One solution to the problem of keeping dilute solutions of aminoglycosides in solution is to use an acidic glycine buffer to dilute patients' samples and controls. This buffer can be conveniently made as a 2-fold concentrated solution (0.2 mol/L glycine, 2.0 mol/L NaCl), to be used as required.

Addition of a relatively small volume of glycine to a large volume of assay buffer then has a relatively small impact on the pH of the assay. Thus glassware commonly used in the laboratory can be used in aminoglycoside radioimmunoassay without the danger of adsorption.

Adsorption of aminoglycosides to glass surfaces may result from electrostatic interactions between negative charges on the surface of the glass and positively charged compounds such as tobramycin and gentamicin. At low pH's, the negatively charged groups on the surface become protonated, while at high pH's, the amino groups of the antibiotic become deprotonated. The sensitivity of adsorption to NaCl and the stability of aminoglycosides in polypropylene containers are in accord with this interpretation.

The precautions regarding aminoglycoside adsorption supplied in the instructions for commercial radioimmunoassay kits vary with the manufacturer but generally underestimate the impact of adsorption on assay characteristics. The results shown in Figure 1 indicate that the incubation interval in glass containers should be precisely controlled under adsorbing conditions. Adsorption in water may well reflect individual characteristics of the patient's serum—such as pH—creating random errors in assay values even in the presence of excellent duplicates for individual sera. Because it is difficult to ensure that adsorption has occurred equally from all samples, analyses of gentamicin and tobramycin can best be performed by eliminating it entirely, by use of the acidic glycine buffer we have described.

References


Assays for Chloramphenicol Compared: Radioenzymatic, Gas Chromatographic with Electron Capture, and Gas Chromatographic-Mass Spectrometric

L. K. Pickering, 1 J. L. Hoecker, 1 W. G. Kramer, 2 J. G. Liehr, 3 and R. M. Caprioli 3

We compared these three techniques for measuring chloramphenicol in serum or urine. Although each has its particular advantages, any of them is shown to be satisfactory and may appropriately be used by clinical laboratories, according to the facilities available.

Additional Keyphrases: serum, urine, cerebrospinal fluid • antibiotics • monitoring therapy • pediatric chemistry

Chloramphenicol, an antibiotic, was isolated in 1947 from Streptomyces venezuelae, an organism found in a soil sample in Venezuela. It is useful in the treatment of typhoid fever, serious infections caused by beta-lactamase-producing Hemophilus influenzae, anaerobic infections with penicillin-resistant Bacteroides fragilis, and life-threatening infections with susceptible organisms in patients who are allergic to penicillin (1). To ensure that therapeutic concentrations are achieved in serum and to help prevent side effects, its concentrations in serum must be monitored. Bioassay and colormetry have been the most commonly used methods of measuring chloramphenicol; however, several problems limit their usefulness.

We have established in our laboratories three methods of determining chloramphenicol concentrations in body fluids: (a) radioenzymatic assay with use of chloramphenicol acetyltransferase (EC 2.3.1.28) prepared by two methods; (b) gas chromatography with electron capture detection; and (c) gas chromatography with mass spectrometric detection. These methods are described, results compared and correlated, and the advantages of each discussed.

Materials and Methods

Analytical Standards

Chloramphenicol base and the internal standard, D(-)-threo-N-(β-hydroxy-α-hydroxymethyl-L-nitrophenethyl) acetamide, used in the gas-chromatographic methods were both obtained from Parke, Davis & Co., Detroit, MI 48232. Methanolic stock solutions (200 g/L) of chloramphenicol and the internal standard were prepared. A working solution of

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1 Program in Infectious Diseases and Department of Pediatrics, The University of Texas Medical School at Houston, 6431 Fannin St., 228 Freeman Bldg., Houston, TX 77030.
2 Department of Pharmaceautics, University of Houston, Houston, TX.
3 Analytical Chemistry Center and Department of Biochemistry and Molecular Biology, The University of Texas Medical School, Houston, TX.

