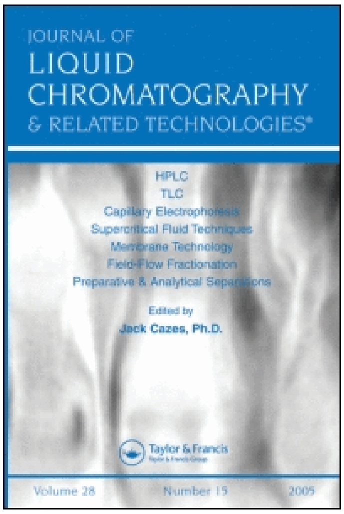

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Determination of Penicillinase-Resistant Penicillins In Serum Using High-Pressure Liquid Chromatography

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DETERMINATION OF PENICILLINASE-RESISTANT PENICILLINS IN SERUM USING HIGH-PRESSURE LIQUID CHROMATOGRAPHY

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ABSTRACT

A method for the determination of methicillin, oxacillin, cloxacillin, dicloxacillin, and nafcillin in serum using high-pressure liquid chromatography (HPLC) is described. The drugs were extracted from serum using a two-step procedure employing acetonitrile followed by methylene chloride. The extraction procedure concentrated the antibiotics in a smaller volume which allows more accurate determinations of low serum levels. The treated sera were analyzed by HPLC on a reverse-phase column and detected by ultraviolet light absorption at 254 nm. Serum concentrations were measurable as low as 0.5 µg/ml. Recovery procedures showed less than 2.5% variation in peak heights when the antibiotics were extracted from different pools of serum. No interfering absorption was found in extracts of serum samples pooled from healthy volunteers, from a commercial source, or from two serum pools from patients receiving a variety of other drugs. Two spiked serum specimens prepared for each antibiotic were assayed four times by HPLC and by the microbiological agar diffusion method. No significant statistical differences between the methods were observed. Control materials were assayed for between-batch and within-batch reproducibility in the presence or absence of an internal standard. Results for between-batch reproducibility demonstrate CV's of about 5%. This procedure provides a sensitive, specific, accurate, and rapid method for determining antibiotic levels in routine clinical specimens.

INTRODUCTION

The penicillinase-resistant penicillins are semi-synthetic drugs consisting of bulky side groups attached to 6-aminopenicillanic acid, the nucleus of penicillin. The side chains protect the β-lactam ring from the action of penicillinases. Methicillin was the first of the resistant penicillins developed. However, the drug is acid sensitive and cannot be given orally. Newer semi-synthetic drugs, the isoxazolyl penicillins (cloxacillin, oxacillin, and dicloxacillin) and the closely related nafcillin are both penicillinase resistant and relatively acid stable.

The toxicity of these antibiotics is similar to the other penicillins. Large doses of methicillin can result in neutropenia, bone marrow depression (1), nephropathy including hematuria, proteinuria, and renal insufficiency (2, 3, 4). The isoxazolyl penicillins may be hepato- (5) or neurotoxic (6). Since large doses of the antibiotics are frequently administered, rapid and accurate determinations of serum levels are clinically important especially when renal failure occurs.

High-pressure liquid chromatography (HPLC) has been successfully applied to the detection and quantitation of the penicillins in pharmaceutical preparations and in urine (7, 8, 9, 10, 11, 12, 13, 14, 15, 16). With few exceptions (17, 18), HPLC has never been used for the determination of the penicillins in biological fluids (serum, cerebro-spinal fluid, joint fluid, and pleural fluid). We have successfully applied HPLC to the clinical quantitation of the β-lactamase resistant penicillins in serum and CSF for one and a half years. This technique offers greater accuracy and speed in comparison to the standard microbiological determinations. Its major advantage over microbiological assays is the ability to quantitate one or more drugs given in combination without interference.

