Quantitation of Antibiotics by High-Pressure Liquid Chromatography: Cephalothin

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A technique for quantitative determination of cephalothin and desacetylcephalothin in serum using a method based on high-pressure liquid chromatography is described. Both compounds were quantitatively extracted from serum by using dimethylformamide. After separation of the drugs by reverse-phase chromatography and detection by ultraviolet light absorption at 254 nm, serum concentrations of cephalothin and desacetylcephalothin as low as 1.0 μg/ml were measured. No interfering absorption was found in extracts of serum samples from normal humans or patients receiving a variety of other drugs, including other antimicrobial agents. Serum specimens obtained after an intravenous infusion of cephalothin to two human subjects and three dogs were assayed by the chemical and microbiological assays. When values, as determined by the chemical method and the microbiological assay in samples from human subjects, were compared, the correlation coefficient (r) was 0.96, whereas, in the dogs, the r value was 0.79. The chemical procedure described in this report for determination of cephalothin and desacetylcephalothin permits future pharmacokinetic investigations of these compounds. This assay is sensitive, specific, accurate, and rapid (approximately 30 min), and appears suitable for routine clinical use.

Cephalothin is a semisynthetic derivative of cephalosporin C that became available for clinical trials in the United States in 1962. Since its introduction, it has been widely used with great success in infections involving a variety of gram-positive and gram-negative bacteria (10, 15, 20). Despite cephalothin’s great popularity and proven efficacy, caution should be exercised in its administration, especially with large doses. Although infrequently reported, renal failure may occur with large doses of cephalothin or in the presence of previous azotemia (3, 14, 16, 18, 19). Therefore, rapid and accurate monitoring of serum levels of this antibiotic becomes essential in these clinical settings. Moreover, a readily available method to determine serum concentrations of cephalothin may be important to properly assess appropriate therapeutic doses.

Recently we have successfully applied high-pressure liquid chromatography (HPLC) to the quantitation of antibiotics in serum and cerebrospinal fluid (12, 13). This technique has advantages over standard microbiological assays with regard to accuracy, specificity, and rapidity. Predicated on these earlier results, the technique of HPLC was applied to the determination of cephalothin concentrations in serum. A one-step quantitative extraction of the drug from serum with dimethylformamide (DMF), followed by separation using reverse-phase HPLC, and, finally, detection of cephalothin in the eluent with ultraviolet spectrophotometry resulted in a rapid assay which also had a high degree of accuracy, sensitivity, and specificity.

MATERIALS AND METHODS

DMF. DMF was reagent grade (J. T. Baker Chemical Co., Phillipsburg, N.J.).

Methanol. Absolute methanol (Mallinckrodt Chemical Works, St. Louis, Mo.) was distilled in a glass apparatus.

Water. Water used in the procedure was deionized and distilled daily.

Acetic acid solution. Glacial acetic acid (J. T. Baker Chemical Co.), reagent grade, was diluted to 1% (vol/vol).

Chromatographic eluent. A mixture of methanol and 1% acetic acid (4:6, vol/vol) was filtered through a 0.5-μm filter (Solvineit; Millipore Corp., Bedford, Mass.) and deaerated with vacuum. This will be designated in the text as methanol-acetic acid.

Chromatographic equipment. Separation was accomplished on an ALC/GPC 200 liquid chromatograph ( Waters Associates, Inc., Milford, Mass.). It was
equipped with a model 6000 solvent delivery system, a model U6K universal injector, and a model 440 absorbance detector. The eluent was monitored at 254 nm, and chromatographic peaks were recorded and their respective areas integrated with a HP3380A integrator (Hewlett-Packard, Avondale, Pa.). Separation was accomplished on a MicroBondapak C18 column (30 cm by 4 mm ID; Waters Associates, Inc.).

In vitro serum samples. Aqueous solutions of sodium cephalothin (USP Reference Standard) were prepared and stored at −70°C for no longer than 1 month. Desacetylcephalothin as the free acid (kindly donated by Robert Furman, Lilly Research Laboratories, Indianapolis, Ind.) was dissolved in DMSO (2 mg/ml) and further diluted with water. A quantity of 0.1 ml of each of the standards was added to 0.8 ml of dog or human sera and incubated at ambient temperature for at least 20 min. Blanks were prepared in the same manner except for omission of the two drugs. Also, controls in which water was substituted for serum were employed.