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the internal standard was prepared by diluting the stock solution 10-fold. Chloramphenicol standards, 0 to 50 mg/L, and mock unknowns were prepared in pooled human sera (shown to be drug-free by testing before adding chloramphenicol) by adding appropriate volumes of the methanol standard to volumetric flasks, evaporating the methanol almost completely, and diluting to volume with blank plasma. Stock solutions, working solutions, and plasma standards, stored at 4 °C, are stable for at least six months. We used the same standards in all assays.

Radioenzymatic Assay

**Enzyme preparation.** We prepared chloramphenicol acetyltransferase by two methods (2-4).

In the first, a mutant strain of *Escherichia coli* (W677/ JR66) was grown overnight in trypticase soy broth (Difco, Detroit, MI 48232) at 37 °C, harvested, and osmotically shocked by the method used by Nossal and Heppel (5) to extract the adenylating enzyme used in the measurement of gentamicin, kanamycin, and tobramycin (6, 7). After centrifugation, the pellet was washed with 5 mmol/L tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl; Sigma, St. Louis, MO 63178) and 1 mmol/L MgCl₂ (Fisher Scientific Co., Houston, TX 77001) at pH 7.8. An equal volume of 10 mmol/L Tris-HCl (pH 7.8) was added, and the suspension, kept on ice, was disrupted by exposure to sonic shock for five 1-min periods, then centrifuged (2 °C, 12 min, 35 000 × g), and the supernatant fluid was used as the source of chloramphenicol acetyltransferase. Its activity was about 10 kU/L (7). Aliquots of it were stored at −70 °C and thawed as needed for an assay.

The second method was more expedient, but did not allow for collection of the aminoglycoside adenylating enzyme (2).

The same *E. coli* strain was harvested after overnight growth, washed twice with Tris-HCl (10 mmol/L, pH 7.8), and resuspended in 40 mL of the same buffer. The bacteria were disrupted by sonication for 3 min at 70 W (Sonifer Cell Disruptor, W185; Heat Systems-Ultrasonic, Inc., Plainview, NY 11803). Cell debris was removed by centrifugation at 30 000 × g for 30 min at 2 °C, and aliquots of the supernatant fluid containing the transferase were frozen at −70 °C until use.

**Enzymatic Assay for Chloramphenicol**

The acetylation reaction was carried out in a final volume of 0.1 mL, in 12 × 75 mm glass test tubes. All ingredients for enzymatic assay except chloramphenicol were prepared on ice to give a reaction mixture containing 10 μL of Tris-HCl (1 mol/L, pH 7.8), 65 μL of water, 10 μL of [1-14C]acetyl coenzyme A (4 Ci/mol; Amersham, Arlington Heights, IL 60005; specific activity, 59.5 Ci/mol), and 5 μL (50 μM) of the transferase. The reaction was initiated by adding 10 μL of a standard or test sample containing the chloramphenicol to 90 μL of the reaction mixture, mixing, and incubating for 30 min in a water bath set at 37 °C. Then the acetylated chloramphenicol was extracted into toluene in one step (4) by adding 4 mL of scintillation fluid [5.5 g of 2,5-diphenyloxazole and 0.1 g of dimethyl-1,4-bis[2-[4-methyl-5-phenyloxazolyl)] benzene per liter of toluene; Fisher Scientific] to each tube and agitating it for 10 s. The extract was poured into scintillation vials containing 2 mL of water and its radioactivity counted in a liquid scintillation spectrometer. Acetate incorporated into acetylated chloramphenicol was measured as counts/min minus control counts obtained with a reaction mixture containing no chloramphenicol. All assays were done in duplicate.

**Gas Chromatographic–Electron Capture Assay**

Plasma samples were analyzed according to a modification of the method of Sams (9). To 0.1 mL of sample in a screw-capped culture tube, 100 μL of internal standard (20 μg/mL), 1.0 mL of phosphate buffer (100 mmol/L, pH 7.0) and 3.0 mL of ethyl acetate (Fisher Scientific) were added. The tubes were rotated for 10 min, centrifuged for 10 min, and the ethyl acetate layer was transferred to a clean tube and evaporated under a stream of filtered air in a water bath set at 40 °C. Derivatization was accomplished by adding 100 μL of “TriSII” (hexamethyldisilazane and trimethylchlorosilane in pyridine; Pierce Chemical Co., Rockford, IL 61105) to the residue and allowing it to stand at room temperature for 10 min. The mixture was then diluted with 1.0 mL of cyclohexane (Aldrich Chemical Co., Milwaukee, WI 53233) and 5-μL aliquots were injected onto the gas chromatograph. Retention times for the internal standard and chloramphenicol were 2.3 and 3.8 min, respectively. All solvents were of “chromatographic” grade or better, and all other chemicals were reagent grade and were used without further purification.