MATERIALS AND METHODS

Oxacillin, methicillin, cloxacillin, dicloxacillin, and nafcillin standard powders were obtained as their sodium salts from Bristol Laboratories (Syracuse, N.Y.). Stock antibiotic solutions were prepared in water at a concentration of 1,000 µg/ml and stored at -20°C for no more than one month. Acetonitrile, methylene chloride, and ammonium acetate were of reagent grade. Water used in the procedure was purified by double distillation. Serum pools were prepared from specimens supplied by a local hospital laboratory, specimens received for fungal immunodiffusion by our laboratory, from a commercial source (Gibco; Grand Island, N.Y.), and from five healthy human volunteers. Serum from patients treated with the various antibiotics were obtained from four hospitals in the Detroit Medical Center.

Sample Preparation. Serum (0.4 ml) was mixed with an equal volume of acetonitrile and vortexed for 10 sec. The serum-acetonitrile slurry was placed on a shaker and slowly mixed for 15 min to facilitate protein precipitation and then centrifuged for 10 min at 3,000 x g at ambient temperature. The supernatant fluid was decanted to a clean screw-cap tube and 4.0 ml of methylene chloride was added. The sample was vortexed for 10 sec, shaken for 15 min and then centrifuged for 10 min at 3,000 x g. A 50 µl aliquot of the aqueous (upper) layer was injected with a microsyringe (#810; Hamilton Co., Reno, Nev.) for chromatography.

Chromatographic Conditions. Analyses were performed on a reverse-phase column (µ-Bondapak C_{18}; Waters Associates, Milford, Mass.) at ambient temperature. Separation was accomplished with a ALC/GPC 204 liquid chromatograph (Waters Associates, Milford, Mass.). It was equipped with a model 6000A solvent delivery system, a model U6K universal injector, and a model 440 absorbance detector. The eluant was monitored at 254 nm and the peaks recorded on a 10 mv chart recorder (Houston Instruments, Houston, Texas) at a chart speed of 0.5 cm/min. Reproducibility studies were completed using an automatic sample injector (Waters Intelligent Sample Processor (WISP) 710B; Waters Associates, Milford, Massachusetts).

The mobile phase consisted of a 62:28:10 mixture of distilled water, acetonitrile, and 0.2 M ammonium acetate, pH 5.6. This mobile phase was previously suggested for determinations of penicillin in pharmaceuticals (19). The buffer was filtered through a 0.5 μm filter (Fluoropore; Millipore Corp., Bedford, Mass.) and was deaerated with vacuum. The flow rate was 1.0 ml/min (approximately 900 psi) for oxacillin, cloxacillin, methicillin, and nafcillin, while the flow rate for dicloxacillin was 3.0 ml/min (approximately 2,800 psi).

Standards were prepared by adding aliquots of the antibiotic stock solutions to 1.0 ml of serum. The drug concentration was corrected for volume and expressed in μg/ml. Standard curves for each drug were generated by plotting peak height vs concentration of the antibiotic.

Microbiological Determination. The assay used for the drugs was a slight modification of the standard agar diffusion technique (20). The indicator organisms were Bacillus subtilis ATCC 6633 and Sarcina lutea ATCC 9341. Two vials of B. subtilis spore suspension (spore suspension # 2; Difco Laboratories, Detroit, Mich.) or 3 ml of an overnight culture of S. lutea were added to 100 ml of cooled (50°C) assay medium (Antibiotic assay medium No 5; Difco Laboratories, Detroit, Mich.). After mixing, 5 ml aliquots were transferred into sterile petri dishes (100 mm diameter) and allowed to solidify. The plates were stored in sealed plastic bags at 4°C until used. Plates containing B. subtilis spores were used up to one month after preparation while unused S. lutea plates were discarded after one week. Three dilutions of each antimicrobial agent were prepared in serum. Twenty sterile paper discs were placed in a sterile petri dish and four discs were inoculated with 0.02 ml of each dilution. Four discs were inoculated with the unknown sample and four with a 1:2 dilution of the unknown. Two discs of each concentration of antibiotic standard, an unknown, and a diluted unknown were placed on a seeded agar plate such that identical concentrations were directly opposite one another. After 4 h of incubation at 37°C, the zone sizes were measured to the nearest 0.1 mm using a ruler and a stereo microscope. The unknown concentration was read from a standard curve generated by plotting log of the antibiotic concentration vs zone size in mm. The standards and unknowns were run concurrently on HPLC and by the microbiological method.