In vivo dog samples. Three dogs (weight, 32, 34, and 35 kg) were given a single intravenous dose (14 mg/kg of body weight over 6 min) of an aqueous solution of sodium cephalothin (Keflin; Eli Lilly International Corp., Indianapolis, Ind.). The dogs were bled at intervals up to 8 h after injection. Sera were stored at −20°C for no longer than 4 weeks before analysis.

In vivo human samples. The human portion of this study was performed on the investigators, who gave informed consent. Two healthy volunteers (investigators I.N.-E. and T.T.Y.) were infused intravenously over 30 min with 1.0 g of sodium cephalothin (Keflin) in 250 ml of 5% (wt/vol) dextrose solution. Blood samples were obtained immediately after and up to 8 h after completion of infusion. Sera were stored at −20°C up to 4 weeks before analysis.

Microbiological assay. The assay employed for cephalothin in both human and dog sera was performed by a standard agar diffusion technique. The test organism was Bacillus subtilis ATCC 6533. To ascertain the relative inhibitory effects of cephalothin and desacetylcephalothin, the minimal inhibitory concentrations of these compounds to the organism were determined. An overnight culture of the organism was grown on solid agar, scraped from the plate, inoculated into standard broth, and adjusted to a concentration of 1.5 × 10⁶ bacteria per ml. Desacetylcephalothin in its free acid form was dissolved in water and DMF 1:1 (vol/vol). No inhibitory effect of DMF on microbial growth was observed. Cephalothin was dissolved in distilled water alone. After appropriate serial twofold dilutions of each drug, 0.01 ml of bacterial suspension was added to each tube and incubated at 37°C for 16 h. Minimum inhibitory concentration was determined as the least concentration of drug that inhibited bacterial growth as judged by lack of visible turbidity.

For microbiological assay, B. subtilis was grown overnight on solid agar, scraped off the plate, inoculated into sterile 0.85% (wt/vol) NaCl, and adjusted to a concentration equivalent to a McFarland 0.5 barium sulfate standard (2.4 × 10⁶ bacteria per ml). The inoculum was prepared by adding 1.8 ml of bacterial suspension to 12 ml of remelted, cooled (50°C) test agar (antibiotic medium no. 2; Difco Laboratories, Detroit, Mich.). The suspension was thoroughly mixed, poured into petri plates (150-mm diameter), and allowed to solidify. Ten wells with diameters of 3.5 mm were punched out of each plate. Standards of cephalothin at concentrations ranging from 0.625 to 10 µg/ml were prepared in dog or human sera. Subsequently, 5 µl of standard antibiotic solution and unknown serum samples were added to the wells. For serum samples with zone sizes larger than the 10-µg/ml standard concentration, appropriate dilutions were made. Plates were incubated at 37°C, and zones of inhibition were read 16 to 18 h later. Zone size was plotted against known cephalothin concentrations on semilog paper, and antibiotic concentrations of unknown samples were determined.

Chemical assay. (i) Extraction. A modification of the procedure described by Hoehn et al. (6) was employed. A quantity of 1 ml of serum was mixed with 1.0 ml of DMSO. The mixture was incubated at 50°C for 10 min and centrifuged at 2,000 × g for 10 min at ambient temperature, and 0.8 ml of the supernatant was mixed with 0.8 ml of water. This mixture was then passed through 0.5-µm filters (Solvinit, Millipore Corp.).

(ii) Separation, detection, and quantitation. A volume of 100 µl of the filtered solution was injected into the liquid chromatograph. Cephalothin and its desacetyl metabolite were eluted with methanol-acetic acid as mobile phase at a flow rate of 2.5 ml/min. The eluent was monitored at 254 nm at an attenuation of 0.01 absorbance unit full scale. Recorder chart speed was 0.5 cm/min.

