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High-Pressure Liquid Chromatographic Assay of Chloramphenicol in Biological Fluids

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Chloramphenicol can be assayed by a sensitive and selective high-pressure liquid chromatographic assay technique. The method described is easily adapted to analysis of many other drugs.

The emergence of *Haemophilus influenzae* strains resistant to ampicillin and the increased recognition of infection due to anaerobic bacteria has led to an increased use of chloramphenicol in recent years. However, chloramphenicol may be toxic, especially in infants. Since the pharmacokinetics of chloramphenicol in newborns and young infants may vary from patient to patient (1) and some of the major toxic reactions to chloramphenicol may be related to blood level (12), a simple method to assay chloramphenicol concentrations is needed. An ideal method would be one in which a small sample size is used and one which is not affected by the presence of other antibiotics.

Methods described previously for measuring chloramphenicol concentrations are either time consuming or laborious. The colorimetric method requires large sample volumes and several extractions to distinguish between the biologically active and inactive forms of chloramphenicol (3). The radioenzymatic assay (2, 8, 10) offers improved sensitivity, precision, and accuracy; however, more technician time and skill are required. The gas-liquid chromatographic method (7) uses 0.5 ml of serum, requires chemical derivatization, and also requires more technician time and skill. Microbiological assays, though used most often, require overnight incubation and are probably the least accurate of all methods due to poor precision, lack of sensitivity, and interferences from other antibiotics. We observed that chloramphenicol eluted late in the high-pressure liquid chromatographic (HPLC) determination of theophylline used in our laboratory. That procedure was modified to provide the clinically useful chloramphenicol method reported here. During the development and evaluation of this method, others have also reported HPLC techniques (6, 9, 11). These HPLC methods are superior to previous methods in speed, precision, and ease of performance. By careful selection of conditions, most of the problems associated with other methodologies can be overcome.

Chloramphenicol was obtained from Parke-Davis Co., Detroit, Mich. Chloroform and isopropanol were reagent grade from Fisher Scientific Co., Pittsburgh, Pa. Acetonitrile and methanol, HPLC grade, were also obtained from Fisher Scientific Co. Sulfamethoxazole was purchased from Burroughs Wellcome Co., Research Triangle Park, N.C.

Analyses are performed on a Waters Associates model ALC 200 HPLC, using a model 440 absorbance detector and U6K Universal injector (Waters Associates, Milford, Mass.). A μ-Bondapak C_{18} reverse-phase column (4-mm ID by 30 cm) is used. The eluting mobile phase is acetonitrile and 0.01 mol of sodium acetate buffer, pH 4.0, per liter (20:80, vol/vol) pumped at a rate of 2.0 ml/min. Detection is at 280 nm. The chart speed is 5 mm/min. Retention time for chloramphenicol is 10 min under these circumstances.

Proteins are precipitated from serum, plasma, or cerebrospinal fluid by mixing (30 s, Vortex) 0.1 ml of sample with 0.4 ml of internal standard/extractant solution. The internal standard/extractant solution contains 2 mg of *N*-acetyl chloramphenicol and 7.5 mg of sulfamethoxazole per liter of chloroform and isopropanol (50:50, vol/vol). The resulting emulsion is centrifuged briefly, and the protein disk and aqueous (upper) layer are aspirated and discarded. The organic phase is transferred to a clean test tube and dried under a stream of air. Fifty microliters of methanol is used to reconstitute the sample, and 6 μl is injected into the HPLC.

Standard solutions containing 5 to 40 μg of chloramphenicol per ml in plasma are prepared using a stock standard solution (10 mg/dl in methanol). Aliquots of the stock standard solution are placed in volumetric flasks and evapo-

rated with a stream of air. Chloramphenicol-free plasma is added to make working standard solutions. The working standards are stable for 6 months when frozen at −20°C. These standards are used in constructing the daily calibration curve.

Peak heights are measured for chloramphenicol and the internal standards. The ratio of the heights (chloramphenicol versus *N*-acetyl chloramphenicol) is plotted on linear graph paper against chloramphenicol concentration. The relative peak heights of the two internal standards are constant in the absence of interfering compounds.

Potential interference from a variety of drugs was investigated, and none was found from any of the following: gentamicin, phenobarbital, diphenylhydantoin, ampicillin, penicillin, acetaminophen, acetylsalicylic acid, thiothixene, methyprylon, diazepam, butabarbital, flurazepam, methylcyclothiazide, hydrochlorothiazide, chlorothiazide, prochlorperazine, glutethimide, propoxyphene, thioridazine, dexamethasone, theophylline, 8-chlorotheophylline theobromine, xanthine, caffeine, uric acid, metronidazole, and amphotericin B. Peaks resulting from any of the preceding compounds are separate from those of chloramphenicol, *N*-acetyl chloramphenicol, and sulfamethoxazole and thus do not interfere with either identification or quantitation of chloramphenicol. The 3-monosuccinate ester of chloramphenicol, commonly used in intravenous preparations, is eluted late in the chromatogram and is therefore not measured under the conditions described.