RESULTS

Choice of Absorbance Detector Wavelength. The ultraviolet absorbance maximums for the five drugs range from 226 - 245 nm. Since the chromatography unit was equipped with a fixed wavelength detector, 254 nm, the closest available wavelength to the maximums was used for all drugs.

Separation of the Drugs by Liquid Chromatography. A typical chromatogram of the drugs extracted from serum is shown in Fig. 1. The retention times for methicillin, oxacillin, cloxacillin, nafcillin, and dicloxacillin were 4.0, 5.7, 6.5, 9.0, and 11.7 min, respectively. Extraction of serum blanks and spiked serums showed that the methicillin peak came off with or very close to the serum peaks. A number of possible mechanisms to change the retention time of methicillin were investigated including alterations in the flow rate and adjustments in the concentrations of the mobile phase components. A decrease in the acetonitrile concentration from 28% to 20% increased the retention time to 6.6 min (Fig. 2) which satisfactorily separated the methicillin and serum peaks. The retention time for dicloxacillin was decreased to 6.8 min by increasing the flow rate of the original mobile phase to 3.0 ml/min.

Standard curves generated for each drug (Fig. 3A-E) passed through the origin and were linear up to 128 µg/ml, the maximum concentration tested. Drug concentrations as low as 0.5 µg/ml could be measured. We have experienced slight variations in the peak heights of the standard curve over a period of time. These

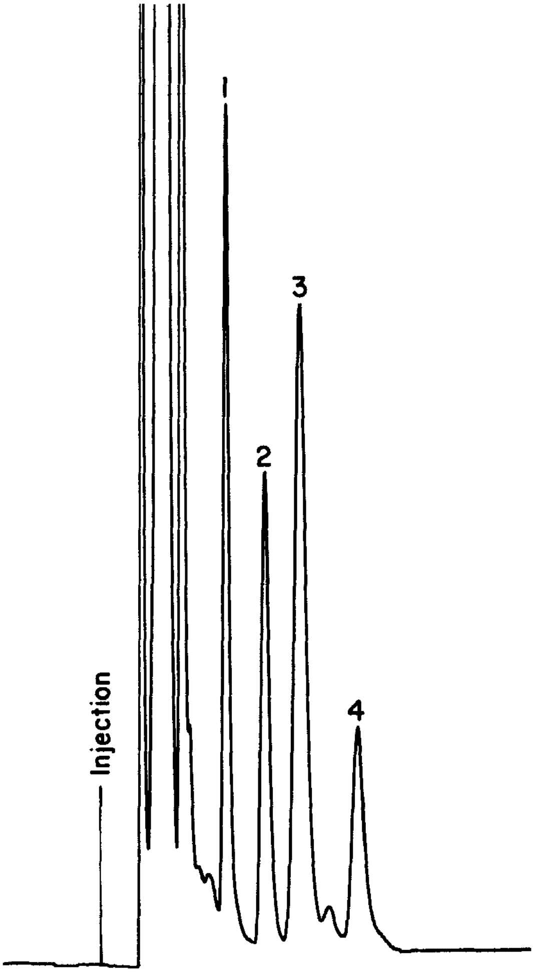

FIGURE 1

Elution profile of the penicillinase-resistant penicillins from a 50 µl injection of an extracted serum containing 20 µg/ml of each drug. The order of elution is: 1) oxacillin; 2) cloxacillin; 3) nafcillin; and 4) dicloxacillin. The flow rate was 1.0 ml/min and the attenuation was 0.02 absorbance unit full scale.