Quantitation was based on integration of peak areas using an HP3380A integrator previously calibrated with known concentrations of cephalothin and desacetylcephalothin.

(iii) Critical steps. Special attention was given to several of the steps in the procedure.

1. The serum sample and DMF were left for 10 min to ensure maximum protein precipitation and quantitative extraction of cephalothin and its desacetyl metabolite.

2. All solvents and extracts were filtered through 0.5-µm filters before chromatography to prevent particulate matter from obstructing filters in the chromatograph.

3. Desalting of the mobile phase was essential because air bubbles caused base line drift and excessive noise in the detector.

RESULTS

Choice of detection wavelength. The ultraviolet absorption maximum for cephalothin was found at 236 nm. The flow detector used with the chromatograph operated with fixed wavelengths, and 254 nm was the available wavelength closest to the absorption maximum. At this wavelength, cephalothin exhibited an absorption that was approximately 2/3 of the maximal absorption at 236 nm.

Separation of cephalothin and desacetylcephalothin by liquid chromatography. With the chromatographic system described, cephalothin and desacetylcephalothin had retention times of 9.99 and 4.44 min, respectively.
Both were well separated from each other and other detectable substances in serum extracts (Fig. 1). Extracts of serum not containing these antibiotics showed no detectable absorption at these retention times when chromatographed (Fig. 1). After elution of cephalothin, other ultraviolet light-absorbing material was observed for approximately 30 min. To elute these latter substances more rapidly, the methanol in the mobile phase was increased to 100%, and the column was washed for 5 min. Finally, the original mixture of methanol-acetic acid was allowed to equilibrate with the column until the baseline became stable. The system was then ready for determination of the next sample.

Recovery. A standard curve was obtained by HPLC using aqueous solutions of cephalothin and desacetylcephalothin at varying concentrations without going through the extraction procedure. Known concentrations of the aqueous standards of cephalothin and desacetylcephalothin were then added to five serum samples. After extraction and chromatography in duplicate, integrated values of peak areas were determined, and recovery was calculated from the standard curve. Table 1 demonstrates that recovery ranges for cephalothin and desacetylcephalothin were 97.0 to 102.2% and 96.8 to 103.4%, respectively.

A linear relationship is demonstrated between cephalothin serum concentrations and the peak area values obtained on the integrator (Fig. 2). An almost identical relationship was found when aqueous solutions of cephalothin were substituted for serum (Fig. 2). This linear relationship was found to extend from concentrations of 1 to 100 μg/ml. Similar findings were noted for desacetylcephalothin.

Sensitivity. The baseline fluctuation in the detector allowed a slope sensitivity setting of 1.0 mV/min on the integrator. This permitted detection of 25 ng of cephalothin or desacetylcephalothin, corresponding to serum concentrations of 1.0 μg/ml. For concentrations of cephalothin below 2.0 μg/ml, a higher concentration of methanol (45%) in the mobile phase, resulting in a shorter retention time and a sharper peak, was necessary for increased accuracy. For subsequent determinations, the column should be cleaned and equilibrated with the original methanol-acetic acid mixture as described above.

Precision. Precision for the chemical assay was determined for two concentrations each of cephalothin and desacetylcephalothin in serum. Six samples of 5.0 μg and six samples of 15.0 μg of cephalothin per ml were extracted separately and chromatographed on the same day. In the case of desacetylcephalothin, six samples each at concentrations of 5.0 and 30.0 μg/ml were

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**Table 1. Recovery of cephalothin and desacetylcephalothin from human serum**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Amount added (μg/ml)</th>
<th>Amount measured in serum (μg/ml)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cephalothin</td>
<td>1</td>
<td>0.97</td>
<td>97.0</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>5.19</td>
<td>103.8</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>9.80</td>
<td>98.0</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>14.85</td>
<td>99.0</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>20.44</td>
<td>102.2</td>
</tr>
<tr>
<td>Desacetylcepha-</td>
<td>1</td>
<td>1.03</td>
<td>103.4</td>
</tr>
<tr>
<td>lothin</td>
<td>5</td>
<td>4.90</td>
<td>98.0</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>10.12</td>
<td>101.2</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>14.52</td>
<td>96.8</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>19.70</td>
<td>98.5</td>
</tr>
</tbody>
</table>

* Values represent the mean of two separate determinations.

