High-Performance Liquid-Chromatographic Assay for Chloramphenicol in Biological Fluids

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We describe a method for measuring chloramphenicol by high-performance liquid chromatography. The assay involves a single extraction of the biological sample with ether, evaporation of the extract, and chromatography of the residue, redissolved in methanol. A reversed-phase column with an eluting solvent of methanol/water (30/70 by vol) is used. Chloramphenicol is eluted from the column in about 4 min and is well separated from the internal standard (mephenesin), which is eluted at 5.5 min. Absorption of the effluent at 278 nm is monitored and measured. As little as 0.1 μg of the antibiotic can be analyzed after its extraction from a 0.1-ml sample. The method is suitable for rapid and specific analysis for the drug in plasma, urine, cerebrospinal fluid, and other biological fluids.

Additional Keyphrases: measurement in plasma, serum, urine, cerebrospinal fluid, ascitic fluid; pediatric chemistry

Chloramphenicol is an effective antibiotic, but has serious side effects (1). Pancytopenia, a fatal reaction, is not related to drug dose but other untoward effects such as anemia and the "gray baby syndrome" are directly related to concentrations of the drug in plasma. Thus, it is often important to monitor plasma concentrations of the drug in patients who are undergoing chloramphenicol therapy.

Several assays have been reported for chloramphenicol in biological fluids. These have been compiled (2) and include microbiological (3, 4), colorimetric (5-9), gas-chromatographic (10-12), fluorometric (13), polarographic (14), and enzymatic (15) methods. One or more of the following disadvantages are associated with each of these methods: lack of specificity, lack of sensitivity, lack of precision, and lengthy analysis time.

We describe here a rapid, specific, and sensitive chromatographic procedure for chloramphenicol. The method is suitable for use in analyzing for chloramphenicol in plasma, urine, and other biological fluids.

Materials and Methods

Instrumentation

The chromatograph we used consisted of a Model 6000 A pump and a U6-K sample injector, both from Waters Associates, Milford, Mass. 01756. A multiple-wavelength detector, Model SF770 (Schoeffel Instruments, Westwood, N. J. 07675) was used. The Partisil 10-ODS (Whatman, Clifton, N. J. 07014) column used in the assay was purchased prepacked from the manufacturer. Mass spectra were recorded with a Model 1015 mass spectrometer (Finnigan Corp., Sunnyvale, Calif. 94086) at 70 eV.

Chemicals

Chloramphenicol was obtained by alkaline hydrolysis of chloramphenicol succinate (Parke, Davis & Co., Detroit, Mich. 48232). The ester was dissolved in 0.1 mol/liter NaOH and the solution was mechanically shaken with 10 volumes of diethyl ether for 30 min. The organic layer was removed and evaporated and the resulting white powder was recrystallized from methanol and water. The dry product, chromatographically pure, had a melting point (uncorr.) of 149-150 °C (lit., 150 °C).

Mephenesin powder, 3-(2-methylphenoxy)-propane-1,2-diol (Sigma Chemical Co., St. Louis, Mo. 63178) was pure by the criterion of liquid chromatography.

All organic solvents were of analytical grade.

Procedure

The experimental sample, 0.1-0.2 ml of serum, plasma, cerebrospinal fluid, or urine, is placed in a 45-ml glass-stoppered centrifuge tube. The internal standard (mephenesin) in methanolic solution is added in about 10-fold the amount of chloramphenicol estimated to be in the sample. For clinical samples, 10 μg of internal standard in 50 μl of methanol is usually added. Tris(hydroxymethyl)aminomethane (0.8 mol/liter, pH 10.4) buffer is then added to produce a volume of 1.0 ml. The solution is extracted with 10 ml of diethyl ether by shaking vigorously for 10 min. The organic layer is separated by centrifugation and 8 ml of it is transferred to a centrifuge tube and evaporated at room temperature under a stream of nitrogen. (Heat should be avoided during this solvent evaporation.) The glass centrifuge tubes used in the assay should be free of detergent film, which will produce peaks that interfere with the chromatographic analysis. Acid-washed tubes or disposable glass tubes have been used successfully. For chromatography, 20-50 μl of methanol is added to
the tube and a 5–10 μl sample is injected into the chromatograph.

