cephalothin and desacetylcephalothin was diluted with an equal volume of dimethylformamide, mixed well, and allowed to stand for 10 min at 25 C or stored overnight at 5 C. The suspensions obtained were then centrifuged to remove the protein precipitate, and the supernatant liquids were spotted on the chromatogram.

Investigators at these laboratories have made numerous attempts to extract and concentrate cephalothin and desacetylcephalothin when these antibiotics are present in plasmas and cerebrospinal fluids. The best method appears to be drying from the frozen state and reconstituting the dried residue in a mixture of equal parts of normal saline and dimethylformamide. It is possible to concentrate this antibiotic in the plasma about fourfold with this

technique. Antibiotics present in cerebrospinal fluids can be concentrated more readily, about 10-fold, because of lower protein and solids content.

Samples of cephalothin and desacetylcephalothin and the test samples of plasma supernatant, cerebrospinal fluid, synovial fluid, or urine were applied to Whatman no. 4 paper (19 by 46.5 cm) impregnated with 0.1 M sodium acetate buffer (pH 4.6). After application of the samples and drying, the sheet was developed for 3 hr by descending chromatography in a solvent system consisting of methyl-ethyl-ketone (MEK), acetonitrile, and water (84:8:8). A mixture of 600 ml of MEK and 400 ml of water was placed in the bottom of the chamber to provide a solvent-saturated atmosphere. After development, the paper chromatogram was air-dried and placed for 15 min on an agar plate of the following composition: Beef Extract (Difco), 1.5 g; Peptone (Difco), 6.0 g; Yeast Extract (Difco), 3.0 g; ion agar, 10 g; and distilled water, 1 liter. Each poured plate (23 by 45 cm) contained 250 ml of agar (pH 6.5) which had been seeded with a 1% spore suspension of *Bacillus subtilis* ATCC 6633. After 15 min, the chromatogram was removed, and the plate was incubated at 37 C for 4 hr and then left at room temperature for 16 hr. Zones of inhibition caused by cephalothin or its metabolite were clearly visible on the bioautograph plate after the 4-hr incubation period.

RESULTS AND DISCUSSION

Binding of cephalothin by plasma proteins can be reversed by the addition of dimethylformamide. This observation was made when ^{14}C-labeled cephalothin (on the thienyl carbonyl) was added to fresh human plasma. The cephalothin-plasma mixture was incubated at 37 C for 10 min at pH 8.1, after which an equal volume of dimethyl-
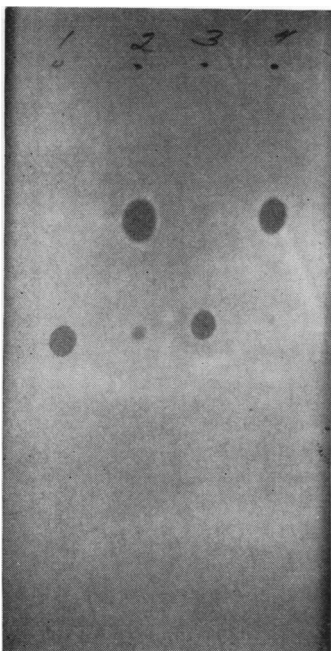
FIG. 1. *Bioautograph of a paper chromatogram of cephalothin and desacetylcephalothin. Lane 1, 0.1 μg of cephalothin; lane 2, 0.5 μg of desacetylcephalothin; lane 3, 0.05 μg of cephalothin; lane 4, 0.2 μg of desacetylcephalothin.*

formamide was added. A precipitate formed immediately. After vigorous stirring and shaking, a 0.1-ml volume of the suspension was removed and treated with 0.4 ml of 0.2 N NaOH solution. This was digested until dissolved, 15 ml of "Diatol" (2) was added, and the sample was submitted for radioactive counting.

The plasma-dimethylformamide suspension was centrifuged, after which 0.1 ml of the clear supernatant liquid was withdrawn and then treated in the same manner as the suspension sample before submission for counting.

Identical radioactivity in the suspension and supernatant liquid proved that the binding of cephalothin by plasma proteins was disrupted by treatment with dimethylformamide.

A bioautograph of a paper chromatogram demonstrating the separation of cephalothin and desacetylcephalothin is shown in Fig. 1. Lanes 1 and 3 represent 0.1 μg and 0.05 μg of cephalothin, respectively, whereas lanes 2 and 4 show the separation of trace amounts of cephalothin from 0.5 μg and 0.2 μg of desacetylcephalothin standards, respectively.

Figure 2 illustrates that 50% dimethylformamide is effective in disrupting protein binding so

that free antibiotic was present in the resulting supernatant. When only 20% dimethylformamide was used (lane 11), desacetylcephalothin did not migrate due to the binding effect of plasma proteins. In the remaining lanes, we were able to show migration of the cephalothin and desacetylcephalothin in the presence of plasma or saline whenever 50% dimethylformamide was used as a diluent.

