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Simplified Radioenzymatic Assay for Chloramphenicol

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A simplified radioenzymatic assay for chloramphenicol was developed by eliminating the need for cumbersome extraction procedures. After the acetylation of chloramphenicol with [^{14}C]acetyl coenzyme A in the presence of chloramphenicol acetyltransferase, the reaction mixture was added to a toluene-based scintillation fluid. Since ^{14}C-acetylated chloramphenicol is more soluble than [^{14}C]acetyl coenzyme A in toluene, the radioactive product could be counted directly. The rapidity of this assay, as well as its accuracy, precision, and specificity, makes it particularly suitable for clinical use. In contrast to previous reports of enzymatic assays for chloramphenciol, we have found that results of the assay of standards prepared in serum were up to 25% higher than those of standards prepared in saline, cerebrospinal fluid, or urine.

Renewed interest in the clinical use of chloramphenicol is based on its value in treating: (i) anaerobic infections, especially those involving penicillin-resistant *Bacteroides fragilis*; (ii) meningitis in which ampicillin-resistant *Haemophilus influenzae* is suspected or proven (1); and (iii) ventriculitis in which penetration of aminoglycosides into the cerebrospinal fluid (CSF) may be inadequate (4).

We have developed a simplified radioenzymatic assay for chloramphenicol. Like that previously described (2, 5), it is based on the specific acetylation of chloramphenicol with [^{14}C]acetyl coenzyme A by chloramphenicol acetyltransferase. Unlike the previously described enzymatic assays, our method allows the direct extraction of ^{14}C-acetylated chloramphenicol into scintillation fluid and circumvents more cumbersome extraction methods. In contrast to the previous reports, we find a substantial difference between the acetylations of chloramphenicol standards in serum and in saline, CSF, or urine.

MATERIALS AND METHODS

Bacteria. *Escherichia coli* W677/HJR66, kindly provided by D. H. Smith, was the source of chloramphenicol acetyltransferase. *Bacillus subtilis* spores (ATCC 6633) from Baltimore Biological Laboratory (BBL) (Cockeysville, Md.) were used in the microbiological assays.

Antibiotics and chemicals. Chloramphenicol base, chloramphenicol succinate, and chloramphenicol palmitate were supplied by Parke, Davis & Co. (Detroit, Mich.); gentamicin was provided by Schering Laboratories (Bloomfield, N.J.); and amikacin and kanamycin were provided by Bristol Laboratories (Syracuse, N.Y.). Ampicillin, cephalothin, clindamycin, tetracycline, and vancomycin were obtained from Ames Co. Div. of Miles Laboratories, Inc. (Elkhart, Ind.), and penicillin G was from ICN Pharmaceuticals, Inc. (Irvine, Calif.). Vacutainer collection tubes (Becton, Dickinson & Co., Rutherford, N. J.) containing the following additives were used: no. 3206PS, 10 mg of potassium oxalate and 10 mg of sodium fluoride; no. 3204KA, heparin sodium, 143 USP units; no. 3204W, 0.5 ml of sodium citrate (38 mg/ml) and potassium sorbate (0.2 mg/ml); no. S3204XF24, 1 ml of trisodium citrate (13.2 mg/ml), citric acid (4.8 mg/ml), and dextrose (14.7 mg/ml); no. S3200XF177, 5.95 mg of sodium polyanetholesulfonate and 14.4 mg of sodium chloride; and no. 3202QS, 10.5 mg ethylenediaminetetraacetate and 0.014 mg of potassium sorbate.

[1-^{14}C]acetyl coenzyme A, 58 mCi/mmol, was obtained from Amersham/Searle (Arlington Heights, Ill.). Acetyl coenzyme A as the trilithium salt was purchased from P-L Biochemicals, Inc. (Milwaukee, Wis.).