A Hewlett-Packard Model 5736A gas chromatograph equipped with a 63Ni electron capture detector was used. The chromatograph was equipped with a 183 cm × 2 mm (i.d.) glass column packed with 5% OV-17 on GasChromQ (Applied Science Laboratories, Inc., State College, PA 16801). Carrier gas (5% methane in argon) flow rate was 40 mL/min and the injection port, column, and detector were maintained at 300, 240, and 300 °C, respectively. Chromatograms were recorded and areas under the peaks of interest calculated by a Hewlett-Packard Model 3385A Automation System. Quantitation was done by using the ratio of the area under the chloramphenicol peak to that of the internal standard.

**Gas Chromatographic–Mass Spectrometric Assay**

To 1 mL of serum sample was added 20 μL of the internal standard solution (1 g/L). After addition of 2 mL of phosphate buffer (100 mmol/L, pH 7.0) and 3 mL of ethyl acetate to each sample tube, the mixtures were agitated and then centrifuged for 10 min. The organic phases were pipetted into test tubes and were evaporated in a stream of nitrogen. The residues were derivatized by adding 190 μL of TriSII and allowing the mixture to stand at room temperature for 10 min. Five microliters of each derivatized sample was then injected into the gas chromatograph–mass spectrometer (a Finnigan Model 3300 gas chromatograph–mass spectrometer interfaced with a Finnigan 6100 data system).

Gas chromatographic separations were achieved on a 152 cm × 2 mm i.d. glass column, packed with OV-1 on GasChromQ, 100/120 mesh. The oven temperature was maintained at 240 °C isothermal and the injector temperature at 250 °C. For quantitative identification of chloramphenicol, the mass spectrometer was operated in the scanning mode (70 eV ionizing energy).

Quantitative measurements were performed by selectively monitoring the ion m/e 225, an intense peak in both the spectrum of trimethylsilylated chloramphenicol and reference material. Calibration curves were obtained by adding known quantities of chloramphenicol (0.5–50 μg) and a fixed quantity of internal standard (20 μg) to 1-mL quantities of serum and subjecting these samples to the same extraction, derivatization, and measuring procedure. Ratios of peak intensity integrations, as obtained by computer, were plotted vs. chloramphenicol concentration. Values for unknown concentrations of chloramphenicol in serum, obtained in the same manner, were calculated from the slope of the standard curve.

**Sample Collection**

Blood was obtained from 50 patients who were receiving chloramphenicol. After clotting, the samples were centrifuged and the sera removed and stored at −70 °C until assayed.
Results

Standard Curves for Enzymatic Assay

By either method of enzyme preparation, a standard curve similar to that shown in Figure 1 was generated for serum and urine. The curve was linear for chloramphenicol concentrations from 1 to 60 mg/L for serum and 1 to 50 mg/L for urine. The correlation coefficient for 14 samples, used with the two enzyme preparations, was 0.999.

Gas Chromatography with Electron Capture

Chromatograms of blank plasma, a standard plasma (chloramphenicol, 10 mg/L), and a representative patient’s sample are shown in Figure 2. There are no interfering peaks in the areas where chloramphenicol and the internal standard elute. Both peaks are sharp, symmetrical, and well defined.

Under the conditions used, calibration curves were linear from 1 to 50 mg/L, corresponding to 0.1 to 5.0 μg of chloramphenicol per tube. Because of the great sensitivity (picogram), volumes of plasma as low as 10 μL have been assayed for concentrations within the normal therapeutic range. Urine and cerebrospinal fluid also have been used in the procedure, with accurate and reproducible results. Blank values for these biological fluids are identical to that from plasma.