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**Fig. 1.** (A) HPLC of an extract of dog serum containing cephalothin and desacetylcephalothin. The cephalothin peak had a retention time of 9.99 min. The desacetylcephalothin peak had a retention time of 4.44 min. Chromatographic conditions were as described in the text. (B) HPLC of an extract of serum with no drug added, under the same chromatographic conditions.
used. Table 2 shows that there was satisfactory precision for these serum concentrations of both compounds. The relative standard deviations for cephalothin were 2.3 and 1.0%, respectively; for desacetylcephalothin, the values were 1.6 and 0.9%, respectively.

**Specificity.** No interfering absorption at the retention times of the two compounds was found in chromatograms of serum extracts from 90 dogs and from four healthy human volunteers (investigators). Serum samples from 10 patients receiving a variety of other drugs were assayed and exhibited no detectable absorption at the retention times of cephalothin and desacetylcephalothin. Antibiotic drugs that did not interfere with the cephalothin and desacetylcephalothin assay were ampicillin, amphotericin B, clindamycin, ethambutol, 5-fluorouracil, gentamicin, methicillin, penicillin G, and tetracycline; non-antibiotic drugs included acetamino-

**Disappearance from the circulation.** Figure 3 illustrates a time curve for the disappearance of cephalothin from the circulation in a human after a single intravenous dose of 1.0 g of cephalothin. Values from the chemical analysis of cephalothin and desacetylcephalothin along with the corresponding microbiological assay are plotted on the same graph. Approximately 85% of cephalothin was removed from serum by 30 min. The drug could not be detected at 2 h and thereafter with either assay. This has been noted by several investigators previously (7, 8, 17). Desacetylcephalothin was detected by the chemical assay at low concentrations immediately after completion of cephalothin infusion, but could not be measured after 0.5 h. Similar data were found with the second human volunteer.

In Fig. 4, the disappearance curve of cephalothin after an intravenous dose in a single dog is depicted. Cephalothin, determined by the chemical assay, rapidly disappeared from serum, as in humans. However, in contrast to humans, the concentrations of desacetylcephalothin in serum exceeded that of the parent compound and remained in the circulation for a longer time.

**Table 2. Precision of cephalothin and desacetylcephalothin determinations**

<table>
<thead>
<tr>
<th>Drug added to serum (µg/ml)</th>
<th>Mean (µg/ml)*</th>
<th>Relative standard deviation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cephalothin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.00</td>
<td>5.00 ± 0.12</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>(4.79–5.14)</td>
<td></td>
</tr>
<tr>
<td>15.00</td>
<td>15.00 ± 0.15</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>(14.90–15.15)</td>
<td></td>
</tr>
<tr>
<td>Desacetylcephalothin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.00</td>
<td>5.00 ± 0.08</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td>(4.92–5.12)</td>
<td></td>
</tr>
<tr>
<td>30.00</td>
<td>30.00 ± 0.28</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>(29.56–3.35)</td>
<td></td>
</tr>
</tbody>
</table>

* Values represent mean of six separate observations at each concentration assayed on the same day ± standard deviation.
period of time (up to 6 h). In the other two dogs, similar disappearance curves for cephalothin and desacetylcephalothin were found.

Correlation with microbiological assay. The minimum inhibitory concentrations of cephalothin and desacetylcephalothin to the test organism were 0.05 and 0.20 μg/ml, respectively. These findings correlate well with findings of other investigators in that the desacetyl metabolite of cephalothin has only 20 to 25% of the biological activity of its parent compound (1, 7).

Serum samples from the two human volunteers were assayed in duplicate by the microbiological as well as the chemical method. When the bioassay values were compared with the corresponding cephalothin concentrations determined by liquid chromatography, the correlation coefficient was 0.96 (Fig. 5). Since the microbiological assay in part reflects activity of the desacetyl metabolite of cephalothin, the correlation coefficient was also calculated by comparing microbiological assay values with the sum of serum concentrations of cephalothin and desacetylcephalothin determined by the chemical assay (Fig. 6). However, the correlation remained basically unchanged.