Preparation of CAT. *E. coli* W677/HJR66 was grown in Trypticase soy broth (BBL) overnight at 37°C, harvested, and subjected to osmotic shock (6) to extract the gentamicin adenylyltransferase used for assays of gentamicin and tobramycin. After centrifugation, the pellet was washed with a solution of 0.005 M tris(hydroxymethyl)aminomethane-hydrochloride and 0.001 M $MgCl_2$, pH 7.8. To the centrifuged pellet an equal volume of 0.01 M tris-(hydroxymethyl)aminomethane-hydrochloride, pH 7.8, was added. The suspension was kept on ice and disrupted by sonic treatment (Artek Systems 300 Dismembrator) at 180 W for five 1-min periods. The supernatant from centrifugation at 2°C for 12 min

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at 39,000 × *g* was used, without further purification, as the source of chloramphenicol acetyltransferase (CAT).

The CAT activity was measured by the colorimetric method of Shaw (8). A unit of enzyme activity represented 1 μmol of coenzyme A liberated per min at 37°C. The sonic extract, prepared as described above, contained 10 U of CAT activity per ml. Aliquots of the sonic extract (CAT) were frozen at −20°C. Vials for current use were thawed and subsequently kept on ice.

Chloramphenicol standards. Chloramphenicol standards and mock unknown samples were prepared with pooled human serum, CSF, and urine, all shown to be normal by testing individual specimens before pooling. Another set of standards was made in 0.9% sodium chloride (saline). A set of standards was prepared in serum, CSF, urine, and saline from stock solutions of 500 μg of chloramphenicol per ml in each diluent. When not in use, standards and patient specimens were kept at −20°C.

Enzymatic assay for chloramphenicol. A reaction mixture containing all ingredients for enzymatic assay except chloramphenicol was prepared on ice: water, 0.065 ml; tris(hydroxymethyl)aminomethane-hydrochloride 1 M, pH 7.8, 0.010 ml; [^{14}C]acetyl coenzyme A (4 μCi/μmol), 1 μmol/ml, 0.010 ml; and CAT, 0.005 ml (50 mU). To this reaction cocktail (0.090 ml) 0.010 ml of test sample or standard was added. The reaction solution was mixed briefly and then incubated in a water bath at 37°C for 15 min.

The reaction step of the assay was a simple modification of the method described by Shaw (8). However, the extraction step was altered considerably. Direct extraction of acetylated chloramphenicol into the toluene phase from the water phase was accomplished in one step by the addition of 4 ml of scintillation fluid {4 g 2,5-diphenyloxazole and 0.1 g of dimethyl-1,4-bis[2-(4-methyl-5-phenyloxazolyl)]}-benzene per liter of toluene) to each tube and agitation on a Vortex mixer for 10 s. The combined scintillation and water phases were poured into scintillation vials containing 2 ml of water and counted by liquid scintillation spectrometry.

In addition to this simplified method for extraction of acetylated chloramphenicol from the reaction mixture, we have used the method of multiple benzene extractions developed by Shaw (8). Two separate 1.5-ml volumes of benzene were added sequentially to each reaction solution and agitated on a Vortex mixer for 10 s. The benzene, containing acetylated chloramphenicol, was pipetted into a vial containing 3 ml of scintillation fluid and counted by liquid scintillation spectrometry.

Microbiological assay for chloramphenicol. A modification of the method of Sabath et al. (7) was used in microbiological assays for chloramphenicol. *B. subtilis* spore plates were prepared with Difco Antibiotic Medium 5 agar. Portions of 0.020 ml from standards or test solutions were absorbed into sterile 6-mm filter paper disks and placed onto the agar plates. Zones of inhibition were read with a zone reader after 18 to 24 h of incubation at 37°C.

Ultrafiltration of human serum. Pooled normal human serum was placed in a stirred filter cell containing a Diaflo ultrafilter UM2 (Amicon Corp., Lexington, Mass.) having a retentivity of greater than 1,000 daltons. Filtration was carried out at 8°C under 70 lb/in^2.