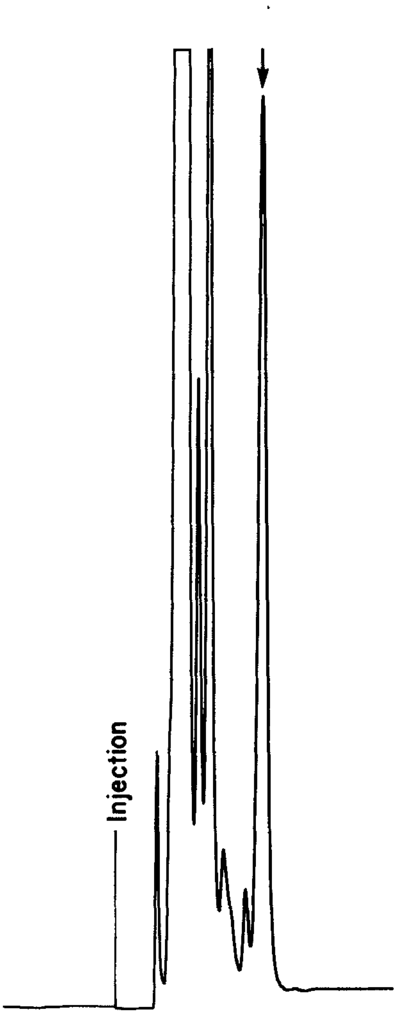

FIGURE 2

Chromatogram of methicillin (20 µg/ml) extracted from serum. The mobile phase was water, acetonitrile, and 0.2 M ammonium acetate, pH 5.6, (70:20:10) at a flow rate of 1.0 ml/min. The attenuation is 0.02 a.u.f.s.