Gas Chromatographic–Mass Spectrometric Assay

Mass spectra of the trimethylsilylated derivative of chloramphenicol and of the internal standard are shown in Figure 3. Ions of significance for chloramphenicol can be seen at m/e 361 (M-CH₃TmsOH), m/e 242 (fragment b), and m/e 225 (fragment a, with a rearranged hydrogen atom). For the internal standard, ions appear at m/e 383 (M-CH₃), m/e 295 (M-CH₂OTms), m/e 293 (M-CH₃-TmsOH), m/e 174 (fragment b), and m/e 225 (fragment a, with a rearranged hydrogen atom).

Sensitivity

The sensitivity of the assays, defined as the least amount of chloramphenicol which could be significantly (p < 0.05) distinguished from no drug, was 500 ng by radioenzymatic assay. Sensitivity of the gas chromatography with electron capture assay was in the picogram range and for the gas chromatographic–mass spectrometric assay the sensitivity was less than 1 ng.

Specificity and Stability

Experiments were designed to determine whether chloramphenicol would decrease in reactivity after incubation with either amikacin, ampicillin, cefazolin, gentamicin, or penicillin G. Chloramphenicol was incubated at both 37 and −70 °C for seven days with each of these antibiotics. Aliquots were removed at 0, 24, and 168 h and tested by radioenzymatic assay for chloramphenicol activity. The results of these incubations (Table 1) showed that chloramphenicol is stable in the presence of these antibiotics at all of these intervals tested.

Precision

Within-assay precision of the radioenzymatic method was determined by measuring in duplicate 10 aliquots of a single serum specimen containing 8 mg of chloramphenicol per liter. The mean and standard deviation were 7.99 ± 0.48 mg/L, with a CV of 6.0%. Between-assay precision for 15 assays showed a CV of 6.9%. The precision of the gas chromatographic–electron capture method was examined by repetitively assaying one patient’s sample. The mean concentration was 10.7 mg/L (CV, 6.5%, n = 6). Between- and within-assay precision of the gas chromatography/mass spectrometry showed a CV of 3.5% (n = 10).

Correlation of Assays

Serum samples from 50 patients were analyzed for chloramphenicol by the three techniques. The results of each assay were compared and the correlations are shown in Figure 4. The best correlation was between results of the radioenzymatic and the electron capture-gas chromatographic assays (r = 0.985, y = 1.03x + 0.80). A good correlation was achieved between the mass spectrometric assay and either the radioenzymatic
Table 1. Concentration of Chloramphenicol Found After Incubation of 30 mg/L Solution with Various Antibiotics for Various Periods at 37 and -70 °C

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Conc., mg/L</th>
<th>Chloramphenicol values, %&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>37 °C for 3 days</td>
</tr>
<tr>
<td>Chloramphenicol alone</td>
<td>30</td>
<td>103</td>
</tr>
<tr>
<td>Plus amikacin</td>
<td>20</td>
<td>97</td>
</tr>
<tr>
<td>Plus ampicillin</td>
<td>20</td>
<td>98</td>
</tr>
<tr>
<td>Plus cefazolin</td>
<td>30</td>
<td>101</td>
</tr>
<tr>
<td>Plus gentamicin</td>
<td>8</td>
<td>98</td>
</tr>
<tr>
<td>Plus penicillin G</td>
<td>20</td>
<td>96</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values expressed as % of original determination.

assay \((r = 0.964, y = 1.07x + 0.98)\) or electron capture assay \((r = 0.971, y = 0.98x + 2.06)\).

**Discussion**

The re-emergence of chloramphenicol as the drug of choice for the treatment of several life-threatening infections in all age groups (10) necessitates the availability of assays that provide precise, specific, and sensitive means of monitoring chloramphenicol concentrations and of performing pharmacokinetic studies. The commonly used microbiological assays have limited sensitivity, variable precision, and are not specific if the sample to be tested contains other antimicrobial agents (11, 12). An additional problem with this assay is the difficulty of interpreting results, especially when variations in support media or doubling zones are encountered, making precise reading of the inhibition zone difficult. In addition the bioassay is often time-consuming and requires that the specimens be sterile.