RESULTS

Acetylation of chloramphenicol. In our simplified radioassay, ^{14}C-acetylated chloramphenicol was extracted directly from the aqueous phase into the toluene phase. [^{14}C]acetyl coenzyme A remained in the aqueous phase. Thus, in blanks lacking chloramphenicol or CAT, less than 2% of the radioactivity added was counted, i.e., 1,000 cpm out of a total of 50,000 to 60,000 cpm. Chloramphenicol itself remained in the aqueous phase where acetylation continued, even after the addition of a nonpolar solvent, such as benzene or toluene.

As shown in Fig. 1, the acetylation of chloramphenicol occurred in two stages, a rapid initial phase and a slower second phase. The heights of the first and second phases were similar with 50 or 1 mU of CAT, but the rate of acetylation in each phase was dependent upon enzyme concentration. The contribution of the slower, second phase of the reaction to extractable radioactivity depended upon the amount of CAT used and the length of the incubation period. Under the conditions we have chosen, using 50 mU CAT and incubating for 15 min at 37°C, about one-third of the extractable radioactivity was generated in the second phase of the reaction.

Standard curves. Using the direct extraction method, a standard curve like that shown in Fig. 2 was generated. Under the conditions of the assay, its range of usefulness was from 5 to at least 50 μg of chloramphenicol per ml. Over this range there was good linearity, typically

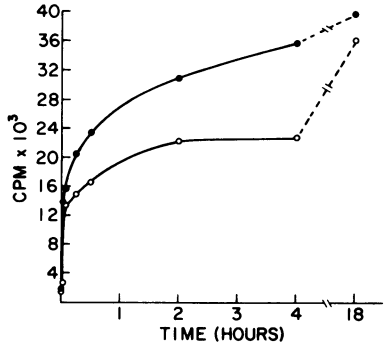

FIG. 1. *Time course of acetylation of chloramphenicol under usual assay conditions with 50-μg/ml serum standard. Symbols: (●) 50 mU of CAT; (○) 1 mU of CAT.*

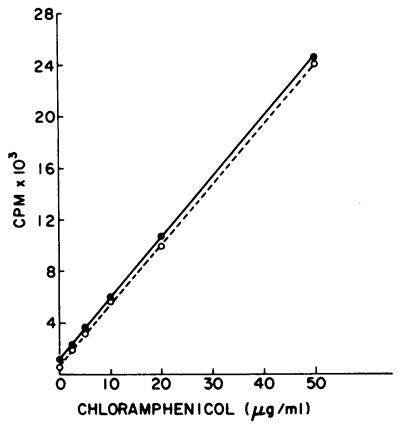

FIG. 2. *Standard curve of chloramphenicol assayed by the direct extraction method. Symbols: (●) Routine assay; (○) same assay after removal of aqueous phase from vials.*

with a coefficient of correlation (r) of at least 0.999. The removal of the water phase from the scintillation vial resulted in a reduction in counts per minute but did not change the linearity or the slope of the standard curve (Fig. 2). In our hands, the use of the benzene extraction method yielded a standard curve almost identical to that of the direct toluene extraction method after removal of the water phase.

Difference between serum and saline/CSF/urine standards. Results from the assay of chloramphenicol standards prepared in serum were as much as 5 to 10% higher than results with standards prepared in saline, CSF, or urine. When less CAT (1 mU) was used in the assay, the difference between standards in serum and those in saline, CSF, or urine was accentuated, ranging up to 25%. Similar differences were seen with the benzene extraction method.

This difference was fully corrected when 0.010 ml of normal human serum was mixed with the saline standard before the addition of the remainder of the reaction cocktail (Fig. 3). Results equivalent to those with serum standards were obtained when 0.010 ml of horse serum was added to the saline standards. The addition to saline standards of 0.010 ml of the concentrate from ultrafiltration of human serum, of hemoglobin (70 mg/ml) obtained by freezing normal erythrocytes, or of bovine serum albumin (10 mg/ml) partially reduced (by more than 50%) the difference in results between serum and saline standards. As with saline standards, when human serum was added to CSF standards, assay results were equivalent to those with serum standards. Results of the assay of urine standards, in contrast to those with saline and CSF standards, were not affected when normal human serum was added.