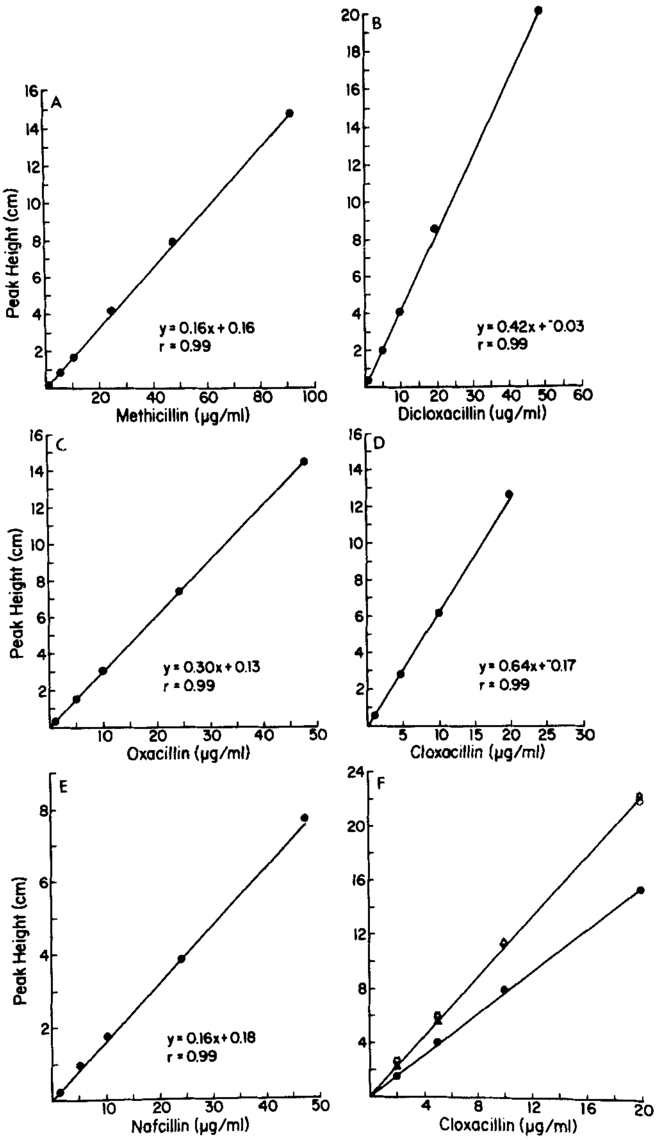

FIGURE 3

Standard curves for the drugs extracted from serum (A) methicillin, flow rate 1.0 ml/min, attenuation 0.1 a.u.f.s.; (B) dicloxacillin, flow rate 3.0 ml/min, attenuation 0.005 a.u.f.s.; (C) oxacillin, flow rate 1.0 ml/min, attenuation 0.1 a.u.f.s.; (D) cloxacillin, flow rate 1.0 ml/min, attenuation 0.02 a.u.f.s.; (E) nafcillin, flow rate 1.0 ml/min, attenuation 0.1 a.u.f.s. (F) Comparison of peak heights as a function of cloxacillin concentration in an aqueous control (•) and extracted from serum pools one (o), two (Δ), and three (□). The attenuation was 0.01 a.u.f.s.

differences may be due to a variety of factors. A change in the concentration or pH of the mobile phase components which causes an upward shift in the retention factor (k') of a drug would result in an increased peak width and a concomitant decrease in peak height. Conversely a decrease in k' would increase peak height and decrease peak width. Similarly as the column ages, decreased efficiency would also result in broader peaks with reduced heights. Despite the changes in peak height on different days, the values reported for the same serum unknowns were always within 5% of the actual concentration. Therefore, it was necessary to make a new standard curve each day. Ideally, the standards and the patient's serum should be extracted at the same time.

To demonstrate the reliability of this technique two 'unknowns' of each drug were prepared in serum by one investigator and assayed by the other. Each specimen was examined by HPLC and by the standard microbiological procedure. The mean of four determinations by HPLC and from four plates for each specimen by the microbiological method are presented in Table 1. There was close overall agreement between the two methods and differences were not statistically significant ($2P > 0.5$; paired t-test).

Recovery. Standard curves for the five drugs were generated by HPLC using aqueous drug solutions of various concentrations without any extraction. Identical amounts of drug were added to three different pools of human serum and extracted. Fig. 3F shows representative data for cloxacillin. In all cases the extracted drugs gave peak heights greater than those observed for the aqueous preparations. These findings indicate a relative concentration of the drugs in the aqueous layer during extraction.

Total protein content in each of the three serum pools was determined on a Technicon SMA 12/60 (Technicon Instruments Corp., Tarrytown, N.Y.). The protein concentrations for the pools were: 6.1, 6.5, and 7.0 g/dl respectively for pools 1, 2, and 3.

TABLE 1. Comparison of HPLC

		Microbiological Assay		
Drug	Amount Added to serum (μg/ml)	Average[a] Recovery (μg/ml) [Range]	Standard Deviation	Coefficient of Variation
Nafcillin	24.6	24.1 [20.3 - 29.5]	3.9	16.2%
	4.97	7.0 [5.9 - 7.5]	0.76	10.9%
Oxacillin	6.95	6.4 [5.9 - 7.1]	0.56	8.7%
	1.99	1.1 [1.0 - 1.1]	0.06	5.5%
Cloxacillin	6.36	7.0 [5.7 - 7.8]	1.12	15.9%
	12.67	11.5 [10.1 - 12.7]	1.1	9.6%
Methicillin	1.99	2.1 [1.9 - 2.4]	0.21	10.0%
	9.9	9.3 [9.2 - 9.3]	0.08	0.87%
Dicloxacillin	4.97	4.8 [4.7 - 4.9]	0.10	2.0%
	9.9	8.5 [6.5 - 10.5]	2.08	24.5%

a) average from four plates on different days
b) average from four injections on different days

and the Agar Diffusion Method

Method of Recovery				
	HPLC			
Recovery %	Average[b] Recovery (μg/ml) [Range]	Standard Deviation	Coefficient of Variation	Recovery %
99.2	23.4 [22.1 - 26.0]	1.74	7.4%	96.5
143	4.65 [4.6 - 4.7]	0.06	1.2%	94.9
91.9	6.71 [6.3 - 7.46]	0.65	9.7%	96.5
52.8	1.79 [1.6 - 2.09]	0.26	14.5%	89.7
111	6.11 [5.75 - 6.58]	0.34	5.6%	96.3
90.7	12.4 [11.1 - 13.2]	0.94	7.6%	97.6
103	1.90 [1.8 - 2.05]	0.11	5.5%	95.3
93.9	9.90 [9.4 - 10.2]	0.36	3.6%	99.9
95.9	4.58 [4.47 - 4.75]	0.13	4.2%	92.2
85.8	10.0 [9.5 - 10.5]	0.42	2.8%	101