Fig. 3. Mass spectrum of the trimethylsilylated derivative of chloramphenicol and the internal standard trimethylsilylated \(\alpha\)-threo-\(N\)-(β-hydroxy-α-hydroxymethyl)-p-nitrophenethyl) acetamide

Ions of significance for chloramphenicol can be seen at \(m/e\) 361, \(m/e\) 242 (fragment b), and 225 (fragment a). Ions of the internal standard appear at \(m/e\) 383, \(m/e\) 293, \(m/e\) 174 (fragment b), and \(m/e\) 225 (fragment a).

Fig. 4. (a) Radioenzymatic and the gas chromatography-electron capture assays compared for chloramphenicol in patients' serum samples \((r = 0.985, y = 1.03x + 0.80)\)

(b) Radioenzymatic and the gas chromatography-mass spectrometry compared for patients' serum samples \((r = 0.964, y = 1.07x + 0.98)\)

(c) Gas chromatograph-mass spectrometry and gas chromatography-electron capture compared for patients' serum samples \((r = 0.971, y = 0.98x + 2.06)\)

Several colorimetric assays are available that measure the amine formed after reduction of the aromatic nitro group of chloramphenicol (13-16). These chemical procedures often co-determine inactive metabolites: chloramphenicol succinate (the intravenous preparation) and glucuronide both contain the nitro group and both are also included in the measurement. Solvent extractions must be performed if only free drug
is to be measured. In addition, the colorimetric tests are not performed on microsamples and suffer the risk of interference by other drugs and bilirubin.

The potential toxicities of chloramphenicol (17, 18) and the need to ensure that a therapeutic concentration is being achieved dictate the need for rapid, precise, and specific assay. We describe and compare three such assays, which provide the capability to conduct pharmacokinetic studies in humans, particularly in newborn infants and young children, for whom there are few data concerning dosing and dosing schedules. The three methods described compare favorably with one another.

The radioenzymatic procedure was first described for assaying gentamicin (19) and subsequently the R-mediated enzyme gentamicin adenyltransferase was partly purified and characterized (20). We previously have used the radioenzymatic assay to measure all aminoglycoside antibiotics (6, 7, 21–23). The acetyltransferase enzyme used in the chloramphenicol assay can be prepared at the same time the aminoglycoside adenyltransferase enzyme is being produced. The chloramphenicol acetyltransferase assay is not affected by other antibiotics except thiamphenicol, a derivative. It is precise, rapid, and detects concentrations as low as 500 μg/L. The assay can be simplified by using the relative solubility of chloramphenicol esters in toluene scintillation fluid, thereby obviating the need for multiple benzene extractions (4). This assay requires the use of liquid-scintillation counting equipment, which is available in most clinical laboratories.

Gas-chromatographic procedures have been described in which flame or electron-capture devices are used as detectors (24, 25). “High pressure” liquid chromatography also has been shown to be a reliable method of measuring chloramphenicol (26). The gas chromatographic-electron capture detection method of measuring chloramphenicol is rapid, technically simple, requires only microsamples, and is sensitive enough to make it well suited for routine use in a clinical laboratory. The specificity of this assay makes it ideal for use in pharmacokinetic studies in adults and children from whom microsamples can be obtained.

The gas chromatographic/mass spectrometric procedure offers a particular advantage over other methods in providing an absolute identification of the molecule being measured (27). Since the complete mass spectrum can be monitored, quantitation is accomplished by measuring specific structural features of the molecule. There is, therefore, virtually no chance of measuring a contaminating molecule that may cochromatograph with chloramphenicol or one that may bind to a receptor in a radioimmunoassay. The disadvantage of this method is that it requires use of relatively complex and costly instruments.

The aminoglycoside antibiotics are biologically and immunologically inactivated by incubation with carbenicillin and ticarcillin in vitro (28). Because other antibiotics are frequently used clinically in conjunction with chloramphenicol, we determined the effect of five antibiotics upon chloramphenicol activity, but saw no change in chloramphenicol values, as measured by radioenzymatic assay, after seven days' incubation at −70 or 37 °C. We find that specimens containing chloramphenicol can be mailed at room temperature to facilities that have the capability to determine chloramphenicol concentrations by one of the methods described, without concern about inactivation.