Freshly prepared solutions of chloramphenicol (containing internal standard) are extracted in duplicate for each day's analysis. For routine analysis of plasma samples containing therapeutic concentrations of the antibiotic, four different standard solutions are used. These contain 0.1, 0.5, 1.0, and 5.0 μg of chloramphenicol and 1.0, 5.0, 10.0, and 50.0 μg of internal standard, respectively, in 1 ml of the buffer. Stock solutions of chloramphenicol and internal standard in methanol are used to prepare the standard solutions.

The chromatographic solvent, methanol/distilled water (30/70 by vol), is delivered at a constant flow of 1.5 ml/min. Detector wavelength is 278 nm, the optimum for chloramphenicol, but if necessary a detector wavelength of 254 nm may be used with little loss in sensitivity. Peak-height measurements are used in constructing standard curves from which to calculate the amount of chloramphenicol in experimental samples.

Assessment of Specificity

Urine and plasma (0.2 ml) from patients receiving various drugs other than chloramphenicol were assayed for chloramphenicol (no internal standard was added). The resulting chromatograms were assessed for the presence of chromatographic peaks that would interfere with chloramphenicol or the internal standard.

The effluent corresponding to the chromatographic peak representing chloramphenicol was analyzed for the possible presence of contaminating substances (drug metabolites, etc.) by mass spectrometry. Urine (0.5 ml) collected during 24 h from patients receiving chloramphenicol therapy was treated with 10 000 U of β-glucuronidase (Glurolase; Endo Laboratories, Chicago, Ill. 60641) for 16 h at 37 °C, to hydrolyze glucuronate conjugates of chloramphenicol. An aliquot of the enzyme-treated urine, equivalent to 0.2 ml of urine, was extracted under the above-described conditions for chloramphenicol assay. The extract was injected into the chromatograph and the effluent corresponding to the chromatographic peak representing chloramphenicol was collected, the elution solvent evaporated under nitrogen, and the resulting residue subjected to mass-spectrometric analysis, with use of the solid probe inlet to the instrument.

Results and Discussion

Chloramphenicol and the internal standard, mephenesin, show excellent liquid-chromatographic characteristics (Figure 1). Chloramphenicol produced a symmetrical, sharp peak having a retention time of 4 min. The internal standard eluted after chloramphenicol and was well separated from it. The data in Figure 1 also indicate that serum is free from substances interfering with these chromatographic peaks, nor were interfering peaks observed with drug-free samples of plasma, cerebrospinal fluid, urine, or ascitic fluid. However, the samples must be extracted at alkaline pH (~pH 10–11) to eliminate interfering peaks. The buffer, added to the samples before extraction, produces the necessary pH adjustment.

Figure 2 shows a typical standard curve produced by analysis for known amounts of chloramphenicol in aqueous solution. The curve is linear for samples containing from 0.1 to 50 μg of the antibiotic. Results identical to those for the standard curves were obtained when the drug was added to plasma, serum, cerebrospinal fluid, or urine and assayed. For convenience, the standard curve determined for each group of clinical samples was obtained by analysis of known amounts of chloramphenicol and internal standard added to the buffer.
The lower limit for detection of chloramphenicol in the liquid chromatograph was 5 ng, and this is more sensitive than is actually required for clinical samples. Comparison of peak heights for chloramphenicol extracted from biological samples to which the drug has been added with those obtained after direct injection of the antibiotic into the chromatograph indicated that the ether-extraction procedure was greater than 80% efficient.

Analytical recovery of chloramphenicol and the reproducibility of the assay were assessed by adding known amounts of the drug to human plasma and analyzing duplicate 0.1-ml samples. The data (Table 1) show that recovery of the drug was adequate. In practice, clinical samples analyzed in duplicate yield values that agree within 6%. Interassay variability was assessed by analyzing a plasma sample containing 25 μg of the antibiotic per milliliter on 18 different occasions during four months. The mean value for the sample was 25.74 (CV = 6%). During this period we saw no trend with time toward higher or lower values.

Some drugs that may be given to patients along with chloramphenicol were tested for possible interference by analyzing serum or urine (or both) from patients receiving the drugs, but not chloramphenicol. Gentamicin, penicillin, ampicillin, furosemide, salicylate, morphine, and clindamycin evidently do not interfere. Chloramphenicol succinate, the common form used for intravenous administration of the antibiotic, is eluted from the column at about 2 min and therefore does not contribute to the chloramphenicol peak.

