High-Pressure Liquid Chromatography Analysis of Antibiotic Susceptibility Disks

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The analysis of antibiotic susceptibility disks by high-pressure liquid chromatography (HPLC) was investigated. Methods are presented for the potency determination of mecinilamin, ampicillin, carbenicillin, and cephalothin alone and in various combinations. Good agreement between HPLC and microbiological data is observed for potency determinations with recoveries of greater than 95%. Relative standard deviations of lower than 2% are recorded for each HPLC method. HPLC methods offer improved accuracy and greater precision when compared to the standard microbiological methods of analysis for susceptibility disks.

The use of antibiotics disks as in vitro diagnostic aids for the determination of the antibacterial activity of various compounds is an established practice and has been well documented. The official method of potency analysis for those disks described in the Code of Federal Regulations (2) is a horizontal agar diffusion assay, using disks of known potency. This technique has the advantage of conveniently confirming the antibacterial activity of the compound during the assay; however, large experimental uncertainties due to difficulties in the preparation of standard disks in the microbiological assay often yield results which are imprecise and which may only approximate actual values. Furthermore, investigators who wish to work with nonstandard antibiotic concentrations must often develop individual sets of appropriate experimental parameters for each antibiotic level to be tested. The use of high-pressure liquid chromatography (HPLC) as a sensitive and specific method for the analysis of antibiotics, especially β-lactam compounds, is becoming more and more accepted. Recent literature contains an increasing number of papers devoted to the HPLC analysis of β-lactam antibiotics including specific assay methods for mecinilamin (7), ampicillin (8, 12), and cephalothin (9, 11), to name a few.

Mecillinam is one of the new class of semi-synthetic β-amidino penicillins whose antibiotic properties have been described in detail (13). Mecillinam shows biological activity against gram-negative organisms and, when tested in combination with acylamino penicillins, also exhibits a synergistic effect against those organisms in vitro (6, 10). The principal β-lactam antibiotics chosen for synergy studies are ampicillin, carbenicillin, and cephalothin. As a supplement in vitro diagnostic aid to physicians, a series of paper susceptibility disks have been formulated containing the antibiotics described above and in various combinations with mecinilamin. Experimentally, a large variety of these disks has been prepared (Table 1) to determine the most clinically effective combinations and levels to be provided to researchers. Although microbiological methods are available for those single-component disks described in the Code of Federal Regulations, mecinilamin is an experimental drug and no official methods of disk analysis have been published for its assay either as a single component or in combination with other antibiotics. Microbiological methods have been developed for mecinilamin alone; however the combination disks containing antibiotic mixtures do not lend themselves to simple microbiological assays for the components. To accomplish these assays, special organisms have to be employed, which are susceptible only to each of the antibiotics and are not affected by synergic combinations. This requires careful laboratory manipulation and considerably increases cost per assay and turn-around time. In an effort to develop an assay which could be used to determine antibiotic potencies for all of the disk samples, it was decided to investigate chemical methods which were both specific and sensitive enough to determine accurately the lowest level of each antibiotic that was present alone or in combination in these disks. The different chemical properties of each of these antibiotics led to the development of different analytical systems for each drug. These systems, however, are similar to each other and can be used for a wide range of drug levels, requiring only minor ad-
adjustments in sample size or reference standard concentrations to accommodate all the samples investigated in this study.

Although it was necessary to develop a separate HPLC system for each compound, we elected to describe these different systems in one report because of their similarity and to demonstrate the special advantages HPLC has to offer in the quantitative analysis of susceptibility disks. Analysis by HPLC offers the advantages of combining both qualitative and quantitative determinations in one method, relatively quick analyses, and a more easily standardized control method for inter-laboratory comparison.

MATERIALS AND METHODS

Disks and reagents. The susceptibility disks used in these experiments were obtained from BBL Microbiology Systems (Cockeysville, Md.) and included mecillinam, ampicillin, cephalothin, and carbenicillin, alone and in combination. Standard potency disks for the microbiological assays were prepared in this laboratory by adding the appropriate weight of antibiotic(s) to plain paper disks and carefully air drying the samples.