Monitoring chloramphenicol in the serum of patients will aid in achieving therapeutic concentrations and minimizing dose-related toxicity such as bone marrow depression and gray baby syndrome in newborns of patients with hepatic dysfunction. The final choice of methods depends upon personnel and equipment available.

References

We evaluated four commercial radioimmunoassay kits for digoxin. We assayed a standard plasma containing digoxin, 2.0 μg/L, and samples from patients receiving digoxin, with use of the kits and of a bioassay, the 86Rb-uptake inhibition technique. Intra-assay precisions differed significantly. Computer-calculated 95% confidence intervals for the radioimmunoassays averaged 0.4 to 0.6 μg/L at the proposed toxic threshold of 2.0 μg/L; the corresponding value of the 86Rb assay was 0.75 μg/L. Digoxin in the standard plasma was overestimated with three of the kits (means: 2.40, 2.56, and 2.59 μg/L) but was assayed accurately by the 86Rb technique and by one kit. This same kit gave a significantly lower mean (1.07 μg/L) for the patients' samples than did the other three kits (1.32, 1.49, and 1.29 μg/L), two of which also differed significantly in accuracy. The 86Rb assay measured glycoside activity corresponding to a mean digoxin concentration of 1.35 μg/L. We conclude that the relatively low precision of digoxin assay and the variations in accuracy between kits from various vendors apparently deserve continual attention.

Additional Keyphrases: 86Rb-uptake inhibition technique • heart disease • computer technique

Several studies have focused on methodological difficulties in connection with radioimmunoassay (RIA) of digoxin (5-4) and considerable differences found in the values measured by kits from various vendors (5, 6). We compared four commonly used commercial kits to see whether important differences in accuracy and precision can still be detected. The samples used in our study were also assayed by the 86Rb-uptake inhibition technique (7). This bioassay was included because its results give a functional dimension: the Na-K-ATPase (EC 3.6.1.3) inhibiting activity of all heart glycosides extractable from the plasma rather than digoxin concentration per se is measured.

Materials and Methods

Patients' plasma samples. We collected 35 blood samples from 33 patients on digoxin maintenance therapy into heparinized Vacutainer Tubes, before the morning dose. The samples were centrifuged within 1 h and the plasma was separated. Plasma from the same sampling occasion of each patient was pooled, apportioned into eight tubes, and stored at -20 °C before analysis, which was performed within four months. Specifications of the patients' samples are available on request to the authors or the Editorial Office of this journal.

Standard plasma containing digoxin, 2.0 μg/L. Stock solutions of digoxin (WHO-quality; WHO Centre for Chemical Reference Substances, AKL, Solna, Sweden) were prepared by weighing the pure substance on a Cahn Gram Electrobalance (Ventrion Instrument Comp., Paramount, CA 90723) and dissolving it in ethanol/water (80/20 by vol) by shaking for 30 min. Two 100 mg/L stock solutions of digoxin were pooled and five separate dilutions to 200 μg/L were then made in ethanol/water (5/95 by vol). These solutions were pooled and 5.00 mL of the pool was diluted to 500.00 mL with plasma. After equilibration for 1 h at room temperature by shaking at low speed, the plasma was apportioned and then stored at -20 °C before analysis, which was performed within six weeks. Heparinized plasma pooled from five healthy, drug-free subjects was used. Values for bilirubin, urea, creatinine, total protein, albumin, electrolytes, and thyroxine-binding globulin in this plasma were within normal limits.

RIA kits. For the analysis of digoxin we used four commercially available kits: the Digoxin RIA Kit 3H and Digoxin RIA Kit 125I from Schwarz/Mann, Orangeburg, NY 10962 (here abbreviated SM3H and SM125I, respectively); the Gammacoat 125I Digoxin RIA Kit from Clinical Assays Inc., Cambridge, MA 02142 (CAG); and the Digoxin 125I RIA Kit from New England Nuclear, North Billerica, MA 01862 (NEN125I). All kits contained buffer material, pre-diluted...