Cerebrospinal fluids were found to contain low concentrations of protein so that protein binding did not present a serious assay problem. Cerebrospinal fluid could be spotted directly without prior treatment with dimethylformamide to give an accurate movement and separation on the paper chromatogram. However, if one is making quantitative measurements from the bioautograph, it is necessary to treat the samples with dimethylformamide or to use reference standards prepared with cerebrospinal fluid. Figure 3 illustrates that, although movement on paper is not
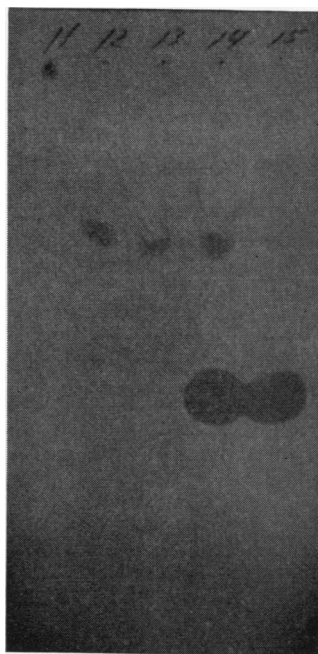

FIG. 2. *Bioautograph of a paper chromatogram of cephalothin (C) and desacetylcephalothin (DAC) in plasma diluted with various concentrations of dimethylformamide (DMF). Lane 11, 0.17 μg of DAC in 20% DMF, and 80% plasma, lane 12, 0.2 μg of DAC in 50% DMF and 50% plasma; lane 13, 0.2 μg of DAC in 50% DMF and 50% saline; lane 14, 0.2 μg each of C and DAC in 50% DMF and 50% plasma; lane 15, 0.2 μg of C in 50% DMF and 50% plasma.*
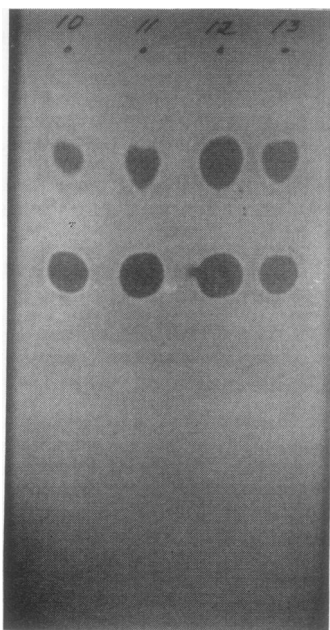
FIG. 3. *Bioautograph of a paper chromatogram of cerebrospinal fluid containing cephalothin (C) and desacetylcephalothin (DAC). Lane 10, 0.05 µg of C and 0.2 µg of DAC in cerebrospinal fluid; lane 11, 0.05 µg of C and 0.2 µg of DAC in water; lane 12, 0.05 µg of C and 0.4 µg of DAC in water; lane 13, 0.05 µg of C and 0.4 µg of DAC in cerebrospinal fluid.*

FIG. 4. *Bioautograph of a paper chromatogram of synovial and cerebrospinal fluids containing cephalothin (C) or desacetylcephalothin (DAC). Lane 1 and 4, 0.05 µg of C and 0.2 µg of DAC in cerebrospinal fluid as a control. Lane 2, a sample of synovial fluid, lane 3, a sample of cerebrospinal fluid.*

inhibited and separation of cephalothin and desacetylcephalothin is achieved, diffusion is inhibited. Quantitative comparisons cannot be made between aqueous standards and cerebrospinal fluid samples.

Figure 4 represents a bioautograph of the controls cephalothin and desacetylcephalothin as well as clinical samples of synovial fluid in which we see cephalothin and a trace amount of desacetylcephalothin. On lane 3 we found only a trace of desacetylcephalothin in cerebrospinal fluid after 4 hr of incubation, but this disappeared after complete incubation. A very low level of antibiotic would be expected in this 4-hr postcephalothin-treatment sample. Cephalothin was detected in samples of cerebrospinal fluid drawn at 2 hr post-treatment.

The variation in concentrations of antibiotic in cerebrospinal fluids from one patient to another is a subject of concern to the clinician. With the development of new detection techniques, it is now possible to detect and measure not only the parent compound but also its metabolites (3, 7).
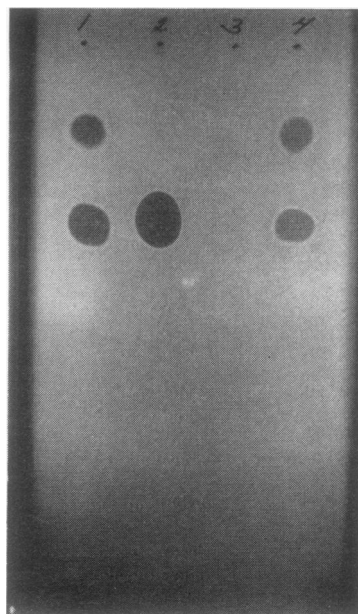
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