The reference standard sample of mecillinam was obtained from Leo Pharmaceutical Products, Ltd. (Copenhagen, Denmark). Ampicillin and cephalothin solutions and standards were prepared from certified U.S. Pharmacopeia reference standards, and carbenicillin reference standard was kindly provided by Pfizer Inc. (New York, N.Y.). All other chemicals were American Chemical Society reagent grade or equivalent except that chromatographic grade solvents were used for all chromatography and were filtered through a 0.5-μm filter before use.

Reference standard and sample preparation. Standard solutions were prepared at appropriate concentrations depending on the expected drug level in the disk by dissolving and diluting the reference standards with filtered distilled water for mecillinam, ampicillin, and cephalothin, and with 1% phosphate buffer, pH 6.0, for carbenicillin.

Disk samples were prepared by extracting the contents of one disk with 1 ml of water or buffer and filtering the resulting solution through a 0.5-μm filter to remove insoluble material. The concentrations of working standards and sample solutions were approximately equal.

Chromatographic equipment. Chromatography was performed using a component chromatographic system composed of a Waters model 600A solvent delivery system (Waters Associates, Milford, Mass.), a Rheodyne model 7120 injector fitted with a fixed 100-μl loop, and an L.D.C. model 1202 variable wavelength detector (Laboratory Data Control, Englewood Cliffs, N.J.). All separations were performed on Chomegabond C18 columns (4.6 mm by 30 cm) packed with 10-μm O.D.S. bonded phase (E.S. Industries, Marlton, N.J.).

Mobile phase. The mobile phases consisted of well-shaken mixtures of acetonitrile and 0.01 M monosodium phosphate solution whose pH was adjusted to 5.0 with sodium hydroxide before mixing. Because of the variety of the antibiotics and combinations under test, the proportions of acetonitrile and buffer were adjusted for each sample to produce useful retention times and separations during analyses. Ratios of buffer and modifier ranged from 97:3 to 85:15 and are reported with appropriate retention data in Table 2 for each of the various compounds.

Microbiological assay. The microbiological assays used in these studies for both single and combination disks were performed by the standard methods described in the Code of Federal Regulations for ampicillin and carbenicillin (2). Cephalothin at 2.5 μg

| Disk component       |   |   |   |
|----------------------|---|---|---|
|                      | A | B | A |
| Ampicillin           | 10| 13.5| 12 |
| Mecillinam           | 10| 12 | 0.97 |
|                      | 10| 13 | 11.5 |
| Ampicillin           | 0.5| 13 | 0.56 |
| Mecillinam           | 0.25| 13 | 0.24 |
| Carbenicillin        | 100| 105| 133 |
|                     | 50| 50.5| 52.5 |
| Carbenicillin        | 50| 2 | 50 | 2.4 |
|                     | 100| 1 | 106 | 1.38 |
| Carbenicillin        | 50| 0.5 | 55 | 0.6 |
| Cephalothin          | 2.5| 2.7| 2.4 |
|                     | 2.5| 2.0 | 0.96 |
| Cephalothin          | 2.5| 2.1 | 0.46 |
|                     | 2.5| 2.4 | 0.32 |
| Mecillinam           | 10| 12.4| 12.3 |
|                     | 2| 2.5 | 2.0 |
| Mecillinam           | 1| 1.0 | 0.9 |
|                     | 0.5| 0.54 | 0.55 |
|                     | 0.25| 0.25 | 0.30 |
could not be assayed by the standard method using *Klebsiella pneumoniae* ATCC 10031 and was assayed microbiologically using *Staphylococcus aureus* ATCC 13150 similar to the ampicillin assay. Standard mecillinam disks at the 1-µg level did not produce zones in the methods used for ampicillin, carbenicillin, and cephalothin, and at that level or below would not be expected to interfere. Mecillinam alone was assayed using *Escherichia coli* ATCC 10536 in a procedure similar to the ampicillin assay.