We did experiments to determine whether metabolites of chloramphenicol would interfere with the analysis for unchanged drug. This was necessary because metabolites of the drug possess little antibiotic activity (16). Urine from patients receiving the drug was used as a source of potentially interfering metabolites. Mass-spectrometric analysis of the chromatographic fraction containing chloramphenicol indicated that metabolites of the drug were not detectable (Figure 3). Urines from three patients, examined in this way, showed no evidence of ions other than those produced by chloramphenicol. This evidence, coupled with the

### Table 1. Analytical Recovery of Known Amounts of Chloramphenicol Added to Human Plasma

<table>
<thead>
<tr>
<th>Known added (mg/liter)</th>
<th>Found* (mg/liter)</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>45.5</td>
<td>46.4</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>44.6</td>
<td></td>
</tr>
<tr>
<td>33.3</td>
<td>31.3</td>
<td>94.4</td>
</tr>
<tr>
<td></td>
<td>31.6</td>
<td></td>
</tr>
<tr>
<td>23.8</td>
<td>22.8</td>
<td>95.0</td>
</tr>
<tr>
<td></td>
<td>22.4</td>
<td></td>
</tr>
<tr>
<td>9.8</td>
<td>9.3</td>
<td>100.5</td>
</tr>
<tr>
<td></td>
<td>10.4</td>
<td></td>
</tr>
<tr>
<td>3.3</td>
<td>3.4</td>
<td>106.1</td>
</tr>
<tr>
<td></td>
<td>3.6</td>
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</table>

* Results of duplicate analyses.
matographic methods involve derivatization of the drug, with its accompanying difficulties. Microbiological methods are adequately sensitive but have disadvantages with respect to reliability, reproducibility, and speed. The present assay is more rapid than any of the previously reported methods and possesses the characteristics of a reliable analytical procedure that can be applied to research or clinical problems.

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References


Table 2. Analysis of Clinical Samples from Three Pediatric Patients (3–7 Months Old) Receiving Chloramphenicol Therapy

<table>
<thead>
<tr>
<th>Dose (l.v.) of chloramphenicol* (mg/kg per day)</th>
<th>Sample</th>
<th>Time after last dose, h</th>
<th>Chloramphenicol concentration, mg/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>45</td>
<td>Serum</td>
<td>0.5</td>
<td>9.8</td>
</tr>
<tr>
<td></td>
<td>Peritoneal fluid</td>
<td>0.5</td>
<td>7.0</td>
</tr>
<tr>
<td></td>
<td>Serum</td>
<td>2.0</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>Peritoneal fluid</td>
<td>2.0</td>
<td>1.0</td>
</tr>
<tr>
<td>150</td>
<td>Serum</td>
<td>5.0</td>
<td>36.0</td>
</tr>
<tr>
<td></td>
<td>Cerebrospinal fluid</td>
<td>5.0</td>
<td>18.0</td>
</tr>
<tr>
<td>75</td>
<td>Serum</td>
<td>5.0</td>
<td>26.0</td>
</tr>
<tr>
<td></td>
<td>Cerebrospinal fluid</td>
<td>10.0</td>
<td>18.0</td>
</tr>
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</table>

* Given in divided doses.

fact that no interfering peaks have been observed in clinical samples, indicates that the assay exhibits the necessary specificity for unchanged chloramphenicol.

The alkaline buffer used in the extraction procedure does not release chloramphenicol from its glucuronide conjugate, which would produce erroneously high values for the antibiotic. This was determined by exposing urine containing unchanged chloramphenicol and chloramphenicol glucuronide to the buffer for periods up to 1 h. The more prolonged exposure times did not result in higher (or lower) values for unchanged chloramphenicol as determined by the assay procedure described here. That the urine samples did in fact contain chloramphenicol glucuronide was verified by observing a large increase in the unchanged drug after hydrolysis of the urine with Glusulase.

The assay described here has been in routine use for more than a year. The sensitivity allows measurement of the drug in as little as 0.1 ml of sample, and so the assay can be readily applied to small samples such as are obtained from pediatric patients. Analyses for chloramphenicol in samples of serum, peritoneal fluid (ascites), and cerebrospinal fluid (CSF) confirm (Table 2) that the antibiotic is well distributed in biological fluids. Concentrations in the cerebrospinal fluid are about half those found in the corresponding plasma; this may reflect the approximately 60% binding of the drug by plasma proteins (16).

Our assay is a definite improvement over other methods available for analysis of the drug in clinical samples. It is more specific than colorimetric methods, particularly those in which the drug is not separated from interfering substances by extraction. Gas-chro-