Figure 1 shows a typical calibration curve constructed with chloramphenicol standard solutions in plasma. Typical variations in peak height ratios of 5.0% or less were obtained during a period of 2 months.

A split-sample analysis was conducted on 21 samples, using HPLC and the enzymological microassay of Leitman et al. (8). No significant difference was found between the two methods using a paired *t* test ($t = 1.875$, $P = 0.08$). Figure 2 shows regression results for the two methods.

Twenty determinations of chloramphenicol concentrations performed by different technologists on a single sample over a period of 1 month yielded a mean concentration of 20.3 μg/ml. The standard deviation was 1.02 and the coefficient of variation was 5.0%.

A recovery study performed on eight samples which were spiked with 10 μg of chloramphenicol per ml showed a mean percent recovery (relative to added chloramphenicol) of 96.3%, with a coefficient of variation of 3.8%.

Chloramphenicol in plasma or other fluids can be assayed rapidly and with excellent selectivity by combining the solvent extraction and HPLC separating power of the present micromethod. Sensitivity is adequate for determining chloramphenicol concentrations within a clinically achievable range. Although our practice is to report chloramphenicol concentrations to the

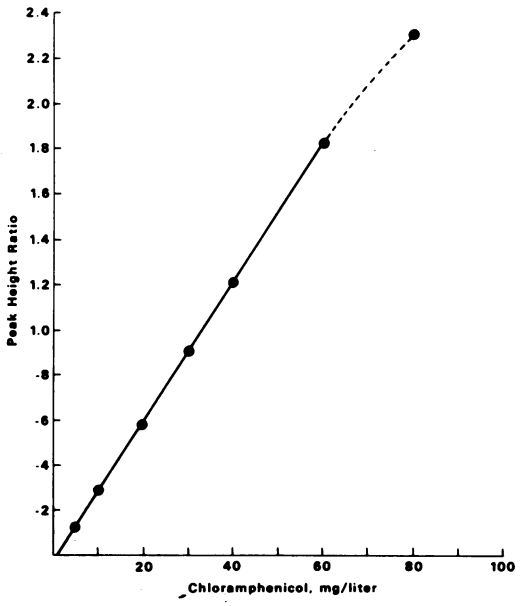

FIG. 1. *Calibration curve of chloramphenicol in plasma. N-acetyl chloramphenicol, 2 mg/liter, was the internal standard. Peak height ratios were calculated as N-acetyl chloramphenicol divided by chloramphenicol peak heights.*

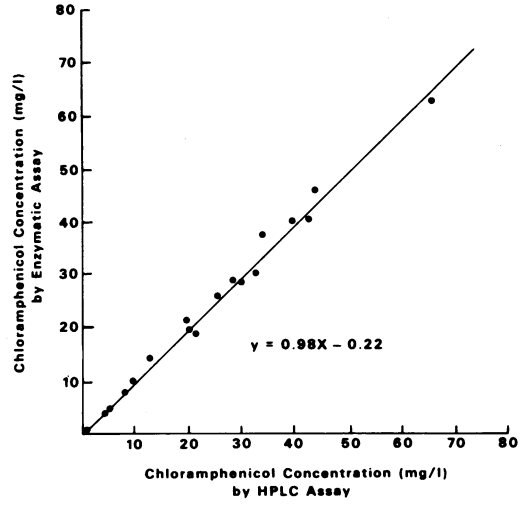

FIG. 2. *Comparison between the radioenzymatic assay and the HPLC assay for chloramphenicol. Coefficient of determination (r^2) for the two assays is 0.99.*

nearest 0.5 μg/ml, peak heights allow quantitation of as little as 100 to 200 ng/ml with a 100-μl sample.

HPLC allows determination of chloramphenicol concentration within 30 min and thus provides an advantage over the microbiological inhibition assay. With this report there are now four methods, all recently described, for the analysis of chloramphenicol using HPLC. One advantage of the method we describe is the ability to quickly change from chloramphenicol to theophylline analysis (M. R. Glick, R. H. B. Sample, and T. O. Oei, J. Ind. State Med. Assoc., in press). Conversion requires only the use of a different internal standard and mobile phase concentration. With variations in only these two parameters, we have been able to expand the technique to include 15 other drugs and metabolites (4).

One of the major differences among published HPLC chloramphenicol methods is the use of a direct (9) versus an extraction (6, 11) technique before chromatography. Although several minutes are required for evaporation of the organic solvent, there are data which suggest that this is a necessary and desirable prechromatographic step. Using a direct method of sample preparation, two cephalosporin antibiotics have retention properties which cause interference in the assay of theophylline (5). Inclusion of a preliminary solvent extraction step removes this interference. Using a direct technique (unpublished data), we have also found interference with the chloramphenicol assay by phenobarbital, whereas this is not found with the method we described. Koup et al. (6) also report interference with chloramphenicol using a direct technique. We therefore recommend the use of the solvent extraction step in future HPLC procedures.

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