Upon comparison of values obtained by bioassay and chemical assay in the dog experiments, the correlation coefficient was less. If the microbiological assay values were compared with those of cephalothin alone, desacetylcephalothin alone, or the sum of cephalothin plus desacetylcephalothin (all determined by the chemical assay), the correlation coefficients were 0.79, 0.84, and 0.80, respectively. This lower correlation coefficient in dogs may be due to the higher concentrations of desacetylcephalothin in the sera of dogs. Since the desacetyl form of cephalothin possesses less biological activity than its parent compound, a greater discordancy between the chemical and microbiological assay is not totally unexpected.

DISCUSSION

Antibiotics are one of the most frequently used classes of drugs in clinical medicine. For serious infections, larger amounts of antibiotics
are often administered to improve therapeutic efficacy. However, the risk of adverse effects and potential toxicity must be carefully avoided. It is important that rapid and accurate determinations of antibiotic concentrations in serum and body fluids be available so that physicians can assess appropriate therapeutic doses and minimize serious, dose-related side effects.

Most clinical laboratories have relied on microbiological assays as the principal method for determining antibiotic concentrations in serum and other body fluids. The major disadvantages to a microbial assay are lack of uniform methodology applicable to most clinically important antibiotics, interference of other antimicrobial agents concurrently present in the biological sample, and prolonged delay in obtaining the result.

Recently, the HPLC technique has been successfully implemented for rapid, accurate, and specific measurements of tetracycline (13) and amphotericin B (12) in serum and cerebrospinal fluid. The complete methodology involved simple extraction of antibiotics from the samples, followed by separation by HPLC and, finally, detection of the compounds by ultraviolet spectrometry.

The application of HPLC to quantitation of cephalothin has been previously reported (2, 4). Cooper et al. (4) determined cephalothin and desacetylcephalothin by HPLC in human serum and urine. However, the extraction procedure was only slightly better than 80% effective with regard to recovery of the drug from serum. Furthermore, the chromatographic separation was based on ion exchange, which required temperature control at 50°C, whereas reverse-phase partitioning is usually performed at ambient temperature. In addition, column life is shorter with the ion-exchange method than with reverse-phase partitioning. The lack of data on precision, correlation with standard microbiological assay, interference from other drugs, and time of performance of test prohibits assessment of the feasibility of this earlier methodology for clinical use. Buha et al. (2) described a method using HPLC to measure cephalothin, cefoxitin, and their deacetylated metabolites. However, their investigations were restricted to examination of urinary concentrations of these compounds in humans.

With the technique of HPLC, the desacetyl metabolite of cephalothin is also accurately measured, which is not possible by microbiological assays. Since its biological activity is known to be much less than that of the parent compound, it may be important to assess quantitatively its distribution in various body fluids to better understand the pharmacokinetics and efficacy of cephalothin therapy. For example, the high rate of failure of cephalothin in the treatment of bacterial meningitis (5) has been attributed not only to its variable penetration into the cerebrospinal fluid but also to the possible presence of the less active metabolite, desacetylcephalothin in the cerebrospinal fluid. These problems can now be investigated by using techniques such as the one described in this paper.

Approximately 75% of circulating cephalothin is bound to serum proteins (11). Therefore, complete dissociation from proteins is essential for determination of total serum concentrations. The chemical procedure reported here is a chemical assay in which cephalothin and its desacetyl metabolite were quantitatively recovered from serum. The lack of interference from a variety of other drugs including antibiotics is extremely useful, since many patients may receive combination antimicrobial therapy (9). The data presented demonstrates that this technique is also precise, reproducible, and well correlated to the microbiological assay. Its sensitivity of 1 ng/ml is more than adequate for routine use. Additionally, the methodology described here is extremely rapid. The entire procedure can be performed in 30 min for a single serum sample.

Thus, this chemical assay appears feasible for routine clinical use and moreover may be implemented for investigation of the pharmacokinetics of cephalothin and desacetylcephalothin.

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LITERATURE CITED