Although there were some differences between the sera, the quantity of antibiotic extracted from each pool is almost identical. At antibiotic levels as high as 100 µg/ml, the differences in peak height for the three pools is less than 2.5%.

Reproducibility. Between-batch reproducibility was assessed by assaying control serums containing three different concentrations of oxacillin 10 times over a three month period. The control serums were also assayed 10 times within the same run to provide within-batch reproducibility data. At the same time, the need for an internal standard was assessed by adding 20 µl of nafcillin (4 µg/µl) to 1.0 ml of serum before extraction. Concentrations of the control serums were calculated in two ways: 1) a ratio of the peak height of oxacillin to the peak height of nafcillin in the serums were compared to a standard curve constructed in the same fashion; and 2) peak heights for oxacillin were compared to the standard curve constructed for oxacillin using peak heights. The concentration of oxacillin was corrected for the added volume of both oxacillin and nafcillin in constructing the standard curves and reading results of the data excluding the internal standard. These results are presented in Table 2 and 3. Results for the other four drugs are similar (data not shown).

Clinical Application. During the past year and a half, we have received approximately 200 patient serum, CSF and joint fluid specimens for assay of the drugs under consideration from hospitals in the Detroit Medical Center. A large number of these patients received a combination of antibiotics. Although this presents little difficulty for the HPLC assay, it makes comparison of HPLC and microbiological assays difficult. Some representative data for dicloxacillin assayed by both methods is shown in Figure 4. Similar results for the other drugs were observed (see discussion).

Interference Studies. We have not observed interfering peaks in the serums of approximately 200 patients receiving other drug therapy. In addition, when several other drugs were added to

TABLE 2

Results for Within-batch Reproducibility Experiments

	With internal standard						Without internal standard				
Oxacillin concentration (μg/ml)	n	$\bar{x}$	SD	CV	average recovery (%)	Oxacillin concentration (μg/ml)	n	$\bar{x}$	SD	CV	average recovery (%)
3.0	10	3.0	0.19	6.3	99.0	2.9	10	2.9	0.15	5.2	97.6
17.0	10	17.1	0.27	1.6	101	16.4	10	16.3	0.28	1.7	99.7
47.0	10	47.9	0.48	1.0	102	44.0	10	43.9	0.71	1.6	99.6

$\bar{x}$, Mean; SD, standard deviation; CV, coefficient of variation.

TABLE 3

Results for Between-batch Reproducibility Experiments

	With internal standard						Without internal standard				
Oxacillin concentration (μg/ml)	n	$\bar{x}$	SD	CV	average recovery (%)	Oxacillin concentration (μg/ml)	n	$\bar{x}$	SD	CV	average recovery (%)
3.0	10	2.9	0.25	8.4	97.6	2.9	10	2.8	0.18	6.3	95.6
17.0	10	16.6	0.71	4.3	97.6	16.4	10	15.9	0.55	3.5	96.9
47.0	10	46.3	1.69	3.7	98.5	44.0	10	42.5	1.53	3.6	96.5

$\bar{x}$, Mean; SD, standard deviation; CV, coefficient of variation.