**RESULTS AND DISCUSSION**

**Separation of antibiotics.** Suitable separations of the compounds of interest—mecillinam, ampicillin, cephalothin, and carbenicillin—from each other and from blank susceptibility disk extracts were achieved using the chromatographic conditions described for each compound in Materials and Methods and in Table 2. Retention data for the antibiotics studied in the various mobile phases are also included in the table. A typical chromatogram demonstrating separation is shown in Fig. 1. The buffer-to-acetonitrile ratio used for this sample was 85:15. The use of several different buffer-acetonitrile ratios in the mobile phases was necessary to achieve complete separation of the disk components and to complete the analyses in reasonable time. The systems described here are designed to give the best results for mecillinam, ampicillin, cephalothin, or carbenicillin alone or in combination. The use of the solvent ratios described in Table 2 for ampicillin and cephalothin in the combination disks of these compounds permits simultaneous analysis of both components. Because of very different retention characteristics, it is necessary to analyze mecillinam and carbenicillin separately.

It was observed previously (7) that reducing the nonpolar component of the mobile phase generally increased the retention times of the types of compounds involved in this study and generally improved separation among all the compounds. When the acetonitrile in the mobile phase was decreased, corresponding increases in separation and retention times were observed for each of the drugs extracted from the disks and for several small peaks attributed to minor amounts of degradation of the antibiotics. These peaks are also observed in intentionally degraded standard solutions, and the presence of these compounds did not affect the microbiological performance of the disks. Plate assays performed at the time of the HPLC analyses yielded potency values which were in good agreement with the HPLC data and were well within the specifications of 67 to 150% of label claim established for disks (3).

**TABLE 2. Mobile-phase composition and retention data**

| Component | Buffer-acetonitrile ratio | k" |
|-----------|---------------------------|----|
| Ampicillin | 90:10                     | 2.3 |
| Carbenicillin | 97:3                    | 4.1 |
| Cephalothin | 85:15                    | 5.25|
| Mecillinam | 85:15                     | 3.6 |
| Mecillinam | 90:10                     | 7.9 |
| Mecillinam | 97:3                      | Does not elute |

"k", Capacity factor.

**Linearity and precision of analysis.** The linearity of the HPLC response for each antibiotic was calculated by a linear regression analysis of data for a series of standard weight injections. Data for the line \( y = mx + b \) for each drug is presented in Table 3, including the range of sample weights injected, parameters for the lines, and correlation coefficients for the regression analyses. In each case, correlation coefficients of 0.999+ indicate that excellent linear response is observed over the working range of analytical sample weights used in the experiments. The second part of Table 3 contains...
averages and standard deviations for a series of six replicate injections of a single disk extract of each compound. Relative standard deviations of less than 2% demonstrate acceptable precision for these methods. Quantitation was achieved by peak area or height measurement with equivalent results for mecillinam, ampicillin, and cephalothin. The two biologically active isomers of carbenicillin produced two peaks in the chromatogram of preparations of this compound. A detailed discussion of these epimers by Butler et al. (1) indicates that they possess indistinguishable antibacterial activity. Therefore, it was necessary to sum the areas of the isomer peaks to calculate the correct potency figure for this drug.

Susceptibility disk assay. Susceptibility disks were assayed by extracting the contents of one disk into an appropriate volume of sample solvent, filtering the solution to remove particulate matter, and injecting the resulting solution. Water was used as the sample solvent rather than mobile phase to reduce analyst exposure to acetonitrile. To demonstrate the equivalence of both sample solvents, a group of mecillinam disks was divided and extracted with either water or mobile phase. The average potencies of these samples determined by HPLC were 0.795 ± 0.088 and 0.794 ± 0.075 μg per disk, respectively. No interfering peaks due to paper extracts are observed in any chromatogram at the various levels of antibiotic present. Similar results were obtained for both ampicillin and cephalothin, and as a result water was selected as a suitable sample solvent for those compounds. Carbenicillin, however, was observed to degrade significantly in water in less than 1 h, leading to scattered potency values and additional peaks in the chromatogram. The recommended solvent for the assay of disodium carbenicillin diagnostic sensitivity powder is pH 6.0 phosphate buffer (4), and use of this buffer extended the sample solution stability to more than 4 h.