Figure 4 shows the kinetics of acetylation of a chloramphenicol saline standard by 1 mU of CAT in the presence and absence of added serum. Whereas the presence of serum had little effect on the rate of acetylation in the initial phase of the reaction, the rate of acetylation in the second phase was enhanced by serum.

Precision and accuracy. The precision of the test was evaluated by assaying each of seven serum standards six times in a single

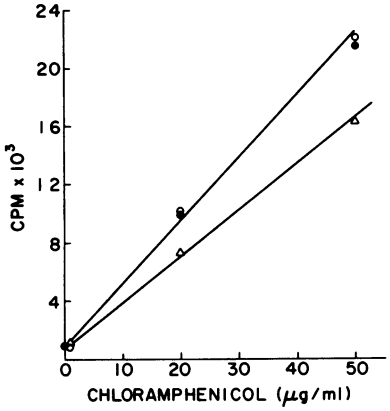

FIG. 3. *Comparison of chloramphenicol standards in serum and saline, using 1 mU of CAT. Symbols: (●) Serum; (△) saline; (ρ) saline and normal human serum.*

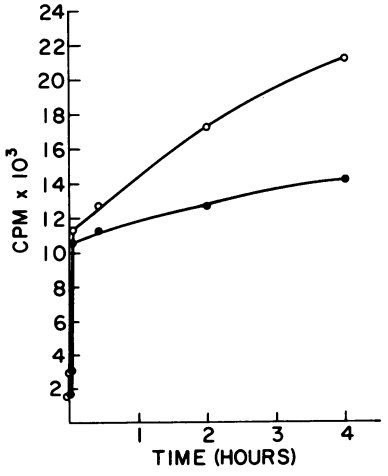

FIG. 4. *Time course of acetylation of chloramphenicol, using 1 mU of CAT. Symbols: (●) 50-μg/ml saline standard; (○) 50-μg/ml saline standard plus an equal volume of normal human serum.*

experiment. Figure 5 shows the standard curve with the average counts per minute and standard deviation for each level of chloramphenicol.

A single serum specimen containing 14.1 μg of chloramphenicol per ml was assayed for chloramphenicol 10 times in a single experiment. The mean and standard deviation were 12.9 ± 0.5 μg/ml. In another assessment of precision and accuracy, a stock solution of chloramphenicol was diluted in serum to 14.3 and 35.7 μg of chloramphenicol per ml. On 10 separate days, these mock unknowns and a set of serum standards were assayed for chloramphenicol. The means and standard deviations of single daily determinations were 14.1 ± 0.9 and 35.0 ± 0.8 μg of chloramphenicol per ml, respectively.

Specificity. The influence on the chloramphenicol assay of antibiotics, azotemia, hyperbilirubinemia, and additives commonly used in blood collection tubes was assessed. When samples were assayed alone, there was no reaction with blood hemolyzed by freezing, with plasma collected by the Vacutainer tubes listed in Materials and Methods, or with the following antibiotics in serum: amikacin, 25 μg/ml; ampicillin, 10 μg/ml; cephalothin, 200 μg/ml; clindamycin, 25 μg/ml; erythromycin, 10 μg/ml; gentamicin, 10 μg/ml; kanamycin, 25 μg/ml; penicillin G, 32 U/ml; tetracycline, 10 μg/ml; vancomycin, 25 μg/ml; and chloramphenicol palmitate suspension, 500 μg/ml. When each of these samples was assayed after the addition of chloramphenicol at a concentration of 23.8 μg/ml, there was less than 10% deviation from this value. Chloramphenicol succinate alone in serum at a concentration of 500 μg/ml contained 7.7 μg of reactive chloramphenicol per ml. Sera from two patients with elevated total serum bilirubin of 6.3 and 20 mg/dl and from two patients with elevated

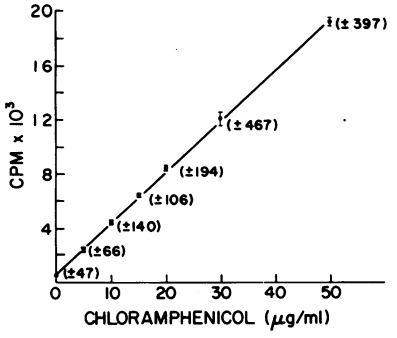

FIG. 5. *Standard curve of chloramphenicol, showing mean and standard deviation of six determinations for each point.*