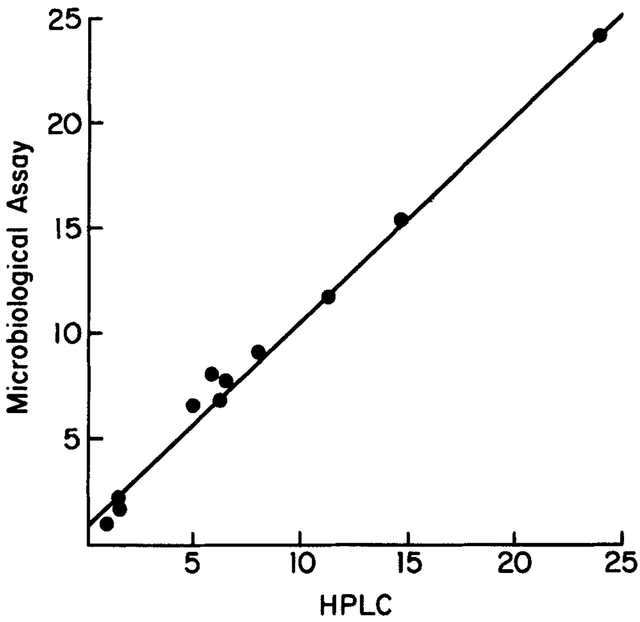

FIGURE 4

Patient comparison between levels (μg/ml) of dicloxacillin obtained with HPLC and the microbiological assay, with a correlation coefficient of 0.99, a slope of 0.97, and an intercept of 0.96.

serum and extracted none of them had retention times the same as any of the penicillinase-resistant penicillins. Those drugs showing no interference include: penicillin, ampicillin, ticarcillin, amoxacillin, mecillinam, pipericillin, carbenicillin, moxalactam, chloramphenicol, vancomycin, cephalothin, cefazolin, cephalexin, cephaloridine, cepharin, cepharadine, cefatoxamine, cefoxitin, cefamandole, co-trimoxazole, metronidazole, clindamycin, 5-fluoro-cytosine, theophylline, tobramycin, gentamicin, amikacin and ceforanide.

DISCUSSION

Antimicrobial agents are one of the most widely prescribed classes of drugs used in modern medicine. Dosage of the drugs is usually tailored to the severity of the infection with more severe infections receiving larger doses. However, as the dosage increases, the possibility of adverse side affects also increases. Thus, it is appropriate to monitor antibiotic levels in patients with severe infections or renal insufficiency. All of the drugs discussed in this report have serum half-lives of under 1 h making it necessary to administer the drug frequently to maintain effective serum levels. With few exceptions, all of the techniques previously employed for determining the levels of the penicillinase-resistant antimicrobials require excessive time and may produce results of only moderate accuracy.

Currently, several methods for the determination of antimicrobials exist. Enzymatic assays and radioimmunoassays have been developed that are rapid and specific (21, 22), but they are rather expensive and at the present time are not available for the penicillins. All the other methods presently used suffer from major disadvantages. The turbidimetric (23, 24), potentiometric (25) and agar diffusion methods are subject to interference caused by other antibiotics and they lack a uniform procedure for all the antibiotics.

HPLC has been applied to the quantitation of penicillins in pharmaceuticals. One procedure for the quantitation of penicillin in biological fluids does exist (17), however the drug is broken down with acid and detected by post-column derivatization. Our procedure requires no derivatization and detects the presence of the intact drug. Soldin et al. (18) have recently described an HPLC procedure for cloxacillin and nafcillin. This procedure utilizes an internal standard and monitors each drug at a different wavelength using a variable wavelength detector. There are no significant differences in between-day coefficients of variation or sensitivity between the two procedures. However, we can quantitate all five drugs at a single wavelength on a less expensive detector. Their procedure requires vacuum evaporation and reconstitution of a chloroform extract with approximately a 95% recovery of the drugs. The extraction procedure we describe requires no evaporation and also concentrates the antibiotics in a smaller volume which allows accurate determination of low serum levels. This concentration is a logical consequence of the extraction procedure. The acetonitrile precipitates the proteins and extracts the lipids resulting in a reduced volume and thus increasing concentration. A significant increase in drug concentration occurs during the methylene chloride extraction step. Acetonitrile is miscible in methylene chloride, while the aqueous material in serum is practically insoluble. Thus the acetonitrile is effectively removed by the methylene chloride while the antibiotic is concentrated in the upper aqueous layer.