Initially, two different analytical procedures were investigated for disk analysis using mecillinam as a model. The first involved the extraction of a sufficient number of disks into 4 ml of water to produce a working mecillinam concentration of approximately 5 μg/ml. The assay value obtained from the average of two replicate determinations was reported as the average potency of the sample. An alternative procedure and the one which eventually was adopted in this laboratory is a single disk assay procedure which calls for the extraction of the contents of 1 disk into 1 ml of water. Five replicate samples are prepared and analyzed, and the data are averaged to yield the appropriate potency value. The single disk assay is more time consuming, requiring five determinations versus two for the first method; however, using the second method the investigator is able to obtain a value for the standard deviation of the series of individual potencies which is an indication of the uniformity of the disk samples. This method provides data similar to that obtained from the microbiological diffusion assay which also calls for individual disk assays for uniformity. Comparative data for both HPLC procedures was generated for a sample of 1-μg mecillinam disks. The average potency determined for 20 disks extracted with 4 ml of water was 1.2 μg per disk whereas the average result of a series of 6 single-disk assays was 1.3 ± 0.08 μg per disk. This standard deviation is slightly larger than that observed for drug standard solutions and, to determine whether the observed variance is due to experimental error or to disk-to-disk variation, a series of six individual disks from one representative lot of each antibiotic was analyzed. Table 4 contains data derived from peak area measurements from solutions prepared by extracting the contents of a single disk in 1 ml of sample solvent. The relative standard deviations are not excessively large compared to the standard single solution analyses described in Table 3 and do not charge the precision of analysis by very much. These data indicate that the content uniformity of disks is well within the range required.
Table 4. Content uniformity of commercial disks by HPLC

| Antibiotic     | Claim (µg/disk) | Potency (µg/disk) | SDb | Relative SD (%) |
|----------------|-----------------|-------------------|-----|-----------------|
| Mecillinam     | 1               | 1.3               | 0.08| 6.0             |
| Ampicillin     | 10              | 13.5              | 0.37| 2.75            |
| Cephalothin    | 50              | 50.5              | 1.53| 3.0             |
| Carbenicillin  | 2.5             | 2.7               | 0.048| 1.8            |

* Average of six values.

* SD, Standard deviation.

For the microbiological assay by the Code of Federal Regulations (3), which allows an absolute deviation of 2.5 mm for a zone of 25 mm and may represent as much as 50% deviation depending on the magnitude of the regression data.

To test the recovery of the extraction procedure, a batch of 1-µg mecillinam disks was prepared carefully in this laboratory by the general Code of Federal Regulations method for preparation of disks (2). This sample contained 1.053 µg per disk and was analyzed versus an external mecillinam standard. The recovery for this sample was 1.02 ± 0.015 or 97.0 ± 1.5% of claim for five replicates.

Finally, a series of disks for which microbiological potency data were available was assayed by HPLC, and both results were compared with manufacturer's claims in Table 1. A typical chromatogram of a combination antibiotic disk sample, 1 µg of mecillinam and 10 µg of ampicillin, is presented in Fig. 2. Chromatograms of all the disks are essentially the same. No interferences with the peaks of interest are observed in chromatograms of control disk extracts or in chromatograms of combination disks. HPLC and microbiological potency figures generally are in good agreement with each other and with the claim. Occasionally, however, discrepancies between HPLC and microbiological values are observed. Carbenicillin 100-µg disks, for example, differed by as much as 28 µg per disk (or approximately 30% of claim). Similar differences between chromatographic and microbiological methods have been reported by Diasio et al. (5) in the determination of 5-fluorocytosine by HPLC. These differences are a function of the precision of the microbiological methods and, in fact, contribute to the specification allowance of 67 to 150% of claim.

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