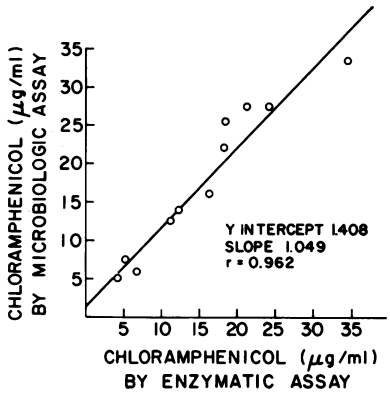

FIG. 6. *Correlation of chloramphenicol concentrations in human serum determined by enzymatic and microbiological methods.*

blood urea nitrogen of 44 and 110 mg/dl resulted in blank values when assayed alone and did not affect the reaction after chloramphenicol addition.

Comparison of enzymatic and microbiological assays. Figure 6 shows graphically the correlation between results of microbiological and radioenzymatic assays of samples prepared by the addition of known amounts of chloramphenicol to normal serum. The coefficient of correlation between known concentrations and the results of the microbiological assay was 0.957; for the enzymatic assay, it was 0.996.

DISCUSSION

Our experience with this radioenzymatic assay for chloramphenicol, like that of previous investigators (2, 5), has shown the method to be specific, accurate, and precise. We have simplified the assay by making use of the relative solubility of chloramphenicol esters in toluene scintillation fluid, thereby obviating the need for multiple benzene extractions. The major disadvantage of this direct extraction method compared with the benzene extraction procedure is the higher counts per minute in blank samples, containing no chloramphenicol. The high blanks could result from the presence of the radioactive water phase in the scintillation vials, emulsification of the water in scintillation fluid, or solubility in scintillation fluid of any radioactive material other than ^{14}C-acetylated chloramphenicol. In spite of the higher blank counts, accuracy and precision were excellent at concentrations in the range of greatest clinical concern.

Our findings regarding the effect of 1 and 50 mU of CAT on the time course of the acetylation reaction are in agreement with Lietman et al. (5). However, the lack of equivalence

between acetylation of chloramphenicol in serum standards and in saline, CSF, or urine standards has not been reported previously (2, 5). Based on the results shown in Fig. 4, the enhancement effect of serum occurs in the second phase of acetylation, during the enzymatic transfer of an acetyl moiety from the 3- to the 1-position of chloramphenicol (8). Despite the discrepancy between CSF and serum, assay results of CSF samples can be compared with saline standards or, alternatively, made comparable with serum standards by the addition of 0.010 ml of serum.

The measurement of chloramphenicol in the serum of patients may aid in minimizing dose-related toxicity, such as bone marrow depression and the gray baby syndrome (9). These problems are of greatest concern in patients with hepatic dysfunction, whether physiological in the neonate or pathological.

Two clinical examples illustrate the value of this assay in the treatment of such patients. The first patient was an infant girl with an estimated gestational age of 32 weeks, weighing 750 g. During the first week of life, she was hyperbilirubinemic. Chloramphenicol, 25 mg/kg per day in four divided doses, was given intravenously during week 4 to treat a mixed infection of a lung cyst. The peak serum level was only 12 μg of chloramphenicol per ml. On the basis of this relatively low level, the dose was doubled to 50 mg/kg per day, with a subsequent rise in peak level to 18 μg of chloramphenicol per ml. The second patient was a 23-year-old man with pneumococcal meningitis, postnecrotic cirrhosis, and penicillin allergy. The trough serum level taken while the patient was receiving chloramphenicol, 100 mg/kg per day in four divided doses, was 153 μg of chloramphenicol per ml. The dosage was adjusted to 25 mg/kg per day to reduce the serum level. In both cases, the determinations of serum levels of chloramphenicol were needed to optimize the dosages.

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