The presence of interfering substances in patient material is always a matter of concern. Despite the fact that no interference occurs when the antibiotics are extracted from normal serum, serum from medicated patients and from pooled sera may contain interfering substances. In our experience with clinical specimens, we found only one specimen we were unable to assay due to interference. This patient was an extreme case of renal failure. Interference in the standards can be detected by making a blank of each new lot of pooled serum or by finding a standard curve that does not pass through the origin. In the case of the patient's serum, there is no practical way to determine interference. This problem can, however, be minimized in two ways: 1) adequate communication between the physician and the laboratory with respect to any and all drug therapy will allow the laboratory to rule out interference by other drugs; and 2) when sufficient patient material is provided (2.5 ml), the standard curve can be constructed by adding antibiotic to aliquots of the patient material. All the samples are extracted and the peak height of the drug in the patient's sample with no added antibiotic is subtracted from those samples containing added antibiotic. A standard curve is then constructed from this data and the antibiotic concentration in the patient's serum read from the graph.

Several procedures routinely use an internal standard to increase accuracy. The addition of internal standard prior to extraction assumes correction for errors in sample and standard manipulation. Although this compensation may not be identical for the internal standard and the compound of interest due to physio-chemical properties, it is assumed that a constant proportion of each is extracted. Tables 2 and 3 clearly show that the addition of an internal standard does not significantly alter the accuracy or precision of this procedure. It would appear that the internal standard may be of some benefit with low drug concentrations. On the whole, an internal standard increases sample preparation and chromatography time and raises the possibility of interference at a second point in the analysis. We do not see the need for an internal standard in this procedure, however, if one is desired, one of the other antibiotics not used in the assay may be included for this purpose. We have found that the addition of a Chaney adaptor to the microsyrine helped to standardize the sample injection, thus increasing precision. For repeated injection of the same sample or group of samples, the automated injection system offers excellent precision.

Active metabolites of the isoxazolyl penicillins are known to be present in both urine and serum (26). These metabolites possess antibacterial activity of approximately the same order of magnitude as the parent compounds. In urine, metabolite activity compared to total antimicrobial activity ranged from 20% for oxacillin to 10% for flucloxacillin with cloxacillin and dicloxacillin being intermediate. However, following a 2 g i.v. injection of cloxacillin the metabolite activity at 90 min was 20% in urine, but only 9% in the serum. Thus it would appear that the metabolite is of lesser importance in the serum. This would also explain the close overall agreement we have observed between HPLC and microbiological methods (Fig. 4). The separation of these metabolites was accomplished by reverse-phase thin layer chromatography (27) and their separation by HPLC was recently described (28). At the present time, quantitation and separation of these metabolites in routine clinical specimens is not practical for three reasons: 1) the *in vivo* activity of these metabolites is unknown; 2) the data suggests that their concentrations may be rather insignificant with the possible exception of patients in renal failure; and 3) sufficient quantities of metabolite for standard curve construction with routine specimens is not available.

Column life is a practical consideration with any procedure. Using the procedure outlined above we are able to make approximately 1,500 injections of extracted serum during the lifetime of a column. We have found that the use of a column guard and regular column regeneration greatly extend column life.

In the legend presented for the antibiotics, we have attempted to indicate which antibiotic levels and attenuations will be most useful for routine clinical specimens. Although these levels will be the most frequently encountered, higher levels can be expected in patients with severe infections or in renal failure. For example, we have seen methicillin levels in excess of 200 μg/ml and nafcillin levels in excess of 100 μg/ml.

The data presented demonstrates the efficacy of HPLC for routine clinical testing of antibiotic levels in human serum. We have also applied this procedure to cerebro-spinal, pleural, and joint fluids. Its sensitivity of less than 0.5 μg/ml and accuracy of greater than 95% is more than adequate for routine testing.

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